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IDENTIFICATION AND CHARACTERIZATION OF EFFECTORS/BINDING MOLECULES FOR THE SMALL GTPASE RAB15

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IDENTIFICATION AND CHARACTERIZATION OF EFFECTORS/BINDING MOLECULES FOR THE SMALL GTPASE RAB15

by

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Dedicated to my Grandmothers, Alice Mackey and Pauline Strick. They are and will always be my biggest fans.

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IDENTIFCATION AND CHARACTERIZATION OF EFFECTORS/BINDING MOLECULES FOR THE SMALL GTPASE RAB15

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Endocytic trafficking is a key mechanism for regulating receptor availability on the plasma membrane as well as receptor degradation. Clathrin-dependent endocytosis involves receptor internalization into early endosomes. Here internalized receptors are sorted for degradation in lysosomes, direct recycling back to the cell surface or indirect recycling via a second recycling compartment called the pericentriolar recycling endosome. Rab GTPases regulate specific membrane trafficking steps including vesicle budding, vesicle transport and fusion with downstream target compartments. Rab function is mediated by the cyclical binding and hydrolysis of GTP, which in turn regulates the recruitment of downstream effector molecules directly involved in membrane transport steps. This dissertation focuses on the endocytic GTPase Rab15. Rab15 localizes to early and pericentriolar recycling endosomes, and differentially regulates receptor transport at these distinct organelles. For example, over expression of GTP-bound Rab15 inhibits internalization of the Transferrin Receptor and inhibits homotypic endosome fusion *in vitro.* Conversely, over expression of Rab15-GDP differentially stimulates Transferrin receptor recycling from the early endosome and pericentriolar recycling endosome respectively. Rab15 may differentially regulate receptor trafficking through these distinct endocytic compartments by binding compartment specific effectors. To test this hypothesis, I performed yeast two-hybrid screens to identify and characterize Rab15 binding partners. This dissertation is the functional characterization of three Rab15 binding proteins; Mammalian Suppressor of Sec4, Rab15 Effector Protein and Rab15 Binding Protein. Using molecular, biochemical and imaging approaches, I demonstrated that interactions between Rab15 and Mss4 modulate the inhibitory effect of Rab15-GTP on receptor entry into early endosomes. The second binding partner, Rab15 Effector Protein, localized specifically to the pericentriolar recycling endosome where it regulated Transferrin receptor recycling back to the cell surface. Finally, Rab15 Binding Protein is a neural specific protein of unknown function, suggesting an important regulatory function for Rab15 in neural receptor trafficking. These results confirm that Rab15 is a bi-functional GTPase, which differentially regulates receptor trafficking through early and pericentriolar recycling

endosomes, by binding specific effector proteins. Moreover, identification of putative Rab15 effector molecules further defines the endocytic pathway, thus providing valuable information for the characterization of trafficking-related diseases and potential drug targets in the future.

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LIST OF ABBREVIATIONS

ANOVA .....Analysis of Variance
CI-MPR.....Cation independent mannose phosphate receptor
EE.....Early Sorting Endosome
EEA1.....Early Endosomal Antigen 1
EGFR .....Epidermal Growth Factor Receptor
ER .....Endoplasmic Reticulum
GAP.....GTPase Activating Protein
GEF .....Guanine Nucleotide Exchange Factor
GFP .....Green Fluorescent Protein
GFAP .....Glial Fibrillary Acidic Protein
GDPβS .....guanosine 5β-3-O-(thio) diphosphate
GTPγS .....guanosine 5γ-3-*O*-(thio) triphosphate
HA.....Hemagglutinin Antigen
Hrs.....Hepatocyte growth factor regulated tyrosine kinase substrate
LE.....Late Endosome
LDL.....Low Density Lipid Protein
LDL-R.....Low Density Lipid Protein Receptor
LTP .....Long Term Potentiation
MVB .....Multi-vesicular Body
MHC1 .....Major Histo-Compatibility I Complex
Mss4.....Mammalian Suppressor of Sec4
NiNTA .....Nickel-Nitrilotriacetic Acid
PBS .....Phosphate Buffered Saline
PI3K .....Phosphotidyl Inositol-3-Kinase
PI3P.....Phosphotidylnositol 3-phosphate
PM.....Plasma Membrane
Rab .....Ras Like protein from Rat Brain
RE .....Pericentriolar Recycling Endosome
RBP15 .....Rab15 Binding Protein
REP15 .....Rab15 Effector Protein
RTKs .....Receptor Tyrosine Kinases
SNARE .....Soluble NSF Attachment Protein Receptor
Tac.....Interleukin-2 receptor
Tfn.....Transferrin
TfR .....Transferrin Receptor
TGFβ.....Transforming Growth Factor β
TGFβ-R.....Transforming Growth Factor β Receptor
TGN .....Trans Golgi Network
X-Gal.....5-bromo-4-chloro-3-indolyl-β-D-Galactopyranoside

CHAPTER 1: THE MOLECULAR REGULATION OF RECEPTOR ENDOCYTOSIS

Endocytic trafficking is a crucial cellular process for nutrient internalization, cell to cell communication, plasma membrane maintenance, embryogenesis and learning and memory (Olkkonen and Stenmark, 1997; Somsel and Wandinger-Ness, 2000; Segev, 2001a; Segev, 2001b; Park et al., 2004). The endosomal membrane system also provides additional area and scaffolding for glycosylation, proteolytic processing, protein degradation and cellular signaling (Di Fiore and Gill, 1999; Ceresa and Schmid, 2000; Di Fiore and De Camilli, 2001; Owen et al., 2004; Owen, 2004). Endocytosis regulates the internalization and recycling of two major types of receptors. Cargo receptors, such as the Transferrin receptor (TfR) and LDL receptor (LDL-R), facilitate internalization of iron and cholesterol, respectively, and have no measurable effect on cellular signaling (Li and Qian, 2002). Conversely, signaling receptors such as the Epidermal Growth Factor receptor (EGF-R) and the Transforming Growth Factor-β receptor (TGFβ-R), control complex mitogenic signaling mechanisms, cell motility and apoptosis (Di Fiore and De Camilli, 2001; Hayes et al., 2001; Hayes et al., 2002; Gonzalez-Gaitan and Stenmark, 2003). Endocytic mechanisms that control receptor internalization allow both spatial and temporal control over receptor-mediated/induced cellular signaling events. This chapter is an overview of the molecular machinery regulating receptor trafficking, including the mechanisms for internalizing and sorting of different receptors for recycling and degradation, and how mutations in the machinery regulating these processes are linked to human diseases.

The Endocytic Pathway

The molecular machinery that regulates endocytic trafficking is bound by three major criteria. First, the proteins involved in this system are generally multimodular – interacting with other molecules through a series of low affinity molecular interactions, permitting a vast web of protein:protein interactions that function to regulate endocytic trafficking (Watson et al., 2004). The complexity of the endocytic machinery was best shown by Zerial and colleagues (Christoforidis and Zerial, 2000). Using an affinity purification scheme, the authors identified 23 potential binding partners for Rab5, a GTPase that regulates membrane trafficking through Early Sorting Endosomes (EEs) (Christoforidis and Zerial, 2000). Second, the proteins in the endocytic network are compartmentalized into distinct

domains on membrane-bound organelles. The Rab GTPase subfamily, which regulate membrane trafficking through interactions with compartment specific effectors, localize to distinct microdomains with their downstream effectors on EEs (Zerial and McBride, 2001; Segev, 2001a; De Renzis et al., 2002; Watson et al., 2004). Finally, the endocytic machinery and organelles are linked to the cytoskeleton, allowing the organelles/vesicles to be transported within the cell (Nielsen et al., 1999; Lanzetti et al., 2001; Lanzetti et al., 2004; Watson et al., 2004).

Endocytosis consists of at least three internalization pathways: Clathrin-dependent endocytosis, caveolae-mediated endocytosis and macropinocytosis (Pelkmans and Helenius, 2003). In clathrin-dependent endocytosis, receptors are sorted into a clathrin-coated pit by binding specific adaptor molecules that link the endocytic machinery to its cargo. After internalization the nascent vesicle merges with other vesicles to form an early sorting endosome (**Fig. 1**). Usually a signaling receptor is marked for degradation upon internalization, and transported from the EE to the inner luminal body of the multi-vesicular body (MVB) and is degraded in the lysosome (Stahl and Barbieri, 2002). Alternatively, cargo receptors and signaling receptors are recycled from the endocytic compartments back to the plasma membrane to be used for further rounds of cargo transport or cellular signaling (Sorkin et al., 1989; Maxfield and McGraw, 2004). Clathrin-mediated endocytosis is the best characterized form of endocytic trafficking and the following section will focus on detailed mechanisms of clathrin-mediated endocytosis, while other internalization pathways will be briefly discussed.

Clathrin-Coated Vesicle Endocytosis and the Classical Pathway

Clathrin forms a triskelia lattice that resembles a basket like structure on the cytoplasmic surface of the plasma membrane (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981; Kirchhausen et al., 1983; Ungewickell, 1983). Receptors destined for clathrin-mediated endocytosis contain intracellular sorting domains that bind to clathrin adaptor molecules thus linking the "cargo" to the coat, and facilitating receptor sorting into clathrin-coated pits on the plasma membrane (Mousavi et al., 2004; Owen et al., 2004; Owen, 2004; Sorkin, 2004). Perhaps the most characterized clathrin adaptor is the clathrin associated protein complex-2 (AP-2 complex), which is a stable heterotetramer recruited to membranes by binding Phosphoinositide 4,5 bis-Phosphate and is crucial for "pit formation" by promoting clathrin polymerization (Owen et al., 2004; Owen, 2004). Multiple clathrin adaptors bind to specific receptor sub-types, regulating receptor sequestration into clathrin-

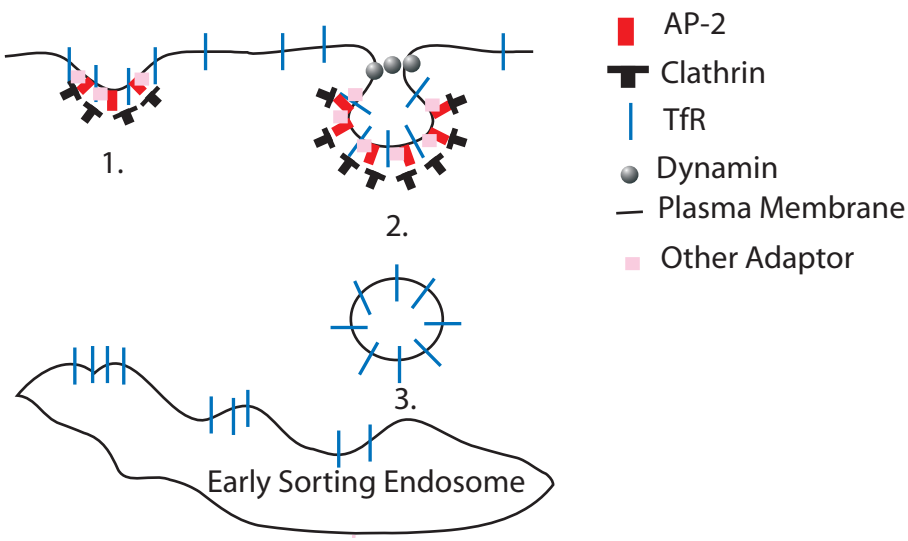

Figure 1: Clathrin-mediated receptor endocytosis of the TfR. 1. Receptors aggregate in clathrin coated pits by binding to specific adaptors that link clathrin to the receptor. 2. The clathrin coated pit invaginates and Dynamin promotes a scission event to cleave the membrane forming a nascent clathrin coated vesicle. 3. The clathrin-coat is shed and the vesicle merges with the early endosome.

coated pits (Lafer, 2002). For instance, Eps15 and Grb2 bind to, and regulate, EGF-R sorting into clathrin-coated pits (Benmerah et al., 1998; Torrisi et al., 1999; Yamazaki et al., 2002; Jiang and Sorkin, 2003; Huang and Sorkin, 2005). Once the receptor is sorted to the clathrin-coated pit, Endophilin associates with the clathrin-coated pit, bending the plasma membrane to facilitate invagination of the vesicle (Ringstad et al., 1999). Once the coated-pit invaginates, Amphiphysin binds to the neck of the clathrin-coated vesicle and recruits Dynamin. Dynamin then promotes a scission event that pinches off the vesicle (Warnock and Schmid, 1996). Dynamin mutants (Dynamin K44A) and the *shibire* mutation (Dynamin knockout) in *Drosophila* block both clathrin-dependent and caveolae-mediated forms of endocytosis, demonstrating that Dynamin is an essential molecule in multiple endocytic pathways (Chen et al., 1991; van der Bliek and Meyerowitz, 1991; van der Bliek et al., 1993; Damke et al., 1995; Warnock and Schmid, 1996; Sever et al., 2000). After scission, the clathrin-coated vesicle is uncoated by interactions with Auxillin and Hsc70 in

an ATP-dependent process (Chang et al., 2002). The uncoated vesicle is available to fuse with other endocytic vesicles, forming the EE (**Fig. 1)**.

Fusion and Sorting of Receptors within the Early Sorting Endosome

The fusion of clathrin-derived vesicles to form classical, early sorting endosomes (EE) is regulated by the small GTPase, Rab5 (Gorvel et al., 1991; Stenmark et al., 1994a; Barbieri et al., 1996). Rab5 functions by recruiting specific effectors that promote attachment and fusion of the vesicle with the EE membrane (Olkkonen and Stenmark, 1997; Somsel and Wandinger-Ness, 2000; Segev, 2001a). The EE is a key sorting organelle for internalized receptors in the endocytic pathway. Here receptors can be sorted for transport to three possible destinations: 1) the receptor could be directly recycled back to the cell surface; 2) the receptor could be sorted to the pericentriolar recycling endosome (RE) for indirect recycling to the cell surface, or 3) the receptor could be marked for lysosomal degradation in a process referred to as "receptor downregulation." Upon binding exogenous ligand, signaling receptors are usually down regulated and marked for degradation. Receptors marked for degradation are ubiquitinated by the E3 ubiquitin ligase Cbl which attaches a single ubiquitin molecule to lysine residues on the cytoplasmic tail of the receptor (Levkowitz et al., 1998; de Melker et al., 2004; Grovdal et al., 2004). Once the ubiquitinated receptors are recognized by the sorting machinery they are transported to the limiting membrane of the MVBs en route to the lysosomes. The MVBs are proposed to be broken down by lipases once they reach the lysosomes and the contents are degraded (Stahl and Barbieri, 2002).

Recently, Harald Stenmark's laboratory determined that Phosphotidyl-Inositol 3-kinase (PI3K) is required for generating distinct microdomains on EEs, that serve as docking or recruitment sites for proteins directly involved in receptor sorting for the degradation pathway (Petiot et al., 2003). PI3K phosphorylates phosphoinositides on the EE surface, facilitating the recruitment of hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) through its phosphoinositide binding FYVE domain (Raiborg et al., 2001c). In addition, Hrs is phosphorylated after ligand binding to receptor tyrosine kinases. Phospho-Hrs binds to multiple proteins on the EE surface including Sorting Nexin 1 (SNX1), Eps15, clathrin, the mammalian ESCRT complex as well as ubiquitinated proteins (Raiborg et al., 2001a; Raiborg et al., 2001b; Bache et al., 2002; Bache et al., 2003). Treatment of cells with the PI3K inhibitor wortmannin results in loss of Hrs and clathrin from EEs, as well as abnormal MVBs which are devoid of internal vesicular membranes leading to defective

EGF-R degradation (Fernandez-Borja et al., 1999; Raiborg et al., 2001b). Similarly, treatment of cells with Hrs siRNA causes a reduction in the degradation of the EGF-R (Bache et al., 2003). Furthermore, *Drosophila* Hrs knockouts have impaired membrane invagination in MVBs, also resulting in defective receptor degradation and enhanced receptor tyrosine kinase signaling (Lloyd et al., 2002; Jekely and Rorth, 2003). Recent work has also demonstrated that the Sorting Nexin 1 (SNX1) protein is involved in early endosomal sorting of receptors toward the degradative pathway (Zhong et al., 2002). SNX1 localizes to endosomal membranes through interactions with its PX domain with phosphoinositides, interactions with Hrs and interactions with the EGF-R directly (Chin et al., 2001; Zhong et al., 2002). Over expression of SNX1 increases the degradation of the EGF-R (Zhong et al., 2002). Based on this data, a model emerges in which internalized EGF-Rs are targeted for degradation by ubiquitination by the E3 ubiquitin ligase Cbl. The ubiquitinated receptor is recognized by Hrs, SNX1 and the mammalian ESCRT complex residing on PI3P enriched domains on EEs. We are just beginning to understand the complex sorting machinery in the EE that regulates down-regulation of receptors; however we still do not understand what regulates the level of receptor ubiquitination.

Receptor Recycling

Receptors that are not targeted for degradation are usually recycled back to the cell surface. Receptor recycling pathways play an important role in maintaining the proper composition of various organelles, and for returning receptors to the cell surface (Maxfield and McGraw, 2004). Furthermore, trafficking rates may be altered in response to cellular signaling responses to outside stimuli. A key example is an increase in Glucose transporter recycling to the cell surface in response to insulin, thus increasing the internalization of glucose (Aledo et al., 1995; Dransfeld et al., 2000). Another example is muscarinic acetylcholine receptor recycling. Upon agonist activation large amounts of the receptor are internalized into EEs. Continual agonist stimulation resulted in the cell surface receptor levels only reaching 65% maximum of the control after 45 min which is most likely a result of a receptor reserve contained in EEs. Most likely receptor recycling regulates the reinsertion of receptor into plasma membrane from this reserve. Fast receptor recycling enables the cell to bring the signaling response up to control levels within 14 min after continued agonist stimulation (Szekeres et al., 1998a; Szekeres et al., 1998b). The molecular mechanisms that regulate sorting of receptors into membrane domains for recycling from the EE or for transport to the RE remain unclear.

Two distinct recycling pathways exist in cells: Direct (fast) recycling from the EE and a slower indirect recycling route from the RE (Stein and Sussman, 1986; Schmid et al., 1988; Maxfield and McGraw, 2004). The EE and the RE are biochemically and mechanistically distinct compartments. EEs are identified by Rab4 and Rab5, while the RE is enriched in Rab11 (van der Sluijs et al., 1992; Bottger et al., 1996; Daro et al., 1996; Ullrich et al., 1996; Trischler et al., 1999). Consistent with their subcellular localization, Rab4 has been shown to function in regulating the direct recycling pathway from the EE, while Rab11 regulates trafficking from the RE (indirect pathway) (van der Sluijs et al., 1991; van der Sluijs et al., 1992; Bottger et al., 1996; Daro et al., 1996; Ullrich et al., 1996; Green et al., 1997). Furthermore, the cellular location of the RE is cell type specific. For example, in CHO cells most of the RE is organized around the microtubule organizing center while in HeLa cells the RE is distributed throughout the cytoplasm (Maxfield and McGraw, 2004).

The mechanisms that govern sorting of receptors in the RE versus the EE are relatively unclear. Cargo receptors such as the TfR and LDL-R are internalized and recycled constitutively, while signaling receptors are recycled under a regulated mechanism. Eighty percent of the TfR recycles using the direct pathway while only 20% of the receptor recycles indirectly through the RE (Sheff et al., 1999; Sheff et al., 2002). In CHO cells EE's recycle the TfR with a $T_{1/2}$ of 2 min, while the TfR recycles through the RE with a $T_{1/2}$ of 12 min, thus providing a mechanism for temporal regulation of receptor availability in response to cellular signals (Maxfield and McGraw, 2004). Furthermore, work by Ira Mellman's group has demonstrated that cells in which the RE is removed using surgical laser micro ablation are still able to recycle TfR from the EE. Eventually, the cell uses existing early endocytic membranes to form a new RE, demonstrating that the cell requires a RE for "optimal" receptor trafficking (Sheff et al., 2002).

Recent reports have characterized roles for CAML and Rab11 FIP2 as adaptors/ regulators of EGF-R sorting to the recycling pathway (Cullis et al., 2002; Tran et al., 2003). CAML is a regulatory protein involved in the direct recycling of EGF-R from the EE. It binds the non-ubiquitinated receptor kinase domain much in the same way as the mammalian ESCRT complex associates with the protein (Tran et al., 2003). CAML may serve as the opposite of the ESCRT-Hrs-Snx1 degradation complex, providing a targeting signal used by the recycling machinery to sort the receptor for recycling back to the plasma membrane (Tran et al., 2003). Recent studies have also implicated that Reps1 and the Rab11 effector

FIP2 as regulatory molecules in EGF-R recycling. Reps1 is a substrate for the EGF-R and also binds to Ral1bp, a binding protein for the Ral1 GTPase (Cullis et al., 2002). In these studies, Reps1 binds to FIP2 and together they bind and mediate sorting of the EGF-R to the RE and subsequent recycling to the plasma membrane (Cullis et al., 2002). These results demonstrate that the endosomal system contains both regulatory molecules that mediate sorting of receptors toward the degradative pathway, and regulatory molecules that regulate the sorting of receptors toward the recycling pathways.

Clathrin-Independent Pathways and Internalization

While clathrin-coated vesicle endocytosis is the most characterized pathway in terms of coated-vesicle internalization, we cannot discount the importance of studies on the clathrin-independent pathways. Several receptors and bacterial toxins are internalized via clathrin-independent mechanisms (Nichols and Lippincott-Schwartz, 2001). First, macropinocytosis is the uptake of nutrients and is synonymous with bulk, fluid phase internalization. Membrane ruffling leads to the formation of large vesicular structures 0.5 to 2 um in diameter called macropinosomes (Johannes and Lamaze, 2002). ADP-ribosylation factor 6 (Arf6), a small GTPase localized to the clathrin-independent pathway, has been implicated in macropinocytosis and phagocytosis because its activation leads to downstream Rac activation and membrane ruffling (Radhakrishna and Donaldson, 1996; Zhang et al., 1999; Donaldson, 2003). However, no known receptors have been shown to internalize via this pathway, rendering the molecular characterization of this pathway extremely difficult.

The second form of clathrin-independent endocytosis occurs through caveolae. Caveolae are flask like structures around 70 nm in diameter that were described structurally by George Palade in the 1950's. Despite their description prior to clathrin-coated vesicles, relatively little is known about receptor internalization via caveolae. Caveolae are coated with caveolins (caveolin-1 and caveolin-2), which are integral membrane proteins that bind to cholesterol (Nichols and Lippincott-Schwartz, 2001; Johannes and Lamaze, 2002). Cholesterol is an essential component of caveolae, and cholesterol-chelating drugs are potent inhibitors of caveolae-mediated endocytosis (Smart and Anderson, 2002). While caveolin-1 and 2 are accepted coat proteins for caveolae invaginations, their exact role in caveolae-mediated internalization is unknown. Caveolae are abundant in certain cell types, usually occurring in endothelial cells such as blood vessels and in smooth muscle (Couet et al., 2001). Knockout mice for caveolin-1 have a disrupted the cell structure and

are depleted of caveolae. Surprisingly, the lack of caveolae was not a lethal phenotype; however, the caveolin-1 null mice had limited pulmonary defects and decreased endurance during extreme physical task (Drab et al., 2001). These results indicate that caveolae are unique membrane domains that could function in the endocytosis of specialized receptors.

Recent work by the Donaldson group demonstrated that clathrin-independent and clathrin-dependent cargo are internalized via different endosomal populations (Naslavsky et al., 2003). The Interleukin-2 (TAC) and Major Histo-Compatibility I Complex (MHCI) receptors are internalized via non-clathrin entry (Radhakrishna and Donaldson, 1997). However, shortly after internalization these vesicles fuse into a non-clathrin derived endosome which then fuses with the classical clathrin-derived endosome (Naslavsky et al., 2003). Internalization of TAC and MHCI via this clathrin-independent pathway requires Arf6. Over expression of Arf6 Q67L (GTP-bound) causes the formation of a pool of PIP_2-positive vesicles containing MHCI and TAC. These vesicles are unable to fuse into a clathrin-independent endosome and hence are unavailable to fuse with clathrin-derived EEs. Furthermore, over expression of Arf6 T22N (GDP-bound) tubulates the MHCI-positive recycling compartment. Moreover recycling of the MHCI receptor to the plasma membrane is blocked in cells over expressing Arf6 T22N (Naslavsky et al., 2003; Naslavsky et al., 2004b). These results demonstrate that the endocytic system is composed of multiple pathways that are dynamic and intersect at distinct points and thus comprise a web of complex, intersecting compartments.

Endocytic trafficking And disease

Endocytosis is a necessary mechanism for the transport of ions and molecules into the cell. For instance the key metabolic effect of insulin is to increase glucose internalization into fat and muscle cells. The two main glucose transporter proteins in mammalian cells are GLUT1 and GLUT4. GLUT1 is ubiquitously localized to the plasma membrane while GLUT4 has been shown to be transported to GLUT4-containing vesicles and ultimately to the cell surface upon insulin stimulation (Uphues et al., 1994; Aledo et al., 1995). In these studies, over expression of Rab4, a GTPase that regulates direct recycling from the EE, inhibited glucose transport and increased the amount of GLUT4 on the cell surface. Furthermore, Rab4 colocalized with GLUT4 containing vesicles, and insulin stimulation increases the guanine nucleotide exchange of Rab4 resulting in a net increase in activated GTP-bound Rab4 (Aledo et al., 1995; Mora et al., 1997). Another example of a receptor

required for nutrient transport is the TfR. The TfR is necessary for the internalization of iron into cells. More than half a billion individuals have iron-related diseases that result in iron deficiency anemia or hemochromatosis (iron over-load) which may be in part due to defective internalization of the TfR (Li and Qian, 2002). Furthermore, recent advances have demonstrated that the TfR can be used to couple chemotherapy drugs as well as for potential gene therapy delivery mechanisms, making understanding of the regulatory mechanisms for TfR endocytosis and recycling extremely valuable (Li and Qian, 2002).

Recently as will be discussed below, several vesicle trafficking proteins have been directly linked to disease and pathogenesis. First, several microorganisms (bacteria and viruses) internalize via existing host cell internalization pathways. The internalization pathways provide a quick route to structures nearby the nucleus and provide an environment that favors virus maturation. Second, several groups have detailed findings that certain vesicle trafficking proteins are mutated or are up-regulated in a variety of cancers. Third, genetic diseases such as X-linked mental retardation and choroideremia have been linked to mutations in key regulatory molecules involved in endocytosis. Finally, endocytic trafficking has been linked to regulating AMPA receptor stores necessary for long term potentiation and ultimately learning and memory. This section will provide a detailed review of how endocytic trafficking plays a role in pathogen invasion, cancer, genetic disease and neurological disease. Understanding the molecular mechanisms of endocytic trafficking will play a role in identifying future disease processes regulated by endocytosis, as well as define future treatments to known diseases.

Pathogen Invasion

Several pathogens have evolved mechanisms to "hijack" cell surface receptors to use for internalization. *Listeria Monocytogenes* infected individuals are prone to fever, muscle aches, gastrointestinal symptoms, meningitis or encephalitis. *Listeria* uses the cells own regulatory systems to internalize into the endocytic compartment. Internalin A (InlA) and Internalin B (InlB) proteins on its cell surface mimic ligands for the E-Cadherin and cMet receptor, respectively (Alvarez-Dominguez et al., 1996; Alvarez-Dominguez and Stahl, 1999; Lecuit et al., 1999; Braun and Cossart, 2000; Bierne and Cossart, 2002). Internalin B protein binding to the cMet receptor results in increased receptor signaling and internalization, thus increasing the internalization of the bacterium (Bierne and Cossart, 2002). Conversely, *Salmonella* inject the host cell with effectors that induce a host response that stimulates macropinocytosis. The bacteria become trapped in a membrane

bound compartment called the Salmonella containing vacuole (Meresse et al., 1999). RILP is a novel Rab7 effector that regulates lysosomal transport from the LE by linking vesicles to the actin cytoskeleton (Jordens et al., 2001). Salmonella effector proteins bind to and impair RILP recruitment to Rab7, a GTPase that regulates trafficking between the EE and LE. This impairment of late endosome trafficking allows the *Salmonella* containing vacuole to avoid the degradative pathway (Meresse et al., 1999; Mukherjee et al., 2002; Harrison et al., 2004). Several bacterial toxins also enter cells using clathrin-independent endocytic pathways. These include cholera toxin, shiga toxin, diphtheria toxin and vero toxin (Nichols and Lippincott-Schwartz, 2001). However recently anthrax toxin was also found to trigger endocytosis of the type 1 membrane protein (anthrax toxin receptor) using a clathrin-dependent process (Abrami et al., 2003).

Virus particles also use endocytic trafficking mechanisms to gain entry into cells and the nucleus, allowing them to mature and replicate. For example, parvovirus, a virus that infects animals of the canine and feline family, has evolutionarily developed to mimic transferrin and thereby use the transferrin receptor to internalize to cells (Hueffer and Parrish, 2001). Furthermore, the potency of infection of adenovirus is lower in cells expressing the dominant negative Dynamin K44A (Duan et al., 1999) or expressing the dominant negative Rab5 S25N (GDP-Rab5) (Rauma et al. 1999). These studies demonstrate that adenovirus internalizes using the clathrin-dependent pathway and this internalization to the EE is required for infectivity and possibly maturation of the virus. Interestingly influenza virus infectivity is lowered by over expression of GDP-bound mutants of Rab5 (S25N) and Rab7 (T22N) which are markers for the EE and LE respectively (Sieczkarski et al. 2003). Pertubation of trafficking between these compartments inhibits virus maturation by not allowing the enveloped virus to reach the acidic environment of the EE and LE and therefore inhibits viral structural changes required for the virus coat formation (Sieczkarski et al. 2003). These examples illustrate how bacterial pathogens are able to invade their respective host cell by hijacking known receptors and regulating components of the endocytic host cell machinery.

Cancer

Current studies have described cellular signaling and endocytic trafficking as a "marriage of convenience" (Polo et al., 2004). Faulty regulatory mechanisms have been described for over 30 different signaling receptors in human cancers (Bache et al., 2004). Signaling complexes that form on the EE allow both a temporal and spatial regulation of

cellular signaling. Endosomes most likely serve as scaffolding for the assembly of receptor tyrosine kinase adaptor complexes on the EE allowing propagation of mitogenic signals. Recent data have demonstrated that several EGF-R adaptor molecules are localized with the EGF-R on the EE (Polo et al., 2004). Furthermore, EGF-R is continually phosphorylated on the EE and downstream signaling molecules are continually active in response to signaling from the EE, even when signaling is inhibited from receptor at the plasma membrane (Di Fiore and Gill, 1999; Carpenter, 2000).

Monoubiquitination of endocytic proteins and cell surface receptors is a key signal for the receptor to be internalized and degraded. The E3-ubiquitin ligase Cbl is a key regulatory enzyme that ubiquitinates signaling receptors, which targets them to the degradative pathway. Cbl itself could be affected in a variety of cancers (Polo et al., 2004). Studies have detailed how Cbl mutations affect the degradation of receptor tyrosine kinases, and therefore turn the cell into a pro-oncogenic form (Polo et al., 2004). Src, a non-receptor tyrosine kinase, is phosphorylated in response to EGF and contributes to the tyrosine phosphorylation of Cbl which increases degradation of Cbl and subsequently up-regulates EGF-R. (Bao et al., 2003; Polo et al., 2004). An alternative pathway includes the GTP-bound CDC42, which binds to β-Pix and Cbl, thus sequestering Cbl from functioning as an E3-ubiquitin ligase for the EGF-R and thus decreases degradation of the EGF-R (Flanders et al., 2003; Wu et al., 2003; Polo et al., 2004).

Direct evidence that impaired trafficking is involved in cancer is that several endocytic proteins are up-regulated or found to be genetically altered in tumor cells. Rabex-5, a known guanine nucleotide exchange factor for Rab5, is up-regulated in colon carcinomas in a number of patients (Nimmrich et al., 2000). Similarly, Mss4, a known guanine nucleotide chaperone for multiple Rabs is up-regulated in pancreatic cancer (Muller-Pillasch et al., 1997). Rabaptin-5, a binding partner for Rab5 and Rabex-5 is found as a fusion protein with the PDGF receptor in chronic myelogenous leukemia (Magnusson et al., 2001). Several other leukemias are affected by clathrin adaptor molecules in genetic fusions with other proteins. For instance Eps15 is in fusion with ALLI/HRX protein as a result of a chromosomal translocation in myeloid leukemias (Rogaia et al., 1997). Moreover, Huntington interacting protein 1 is expressed as a genetic fusion with the PDGF receptor in chronic myelomonocytic leukemia (Ross and Gilliland, 1999). Recently, Rab11a was found to be up-regulated in squamous cell carcinoma of the esophagus (Ray et al., 1997; Goldenring et al., 1999). Up-regulation of Rab11a is thought to play a role in integrin

remodeling, thus affecting cell motility and metastasis (Powelka et al., 2004).

Trafficking and Genetic Disease

Choroideremia

Choroideremia is a form of X-linked recessive retinal degeneration characterized by the progressive degeneration of choroids, retinal pigment epithelium, and retina resulting in noticeable vision loss at the time of adolescence and complete vision loss by 30 to 40 years of age (van den Hurk et al., 1997a; van den Hurk et al., 1997b; Anand et al., 2003). Patients suffering with Choroideremia could have a number of chromosomal translocation, deletions and/or point mutations that could affect the transcription of the Choroideremia gene product. Accordingly, this gene corresponds to the REP1, a protein responsible for the prenylation and localization of nascent rabs to their respective target membranes (Alory and Balch, 2001). Seabra and coworkers identified that Rab27a is involved in Choroideremia and that faulty prenylation of Rab27a might be the cause of the disease (Ramalho et al., 1999; Seabra et al., 2002). Recent structural studies showed that in the absence of REP1, REP2 can bind to Rab27a and facilitate prenylation of the protein. However, REP2 has a higher affinity to Rab7 (and other rabs) than Rab27a. Therefore, defective Rab27a prenylation and defective delivery of Rab27a to donor membranes is a result of competition of Rab27a with other Rab GTPases for REP2. The end result is a pool of unprenylated Rab27a that cannot be delivered to its donor membrane making the protein essentially useless (Rak et al., 2004). Mutations in REP1 protein are also responsible for genetic deafness type 3. Recently, REP1 zebrafish knockouts have developmental defects in hair cells thus conferring hearing loss (Starr et al., 2004).

Familial Hypercholestoremia

The LDL-R binds to low density lipoprotein in the extra cellular space and internalizes the lipid into the cell to be used for cellular architecture (Kurten, 2003). The LDL-R is constitutively endocytosed and recycled back to the plasma membrane for multiple rounds of use. Initially defective internalization of LDL-R was observed in fibroblasts isolated from a patient with familial hypercholesterolemia, a condition characterized by extremely high cholesterol counts and secondary heart disease. This patient had a fifty amino acid deletion in the cytoplasmic domain of the LDL-R (Lehrman et al., 1985). Subsequent genetic analysis determined that amino acid 807 resulted in decreased internalization of the receptor. Amino acid 807 is a key residue in defining the first clathrin adaptor binding

domain (Davis et al., 1986). The receptor is unable to be detected by the sorting machinery in clathrin-coated pits and therefore is unable to be internalized, thus demonstrating the importance of the clathrin coated pit sorting mechanism.

Griscelli Syndrome

Griscelli syndrome is a rare autosomal recessive disorder characterized by pigment dilution of the skin and hair because of impaired trafficking in melanocytes (Sheela et al., 2004). Furthermore, patients have cellular immunodeficiency and acute T-lymphocyte and macrophage activation leading to fatal hemophagocytic syndrome. Currently 60 cases have been reported in the world (Sheela et al., 2004). Several mouse models have been used to study Griscelli syndrome including *dilute, ashen* and *leaden* mice. These mice all have an identical phenotype in which they have normal pigment synthesis but the pigment transport is abnormal. *Dilute* mice have been found to have a genetic mutation in the gene that encodes a defective MyosinVa protein while *ashen* mice have a defect in the gene that encodes Rab27a (Seabra et al., 2002). There are two types of Griscelli syndrome (I and II) characterized by distinct symptoms due to mutations in Rab27a or Myosin5b respectively. The majority of Griscelli syndrome patients have a mutation in Rab27a and are characterized by diluted pigments in the hair and reduced T-cell cytotoxicity (Menasche et al., 2000; Wilson et al., 2000; Haddad et al., 2001). Patients with Griscelli syndrome type 2 have a defect in the MyosinVa gene. Type 2 patients have primary neurological defects stemming from leukocyte infiltration into the brain but have normal immune function (Anikster et al., 2002; Hume et al., 2002; Seabra et al., 2002; Menasche et al., 2003). Melanosome transport occurs from the tip of the melanocyte "dendrite" to the keratinocyte for normal skin and hair pigmentation to occur. Genetic studies have linked both Rab27a and MyosinVa to this disease. Rab27a binds to MyosinVa, thus linking melanosome transport to the microtubule motor machinery. These data suggests that Rab27a recruits the microtubule motor MyosinVa to melanosomes, linking the transport machinery directly to the organelle.

Endocytosis and Neuronal Function

Charcot-Marie-Tooth Disease

Charcot-Marie-Tooth Disease is characterized by impaired detection of pain stimuli due to demyelination of axons in the nervous system (Stein et al., 2003). This is a sensory motor-neuron impairment, distal muscle weakness and atrophy followed

by severe ulcerations requiring the amputation of limbs (Stein et al., 2003). Genetic analysis demonstrated a missense mutation in exons 3 and 4 of the gene encoding Rab7. This mutation disrupts the GTP/GDP bound state of the protein and further disrupts late endosomal trafficking and degradation of receptors – resulting in an impaired detection of pain stimuli (Verhoeven et al., 2003).

X-Linked Mental Retardation

Mental retardation is a chronic condition affecting about 3% of the general population. In some cases, mental retardation is part of a complex syndrome such as Downs syndrome, however, in many cases mental retardation is the only symptom, resulting in non-specific mental retardation. Mutations were found in patients with X-linked non-specific mental retardation from two families at locus Xq28 which encodes Rab GDIα, a GDI expressed specifically in brain (D'Adamo et al., 1998; D'Adamo et al., 2002; D'Adamo et al., 2004)). Rab GDI is a chaperone molecule that recognizes GDP-bound forms of "spent" rabs on the target membrane, extracts them from the membrane, and through interactions with PRA1, returns the rab to the donor membrane (Seabra and Wasmeier, 2004). Further examination of the protein demonstrated that a missense mutation altered the ability for Rab3a to bind this protein. Developmental studies determined that Rab3a and Rab GDIα are highly expressed during brain development, and mutations in GDIα cause abnormal brain development because of not being able to interact with Rab3a and regulate neurotransmitter release (D'Adamo et al., 2002; D'Adamo et al., 2004). Furthermore, loss of Rab GDIα in mice leads to changes in behavior and memory similar to mental retardation (D'Adamo et al., 2002). These data suggest that mutations in Rab GDIα lead to non-specific mental retardation.

Parkinson's Disease

Disturbances in dopaminergic signaling play a role in numerous neuropsychiatric disorders, including Parkinson's disease. Dopamine or synthetic agonist binding induces rapid receptor endocytosis via clathrin coated vesicles (Ariano et al., 1997; Dumartin et al., 1998; Vickery and Von Zastrow, 1999). Receptors have been shown to recycle to the plasma membrane and truncation of the C-terminal cytoplasmic domain inhibits recycling of the receptor (Vargas and Von Zastrow, 2004). Furthermore fusion of the cytoplasmic tail of the D1 dopaminergic receptor and the delta opioid receptor, a receptor that does not recycle, resulted in recycling of the delta opioid receptor indicating that the carboxyl

terminal cytoplasmic domain is important in recycling of the dopamine receptor to the cell surface (Vargas and Von Zastrow, 2004).

Role of Recycling in Long Term Potentiation and Synaptic Plasticity

AMPA receptors localize to postsynaptic membranes in the CA3-CA1 synapses and control excitatory synaptic transmission in the mammalian brain. AMPA receptor localization to these synaptic membranes leads to long term potentiation (LTP) whereas removal leads to long term depression (Lin et al., 2000; Lee et al., 2001; Sheng and Lee, 2001). The AMPA receptor is a heteromeric complex composed of GluR subunits. GluR1 – GluR3 subunits are expressed in the normal adult brain while GluR4 is only expressed during development (Sheng and Lee, 2001). GluR1 drives AMPA receptors to cell surface in response to activation of NMDA receptors and CAMKII while GluR2 is delivered constantly to synapses with no difference in synaptic strength. In the hippocampus, GluR1/R2 is delivered to synapses during activation dependent synaptic potentiation thus activating LTP while GluR2/R3 is recycled continuously (Lee et al., 2004).

After endocytosis, AMPA receptors undergo endosomal sorting and may be sorted to lysosomes for degradation or recycled back to the plasma membrane (Ehlers 2000; Lin 2000; Gruenberg 2001). Recently, Sheng and colleagues determined that subunit composition regulates the internalization and recycling of the AMPA receptor. In particular the GluR2 subunit regulates the distribution of the AMPA receptor to internal membranes. Furthermore, the GluR2 recycles back to the cell surface more than the GluR1 and GluR3 subunits (Lee et al., 2004). These results indicate that the GluR2 subunit may be involved in long term depression because of its dominant localization to the plasma membrane. Ehlers and colleagues recently detailed how alterations in AMPA receptor recycling from the RE control LTP (Park et al., 2004). They determined that AMPA receptors are stored intracellularly in the RE and are reinserted into the plasma membrane surface upon NMDA receptor stimulation. Furthermore, inhibition of recycling from the RE results in decreased LTP in hippocampal brain slices indicating that receptor recycling is important for modulating responses that affect learning and memory (Park et al., 2004).

Rab GTPases - Regulators of Membrane Trafficking Events

The previous sections have given a brief overview of clathrin-dependent endocytosis of cell surface receptors. Upon internalization, endocytic vesicles containing these receptors fuse with the EE and are sorted toward the degradative pathway or recycled back to the

cell surface. Diseases such as cancer, choroideremia and X-linked mental retardation can result from defects in endocytic regulatory molecules. The next section will detail the major group of endocytic regulatory proteins, the rab GTPases. I will provide a general overview of general regulatory mechanisms for these GTPases as well as an overview of each GTPase's specific downstream effectors. Moreover, this section will lead into the next section which describes the function of Rab15 and the overall goal of this dissertation.

Rab GTPases – General Information

The Ras superfamily of small (21-25 kDa) Ras related GTPases consist of three major subgroups: Ras, Rho, and Rab proteins. These proteins share around 30% sequence identity between subgroups with the highest amount of sequence homology in areas necessary for guanine nucleotide binding and hydrolysis (Olkkonen and Stenmark, 1997; Somsel and Wandinger-Ness, 2000). The human Rab protein subfamily consist of 63 members, some of which are cell type specific and responsible for regulating specialized vesicle trafficking events in these cell (Bock et al., 2001).

The rab protein domain structure consists of three guanine nucleotide binding motifs, a specific region termed the effector domain and two domains called the switch 1 and switch 2 regions (Olkkonen and Stenmark, 1997) (**Fig. 2A)**. The switch 1 and 2 domains undergo drastic changes in conformation, depending on the guanine nucleotide state of the protein (Stroupe and Brunger, 2000). The effector domain, which is highly conserved among rabs, consists of a TIG(I/V/A)(D/E)F(K/G/L) motif that physically interacts with GTPase regulatory molecules such as GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) (Becker et al., 1991). Furthermore, rabs contain a carboxyl terminal, hypervariable domain; a carboxyl terminal, prenylation motif (CXC, CWC for Rab15) for linkage to a membrane bound compartment; and a carboxyl methylation site (**Fig. 2A**) (Khosravi-Far et al., 1992; Peter et al., 1992; Olkkonen and Stenmark, 1997).

Rabs associate with specific membrane bound organelles, thus conferring a unique spatial level of specificity to membrane trafficking steps (Somsel and Wandinger-Ness, 2000; Takai et al., 2001). For instance, rabs 1, 2 and 3 associate with the secretory pathway and regulate secretion while rabs 4, 5, 7, 11 and 15 associate with the endosomal membrane system and regulate endocytic trafficking (Olkkonen and Stenmark, 1997; Somsel and Wandinger-Ness, 2000). Recent work by Zerial and colleagues has shown that the endocytic rabs 4, 5 and 11 localize to distinct microdomains on the EE, thus sub-dividing the EE into

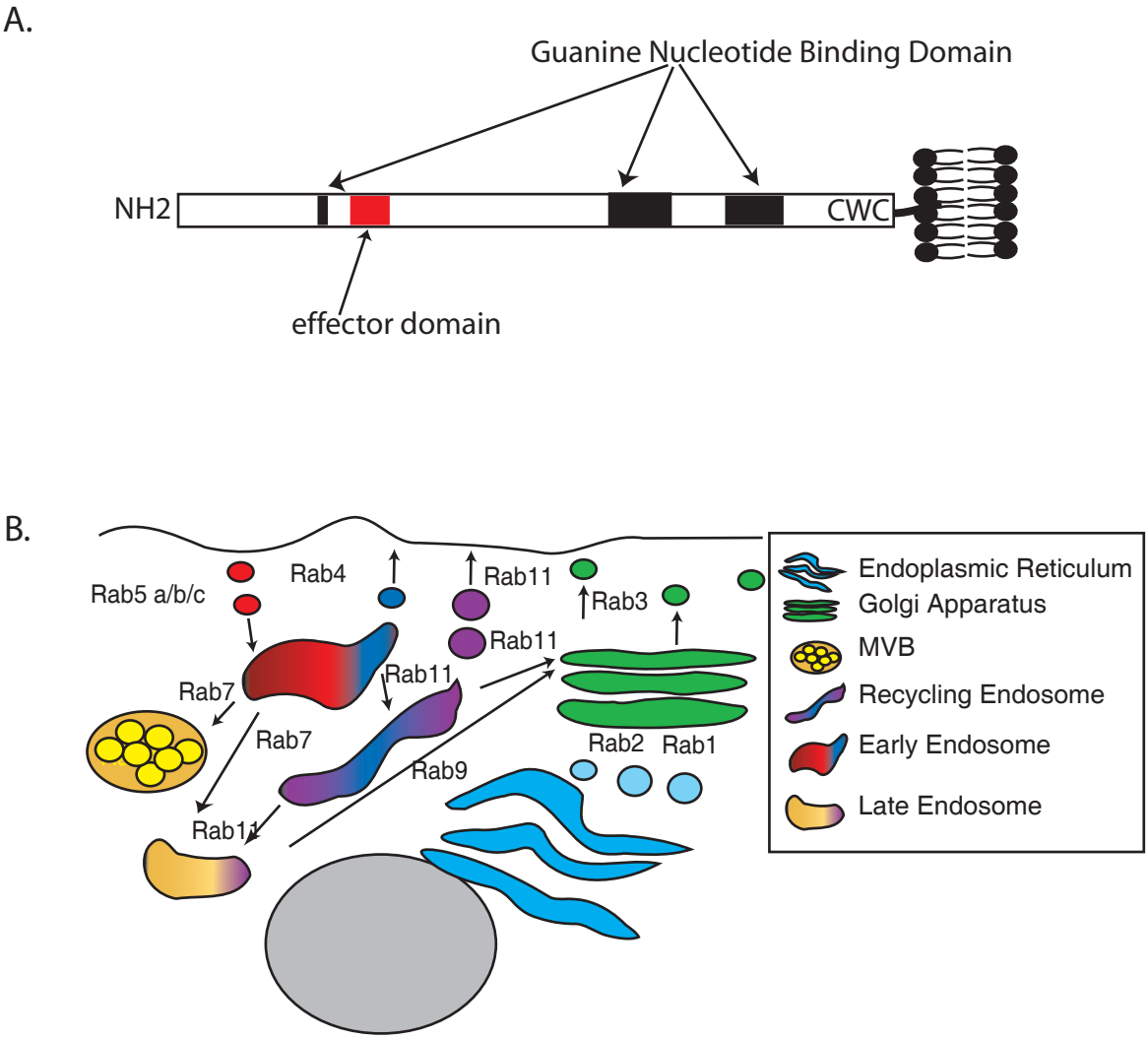

Figure 2: A, Rab protein domain structure and localization. Rabs contain an effector domain, 3 guanine nucleotide binding pockets and a carboxyl terminal prenylation motif. B, Subcellular localization of several Rab GTPases.

specific functional microdomains, competent for internalization as well as recycling from the EE (Sonnichsen et al., 2000; Zerial and McBride, 2001). Moreover, Rab9 stabilizes a domain enriched in mannose-6 phosphate receptor and TIP47 (a Rab9 binding protein that may function as a GEF) on the LE thus providing a distinct microdomain for the trafficking of the mannose-6-phosphate receptor (Pfeffer, 2001; Ganley et al., 2004) (**Fig. 2B**).

Rab proteins cycle between an active GTP-bound and an inactive GDP-bound conformation. Until recently, the central dogma of rab function was that the GTP/GDP cycle is the key mechanism for regulating membrane trafficking events. However, because Rabs are prenylated and linked to membranes, recent theories have indicated that the "membrane on/membrane off" cycle is also important for the regulatory control of Rab GTPases (Dirac-Svejstrup et al., 1997; Seabra and Wasmeier, 2004). For example a non-prenylated rab GTPase is non-functional (Seabra, 1998). Thus, the GTPase cycle coupled with the "membrane on/membrane off" cycle allows both spatial and temporal control of rab activity (Seabra and Wasmeier, 2004).

Rab binding proteins can be divided into two distinct subclasses. The first class consists of Rab regulatory factors which includes Rab Escort Protein (REP), Rab Guanine Dissociation Inhibitor (Rab GDI) and rab GTPase Activating Protein (GAPs). The second class of binding proteins consists of "Effector Proteins" which usually bind to the GTP-bound rab. Rab Effectors may bind to and regulate vesicle fusion, mediate interactions with the cytoskeleton and may function to regulate receptor sorting (Olkkonen and Stenmark, 1997; Somsel and Wandinger-Ness, 2000; Segev, 2001a; Segev, 2001b; Stein et al., 2003). Initially, I will describe in detail how rab regulatory factors regulate the GTP/GDP state and the membrane association of rab proteins in general. I will then specifically describe functions for the endosomal rab GTPases and their putative downstream effectors

The Rab GTPase Cycle

Newly synthesized rabs bind to Rab Escort Protein (either REP1 or REP2) which present the rab to geranyl-geranyl transferase, that in turn adds two geranyl groups to the carboxyl terminus of the target rab (Seabra et al., 1992; Alexandrov et al., 1994). REP1/2 then delivers the prenylated rab to the specific donor membrane where it associates with cytosolic GTP (Alexandrov et al., 1994). Upon movement from the donor membrane to the acceptor membrane, the active GTP-bound rab interacts with a variety of downstream effectors to regulate a specific membrane trafficking step (Somsel and Wandinger-Ness, 2000; Segev, 2001a) **(Fig. 3)**. After the trafficking step is completed, GTP is hydrolyzed

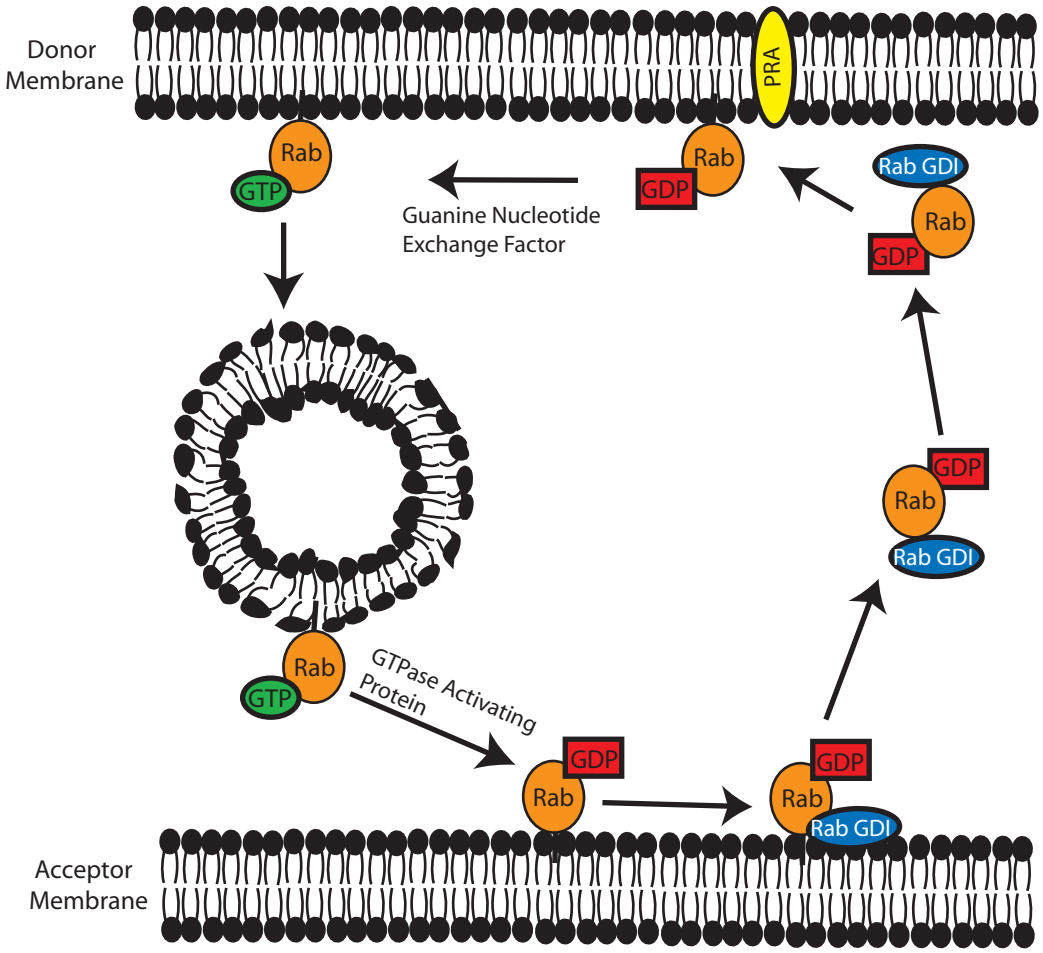

Figure 3: The Rab GTPase Cycle. Rab-GTP is localized to an early endocytic vesicle budding from the donor membrane. Upon fusion with the Acceptor membrane Rab-GTP interacts with a GTPase activating protein that promotes GTP hydrolysis. Rab-GDP is extracted from the Acceptor membrane by Rab-GDI and escorted back to the Donor Membrane and presented to a "Prenylated Rab Acceptor" (PRA). PRA shuffles the hydrophobic prenyl group from Rab GDI to the lipid bilayer of the donor membrane. Rab-GDP interacts with a Guanine nucleotide exchange factor which removes the GDP and stabilizes the nucleotide free conformation for GTP to reassociate.

to GDP by interactions with a specific GTPase Activating protein (GAP). Over expression or up-regulation of a GAP such as RN-Tre for Rab5, results in a net cellular increase in inactive GDP-bound Rab5 (Lanzetti et al., 2000; Somsel and Wandinger-Ness, 2000; Segev, 2001a). The GDP-bound rab is removed from the acceptor membrane by cytosolic Rab GDI and is returned to the initial donor membrane (Soldati et al., 1993; Takai et al., 1993; Dirac-Svejstrup et al., 1994). Upon return to the donor membrane, the Rab GDI/Rab-GDP complex interacts with a "prenylated rab acceptor" protein which extracts the hydrophobic prenyl group from Rab GDI and shuffles the rab back into the lipid bilayer (Dirac-Svejstrup et al., 1997; Hutt et al., 2000; Sivars et al., 2003; Pfeffer and Aivazian, 2004). Upon return GDP-bound rabs bind to a guanine nucleotide exchange factor (GEF), which binds to the rab and catalyzes the release of GDP, allowing free cytosolic GTP to bind and reactivate the rab (Horiuchi et al., 1997; Tall et al., 2001).

The mechanism of how a rab localizes to a distinct membrane-bound organelle is not well understood. Earlier work in Harald Stenmark's laboratory demonstrated that replacement of the Rab5 hypervariable domain with the corresponding domain of Rab6 (a rab that localizes to the cis-Golgi), results in relocalization of Rab5 to the Golgi apparatus and relocalization of Rab6 to the early endosomal network (Stenmark et al., 1994a). However, Miguel Seabra's group recently demonstrated that the once thought hypervariable domain is not responsible for the localization of the rab, but rather the rab "family specific" and Rab "sub-family specific" sequences are responsible for the proper localization of Rabs to their target membrane (Ali et al., 2004). It is currently unclear why differences in these studies exist. One proposal is that Stenmark's use of rabs that are "endocytic in nature" may have influenced the localization of the Rab5/Rab6 chimeras (Ali et al., 2004). Further studies will have to be performed to determine the exact mechanism of Rab localization.

Endosomal Rab GTPases

Six main GTPases to date have shown that localize to and regulate trafficking steps in the endocytic pathway. Rabs 4 and 5 localize to the EE and regulate receptor recycling and homotypic endosome fusion respectively (Gorvel et al., 1991; van der Sluijs et al., 1991; Bucci et al., 1992; van der Sluijs et al., 1992; Stenmark et al., 1994a; Barbieri et al., 1996). Rab11 is enriched on the RE and regulates the recycling of receptors from the RE to the plasma membrane (Ullrich et al., 1996; Green et al., 1997). Rabs 7 and 9 are localized to the LE and regulate trafficking to and from the LE (Soldati et al., 1995). Rab15 localizes to EEs and the RE and differentially regulates TfR internalization and

recycling through these compartments (Zuk and Elferink, 1999; Zuk and Elferink, 2000). This section will summarize the functional roles for each of these GTPases as well as their regulatory proteins and downstream effector molecules (**Table 1**).

Rab5- Regulator of Endosome Fusion

Rab5 is perhaps the most characterized GTPase in the early endocytic pathway. Identified in 1990, Zerial and colleagues demonstrated using immuno-electron microscopy that Rab5 localized to small discrete vesicles around 100 – 200 nm in size, surrounding the nucleus. Further studies showed that Rab5 functions to regulate trafficking from the plasma membrane to the EE (Ullrich et al., 1994; Stenmark et al., 1994a; Rybin et al., 1996). Over expression of a guanine nucleotide binding deficient mutant (Rab5 N133I) or the GDP-bound mutant (S34N) resulted in a decrease in endocytosis of the TfR and the appearance of small disrupted endosomes. Conversely, over expression of GTP-bound Rab5 Q79L resulted in accelerated internalization of the TfR as well as increased endosome-endosome fusion resulting in enlarged donut shaped endosomes (Bucci et al., 1992; Barbieri et al., 1994; Stenmark et al., 1994a; Stenmark et al., 1994b; Stenmark et al., 1995; Barbieri et al., 1996; Barbieri et al., 1998). Furthermore, Stahl and colleagues demonstrated that immuno-depleting cytosol of Rab5 inhibited endosome-endosome fusion demonstrating a requirement for Rab5 in the homotypic fusion of endosomes (Barbieri et al., 1996).

Rab5 interacts with a variety of downstream effectors – many of which control endosome fusion. For instance Rab5ip is a transmembrane domain protein that interacts with GDP-bound Rab5. Depletion of Rab5ip using antibody blocking results in decreased homotypic endosome fusion (Hoffenberg et al., 2000). Rab5 also interacts with several proteins that contain a phosphoinositide binding FYVE domain. The FYVE domain is perhaps the most common domain found in EE effector proteins (Gaullier et al., 1998). Rab5 mediates the recruitment of PI3K to the endosomal membrane which catalyzes the local production of PI3P. FYVE domain proteins localize to phosphinositides thus forming stable protein domains for membrane trafficking events (Gaullier et al., 1998; Gaullier et al., 1999). An endosomal FYVE domain protein, Early Endosomal Antigen 1 (EEA1) is a 180 kDa protein that dimerizes and can bind to GTP-bound Rab5 at both the amino and carboxyl terminus (Simonsen et al., 1998; Callaghan et al., 1999; Christoforidis and Zerial, 2001; Lawe et al., 2002). Over expression of EEA1 increases endosome fusion in cells (Simonsen et al., 1998; Gaullier et al., 1999; Gaullier et al., 2000). EEA1 is considered a “linking” protein that couples Rab5 on the vesicle membrane to the endosomal fusion

Table 1: Endosomal Rab Effectors

Rab	Rab effector	Function	References
Rab4-GTP	Rabip4	TfR recycling	(Mari et al., 2001; Cormont et al., 2001a; Cormont et al., 2001b)
	Rabaptin-5	Endocytosis and Recycling from the EE	(Vitale et al., 1998; Pagano et al., 2004)
	Rabaptin-4	Recycling from the EE	(van der Sluijs et al., 2001; Deneka et al., 2003)
	Rabenosyn-5	TfR recycling	(De Renzis et al., 2002; Naslavsky et al., 2004a)
Rab5-GTP	EEA1	Docking and Fusion	(Mu et al., 1995; Simonsen et al., 1998)
	Rabaptin-5	Binds Rabex-5 (GAP) and Tuberin (GAP). Proposed "Velcro"molecule that also binds to clathrin adaptors	(Stenmark et al., 1995; Rybin et al., 1996)
	Rabenosyn-5	Docking and Fusion	(Stenmark et al., 1995)
	Rab5ip	Endosome fusion	(Hoffenberg et al., 2000)
	Appl1/2	Nuclear transcription	(Miaczynska et al., 2004)
	SARA	TGFβ-R Endocytosis	(Hu et al., 2002; Hayes et al., 2002)
Rab5-GDP	Rin1	GEF	(Tall et al., 2001)
Rab7-GTP	RILP	Binds dynein and links regulates trafficking to the Lysosome	(Jordens et al., 2001)
Rab9-GTP	Tip47	Binds to M6PR and Rab9 linking cargo to rab	(Krise et al., 2000; Carroll et al., 2001; Hanna et al., 2002)
Rab11-GTP	RCP	RE recycling and sorting	(Lindsay et al., 2002; Peden et al., 2004)
	FIP2	RE recycling, EGF-R sorting	(Lindsay and McCaffrey, 2002; Cullis et al., 2002)
	FIP3	cleavage furrow formation	(Horgan et al., 2004)
	FIP4	Cleavage furrow formation	(Hickson et al., 2003)
	Rab11BP	TfR recycling	(Mammoto et al., 1999)

machinery (Callaghan et al., 1999; Simonsen et al., 1999; McBride et al., 1999; Lawe et al., 2000; Lawe et al., 2002; Merithew et al., 2003). Another FYVE domain protein Rabenosyn-5, binds to Rab4 and Rab5. Consistent with these binding properties, Rabenosyn-5 regulates both endosome fusion and the endocytic recycling of proteins, and could function to link these processes in EEs (Nielsen et al., 2000; De Renzis et al., 2002; Naslavsky et al., 2004a). Depletion of Rabenosyn-5 using siRNA delayed the recycling of TfR to the plasma membrane, indicating that Rabenosyn-5 may also mediate a sorting event between the EE and the RE, possibly targeting receptors to the indirect recycling route versus the direct route (Naslavsky et al., 2004a). SARA (Smad anchor for receptor activation), is another FYVE domain protein that binds to Rab5. Over expression of SARA did not alter TfR internalization, but did decrease TfR recycling from the EE. Furthermore, over expression results in enlarged endosomes is reminiscent of GTP-bound Rab5 over expression (Hu et al., 2002). SARA has been directly linked to the endosomal localization of Smad proteins in response to TGFβ-R activation, thus functioning directly in TGFβ-mediated signaling events (Hayes et al., 2002).

Rab5 also interacts with a variety of regulatory proteins including Rin1 as well as the Rabaptin-5/Rabex-5 complex (Horiuchi et al., 1997; Tall et al., 2001). Cells over expressing Rabaptin-5 also have enlarged endosomes (Stenmark et al., 1995). Rabaptin-5 is considered a "velcro" molecule, localizing to both the EE membrane and the endocytic vesicle membranes. By coupling these membranes, Rabaptin-5 makes its binding partner Rabex-5 available to Rab5, which in turn maintains Rab5 in an active GTP-bound state (Horiuchi et al., 1997; Xiao et al., 1997). Recent studies have also identified that Rabaptin-5 binds to the GAP Tuberin. Rabaptin-5 binding to either the GAP Tuberin or the GEF Rabex-5 and may serve to regulate a balance between the GDP-bound and GTP-bound state of Rab5 (Xiao et al., 1997). Recent structural studies using X-ray crystallography data of the Rabaptin-5:Rab5 complex identified residues in Rab5 necessary for the Rabaptin-5/Rab5 interaction. Over expression of Rab5 mutants unable to bind to Rabaptin-5 decreases endosome fusion in cells, thus demonstrating the importance of these protein-protein interactions (Zhu et al., 2004). Rabaptin-5 has also been shown to bind to γ-adaptin, a subunit of the AP-1 complex. Recent reports indicate the AP-1 complex binds to Rabaptin-5 and Rab4, recruiting clathrin to the EE membrane for the formation of recycling vesicles. These data functionally link Rabaptin-5 to receptor recycling (Pagano et al., 2004)

Rin1, a Rab5 GEF, binds to GDP-bound Rab5. Over expression of Rin1 stimulates

EGF-R internalization and EE fusion by increasing the intracellular pool of Rab5-GTP (Tall et al., 2001). Furthermore, coexpression of dominant negative Rab5 (Rab5 S34N) and full-length Rin1 result in decreased EGF-R activation of the Erk1/2 kinase pathway indicating that Rab5 GDP/GTP exchange is essential for EGFR internalization and signaling (Tall et al., 2001). Rab5 also interacts with GAPs such as RN-Tre (Liu and Li, 1998; Lanzetti et al., 2000). When over expressed in cells, these GAPs increase the GDP-bound pool of Rab5 thus inhibiting Rab5 activity and Rab5-mediated endocytic events. Furthermore, RN-Tre binds directly to Eps8, linking Rab5 function directly to EGF-R internalization in response to EGF (Lanzetti et al., 2000). A recent study has demonstrated that Rab5 binding is required by APPL1 and APPL2, proteins that translocate to the nucleus and bind to nucleosome remodeling proteins and histone deacetylases (Miaczynska et al., 2004). These data have identified regulatory roles for Rab5 not only in constitutive endocytosis but the regulated internalization of signaling receptors, suggesting that Rab5 likely controls mitogenic signaling via receptor trafficking. The recent identification of the APPL1 and APPL2 proteins, indicate that Rab5 may play a direct role in activating proteins that can translocate directly to the nucleus and affect gene expression.

Rab4 – Regulator of Endocytic Recycling

Rab4 was identified by Mellman and colleagues in 1991 as a GTPase that regulates endosomal recycling of the TfR from the EE. Over expression of Rab4 redistributes the TfR from intracellular stores to the plasma membrane (van der Sluijs et al., 1991; van der Sluijs et al., 1992). Rab4 reduces the steady state levels of TfR in the EE and also reduces the intracellular release of iron from Tfn (Daro et al., 1996). Furthermore, fluid phase internalization of the fluid phase marker HRP is inhibited in cells over expressing Rab4. Recent studies have also indicated that over expression of Rab4 results in increased degradation of LDL-R and TfR in addition to increasing receptor recycling. These results indicate that Rab4 is not only involved in the recycling of GLUT4 and TfR but also controls the degradation of the TfR and the LDL-R possibly by regulating receptor sorting (McCaffrey et al., 2001). Consistent with this idea, a study using Rab4-siRNA-mediated knockdown demonstrated that knockdown of Rab4 increased TfR recycling from the EE, indicating that Rab4 may function to sort proteins toward the recycling or degradative pathways (Deneka et al., 2003).

Several Rab4 effectors have been isolated and characterized. Rabip4 is a FYVE domain, RUN domain (Ras family interaction domain) containing protein that regulates

the trafficking and stability of EEs (Cormont et al., 2001a). Rabip4 binds simultaneously to both Rab4 and Rab5 and forms a high molecular weight complex in cells. Deletion mutants of Rabip4 lacking the RUN domain block TfR recycling in cells (Cormont et al., 2001a). Rabaptin4 (aka Rabaptin 5α) is ubiquitously expressed and inhibits the intrinsic GTPase ability of Rab4. Rabaptin4 also interacts with both Rab4 and Rab5 and delays TfR receptor recycling from the EE (van der Sluijs et al., 2001; Deneka et al., 2003). Rabenosyn-5 also interacts with both Rab4 and Rab5 and when over expressed delays TfR recycling (De Renzis et al., 2002; Naslavsky et al., 2004a). Recently CD2AP/CMS (ubiquitously expressed protein named CMS for Cas) links Cbl to Rab4 (Cormont et al., 2003). These data demonstrate that Rab4 may not regulate in the assembly of recycling complexes directly, but the assembly of functional Rab4 domains important for sorting receptors for recycling or degradation.

Rab11 –Functions in Recycling from the Recycling Endosome

In 1993, Parton and Zerial identified Rab11, a ubiquitously expressed protein, which is expressed at higher levels in tissues with high levels of secretion. Using sucrose flotation gradients, this group determined that Rab11 was associated with the TGN (Urbe et al., 1993). Later, Rab11a was reported to distribute between the TGN and internalized FITC-labeled Tfn, in a perinuclear distribution following a 30 min chase (Ullrich et al., 1996; Green et al., 1997). They reported differential effects of constitutively GTP-bound Rab11 (Q70L) and GDP-bound Rab11 (S25N) on Tfn trafficking in BHK cells. Expression of Rab11 wt, Q70L, and S25N had little effect on the initial internalization of ^{125}I-Tfn into the EE (Ullrich et al., 1996). BHK cells expressing either Rab11 wt or Rab11 Q70L (GTP-bound forms of Rab11) showed a decrease in recycling of ^{125}I-Tfn from the RE, while Rab11 S25N (GDP-bound) showed a marked inhibition of ^{125}I-Tfn recycling from the RE, thus demonstrating that Rab11 controls recycling from the RE (Ullrich et al., 1996). The actual effects of Rab11 in secretion may be due to antibody recognition of both Rab11a and the uncharacterized Rab11b protein which colocalizes with Rab11a on the TGN (Khvotchev et al., 2003; Lapierre et al., 2003). These data indicate that Rab11a affects recycling of receptors from the RE to the plasma membrane.

Rab11 binding proteins have been identified and characterized, and are generally refered to the Rab11 Family of Interacting Proteins or FIPs. The FIPs all contain a Rab11 binding domain, composed of 20 amino acid carboxyl terminal alpha helical domain, containing highly conserved hydrophobic residues (Prekeris et al., 2001; Prekeris, 2003;

Junutula et al., 2004b). Based on sequence homology, the FIPs are divided into three classes: Class I, II, or III. Class I proteins contain the RBD as well as an N-terminal C2 domain that binds to PI3P. Members of this class include Rip11b/Gaf1b, Rip11a/Gaf1a, FIP2/nRip11 and RCP and these classes have been shown to form strong complexes with GTP-bound Rab11a or Rab11b (Prekeris, 2003; Junutula et al., 2004b). Class II FIPs are FIP3 and FIP4 and are characterized by the presence of the RBD and two EF hand domains which are putative calcium binding proteins (Prekeris, 2003) Class III contains only one described protein, FIP1. FIP1 contains the RBD and no other putative domains (Prekeris, 2003).

Class I FIP proteins are poorly understood. Perhaps the best characterized protein is Rab11 FIP2 (Lindsay et al., 2002). McCaffrey and others demonstrated that FIP2 binds to GTP-bound Rab11 specifically and when over expressed in cells results in tubulation of the Tfn containing compartment as well Tfn accumulation in the RE (Lindsay et al., 2002). Recently, data demonstrate that FIP2 binds to Reps1, a known EGF-R substrate and may regulate sorting of the EGF-R towards the recycling pathway through interactions with Rab11 on the RE (Cullis et al., 2002). Moreover, FIP2 also binds to Rab8, a GTPase involved in the secretory pathway and may also be an effector for the GTPase Rab11b (Hattula and Peranen, 2000). These data indicate that FIP2 may be a key regulator in sorting of receptors from either the RE or the TGN to the recycling pathway through the RE.

The class II FIP protein consists of FIP3 and FIP4 which minimally affect TfR recycling (Wallace et al., 2002; Horgan et al., 2004). Recently, the FIP3 and FIP4 proteins have also been identified as Arf5 and Arf6 binding proteins and are also known as Arfophilin-1 and Arfophilin-2 (Hickson et al., 2003). Arfophilins 1 and 2 have been shown to regulated cell motility, cytokinesis, and phagocytosis (Hickson et al., 2003). These cellular functions require large amounts of membrane reshuffling as well as cytoskeletal rearrangements. *Drosophila* knockouts of Rab11 and the homolog of Rab11 FIP4 (Nuf), have severe defects in cytokinesis and cleavage furrow formation resulting in an embryonic lethal phenotype (Riggs et al., 2003). Furthermore, Rab11 FIP3 is localized with Rab11 in a pericentrosomal organelle during interphase however, during cytokinesis Rab11 and FIP3 localize to the cleavage furrow (Horgan et al., 2004). This data indicate that the Rab11 effectors FIP3 and FIP4 play a role in cytokinesis during development and cell division, thus uncovering a whole new role for the Rab11 positive RE as a membrane store

for cleavage furrow formation and cellurization (Hickson et al., 2003; Riggs et al., 2003; Horgan et al., 2004)

Rab7 and Rab9 – regulators of the late endosome and degradation

Rab7 and Rab9 were characterized by Suzanne Pfeffer's group by using a cell-free assay that reconstitutes the transport of cation independent – mannose phosphate receptor (CI-MPRfrom the LE to the TGN (Lombardi et al., 1993; Meresse et al., 1995; Soldati et al., 1995; Bottger et al., 1996; Vitelli et al., 1997). Furthermore, the GDP-bound mutant Rab9 S19N inhibited recycling of the CI-MPR from the LE to the TGN whereas fluid phase endocytosis was unaffected (Lombardi et al., 1993; Shapiro et al., 1993). Pfeffer and colleagues also demonstrated that Rab9 and Rab7 define distinct domains on the LE. Quantitative analysis of Rab7 and Rab9 colocalization demonstrated that there is only a 15% overlap between Rab7 and Rab9 . Furthermore MPRs are only on Rab9 enriched domains and that a Rab9 effector TIP47 binds specifically to the cytoplasmic domains of the CI-MPRs and that Rab9 binds to TIP47 via physically distinct domains thus linking Rab9 directly to the cargo (Krise et al., 2000; Carroll et al., 2001; Hanna et al., 2002; Ganley et al., 2004). There is currently no evidence that Rab7 regulates membrane traffic between the LE and TGN and it appears to regulate traffic between the EE and the LE as well as homotypic fusion of the LE (Feng et al., 1995; Bucci et al., 2000). Over expression of Rab7 T22N traps internalized Vesicular Stomatitis Virus-G in the EE thus inhibiting transport between these endosomes (Feng et al., 1995). RILP was identified as a Rab7 effector using a yeast two-hybrid screen. RILP interacts with Rab7 and not Rab9 and is localized to lysosomal membranes (Cantalupo et al., 2001; Jordens et al., 2001). Over expression of the carboxyl terminal portion of RILP inhibits EGF and LDL degradation (Cantalupo et al., 2001). RILP is thought to organize the LE and lysosomal vesicles around the microtubule organizing center by linking the organelle to the dynein/dynactin complex (Jordens et al., 2001).

How does Rab15 affect endocytic trafficking?

Identifcation and Characterization

Rab15 is a monomeric GTPase originally isolated from a rat brain cDNA library (Elferink et al. 1993). The Rab15 protein contains three domains corresponding to the GTP binding pockets, an effector domain, switch 1 and 2 regions and a carboxyl terminal

prenylation motif. Rab15 is expressed at high levels in brain tissue, specifically in the cortex, hippocampus, thalamus, hypothalamus, midbrain and striatum while at lower levels in the cerebellum, brainstem, and spinal cord (Elferink et al., 1992). Detailed expression analysis using RT-PCR demonstrated that Rab15 is ubiquitously expressed at low levels in all major tissues and cell types, while being expressed at 10-fold higher levels in nervous tissue (Zuk and Elferink, 1999).

Rab15 – A novel Inhibitory GTPase localized to the Endocytic Pathway

Studies by Zuk and Elferink demonstrated that Rab15 localized to the early endocytic compartments and participates in early endocytic trafficking (Zuk and Elferink, 1999; Zuk and Elferink, 2000). First, membrane binding studies demonstrate that Rab15 localized to membrane fractions via the carboxyl terminal (CWC) prenylation motif (Zuk and Elferink, 1999). Second, when over expressed in cells, Rab15 colocalized with Rab4 and Rab5 on EEs as well as with Rab11 on the RE (Zuk and Elferink, 1999). Third, functional studies using TfR as a model system, demonstrated that Rab15 differentially regulated the trafficking of TfR through EEs and the RE (Zuk and Elferink, 2000). Together these data demonstrated that Rab15 is a small GTPase important for the trafficking of the TfR through EEs and the RE.

Using mutant Rab15 proteins that were constitutively bound to GTP or GDP, functional studies revealed that Rab15 functioned as a novel inhibitory GTPase, which inhibits trafficking of the TfR (Zuk and Elferink, 2000). For example the Q67L mutation abolishes the endogenous GTPase ability of Rab15, thus locking the protein in a GTP-bound state. Conversely, the mutant T22N locked the protein in a conformation that preferentially binds GDP. Finally, the mutation N121I locks Rab15 in a nucleotide-free state. These mutations did not interfere with the ability of Rab15 to bind endocytic membranes in TRVB-1 cells (CHO cells expressing human TfR), but rather affected the guanine nucleotide bound state of the protein (Zuk and Elferink, 2000). GTP-bound Rab15 (wt and Q67L) inhibits TfR internalization at the level of endosomal fusion. Conversely, over expression of the inactive Rab15 mutants (T22N and N121I) stimulate TfR internalization (Zuk and Elferink, 1999; Zuk and Elferink, 2000). Furthermore, Rab15 T22N stimulates the recycling of the receptor from both the EE and the RE while Rab15 N121I stimulates TfR recycling from the RE only. These results indicate that Rab15 is a novel inhibitory Rab GTPase that functions to inhibit TfR internalization and differentially affect recycling from the EE and the RE.

Experimental Rationale and Hypothesis

The inhibitory phenotype on TfR endocytosis in the over expression studies of Rab15 mutants is opposite to similar studies using Rab5 mutants (Gorvel et al., 1991; Bucci et al., 1992; Stenmark et al., 1994a; Barbieri et al., 1998). In these studies, GTP-bound Rab5 increased TfR endocytosis whereas GDP-bound Rab5 blocked receptor trafficking. Given these results, Rab15 could inhibit endocytic trafficking of the TfR by two possible mechanisms. First, Rab15 may inhibit Rab5-mediated TfR internalization by binding and sequestering Rab5 effector molecules, thereby inhibiting Rab5 activity and inhibiting endocytosis of the TfR. Second, Rab15 inhibits Rab5-mediated endocytic trafficking by using a unique set of effector interactions. Furthermore, because Rab15 may differentially regulate internalization and recycling at the EE and the RE, Rab15 may interact with binding proteins that are compartment specific. Given these possibilities, the goal of my dissertation is to further elucidate a role for Rab15 by identifying and characterizing specific Rab15 effectors/binding proteins. I demonstrate that GDP-bound Rab15 interacts with Mammalian Suppressor of Sec4, and that these interactions functionally regulate Rab15 activity on early endocytic trafficking (Chapter 2). Furthermore, I have identified REP15, a novel protein that specifically localizes to and regulates TfR recycling from the RE (Chapter 3). Finally, I have identified RBP15, a novel neuron-specific binding partner for Rab15 (Chapter 4). Together, these data indicate that Rab15 regulates TfR internalization and recycling by binding to unique effectors that localize to these functionally distinct compartments.

CHAPTER 2: MAMMALIAN SUPPRESSOR OF SEC4 MODULATES THE INHIBITORY EFFECT OF RAB15 DURING EARLY ENDOCYTOSIS[1]

Introduction

As previously stated in Chapter 1, endocytosis of cell surface receptors regulates both the intensity and duration of receptor signaling by controlling the location of signaling interactions and the desensitization and recycling of activated receptors (Ceresa and Schmid, 2000; Leof, 2000). Accordingly, endocytic compartments are highly specialized both in terms of their organization and function. The early/sorting endosome is a major trafficking compartment from which several trafficking pathways emerge. Rab GTPases have emerged as potent regulators of membrane trafficking through early/sorting endosomes. Rabs do not regulate membrane trafficking *per se*, but function as regulatory throttles impacting the kinetics of membrane transport steps through the recruitment of specific effectors which in turn mediate membrane transport (Zerial and McBride, 2001; Deneka and van der Sluijs, 2002; Pfeffer and Aivazian, 2004). For example, Rab5 mediates the internalization and fusion of incoming endocytic vesicles *in vivo* (Bucci et al., 1992; Li and Stahl, 1993; Stenmark et al., 1994a) and the homotypic fusion of endosomes *in vitro* (Gorvel et al., 1991; Barbieri et al., 1994; Stenmark et al., 1994a; Barbieri et al., 1996; Barbieri et al., 1998). Over expression of the constitutively active GTP-bound mutant Rab5 Q79L in BHK cells results in a dramatic increase in fluid phase and receptor mediated endocytosis and leads to formation of enlarged early/sorting endosomes. Conversely, over expression of GDP-bound Rab5 (S34N), reduces endocytic uptake and results in the formation of a diffuse network of small endocytic vesicles (Bucci et al., 1992; Li and Stahl, 1993; Stenmark et al., 1994b).

Following activation on endosome membranes, Rab5-GTP drives the organization of a specialized membrane domain with distinct functional characteristics (McBride et al., 1999; Sonnichsen et al., 2000; Zerial and McBride, 2001). Rab5-GTP forms this domain by recruiting the phosphatidylinositol3 kinase hVPs34, which catalyzes the local production of PI3P (Li et al., 1995; Simonsen et al., 1998; Christoforidis et al., 1999b). Rab5-GTP

[1] Strick, D.J., Francescutti, D.M., Zhao, Y., and Elferink, L.A. (2002) J. Biol. Chem. 277(36): 32722-32729.

and hVPs34 activity are essential for the subsequent recruitment of Rabenosyn-5 and the docking protein, Early Endosome Antigen (EEA1) to early endosomal membranes through PI3P (Simonsen et al., 1998; Christoforidis et al., 1999a; Christoforidis et al., 1999b; Nielsen et al., 2000). EEA1 also interacts directly with Syntaxin 13, a SNARE implicated in the fusion of early endosomes (Prekeris et al., 1998; McBride et al., 1999). Thus a model is emerging in which Rab5-GTP functions as a regulatory protein, driving assembly of specific effector complexes on endosomal membranes leading to membrane fusion. Consistent with this model, the early endocytic GTPases, Rabs 4 and 11 have also been shown to organize into distinct domains on early endosomes through the local recruitment of effectors (Sonnichsen et al., 2000; De Renzis et al., 2002). Moreover, Rab4 and Rab5 function are linked through the shared effectors Rabaptin-5 (Vitale et al., 1998) and Rabenosyn-5 (De Renzis et al., 2002). Thus rab specific domains appear to coordinate endosomal trafficking directly by communicating via shared effector complexes.

The early endocytic GTPase Rab15 exhibits distinct endocytic localization and activity. Rab15 distributes between two early endosomal compartments, colocalizing with Rabs 4 and 5 on early/sorting endosomes and with Rab11 on pericentriolar recycling endosomes (Zuk and Elferink, 1999). Over expression of activated Rab15 (Rab15-GTP) inhibits both fluid phase and receptor-mediated endocytosis *in vivo* and the homotypic fusion of early endosomes *in vitro*. Conversely, mutations that constitutively inactivate Rab15 (Rab15-GDP) stimulate early endocytosis and fusion of homotypic endosomes *in vitro* (Zuk and Elferink, 2000). These data suggest that Rab15 functions to reduce endocytic trafficking, primarily at the level of early/sorting endosomes. Consistent with an inhibitory role, over expression of Rab15-GTP reverses the stimulatory effect of Rab5-GTP on early endocytosis, whereas coexpression of Rab15-GDP with activated Rab5 increased internalization of the fluid phase marker HRP relative to cells expressing activated Rab5 alone (Zuk and Elferink, 2000).

Given the opposing effects of Rab15 and Rab5 on early endocytosis, the transport steps regulated by these GTPases likely intersect at some point within the endocytic network, presumably through the action of a shared effector or accessory protein. Rab15 may interfere with Rab5 function directly by sequestering Rab5 effectors or, indirectly through a unique set of effector interactions. To distinguish between these possibilities, we examined the effector binding properties of Rab15. Using a yeast two-hybrid binding assay we demonstrate that Rab15 does not directly interact with the Rab5 effectors Rabaptin-5

or Rabex-5. Rather, Mammalian Suppressor of Sec4 (Mss4) was identified as a binding partner for Rab15. Mss4 specifically binds GDP-bound Rab15 (T22N) and the nucleotide-free mutant N121I, consistent with the proposed role of Mss4 as a chaperone mediating GDP removal, stabilizing its target rab in a nucleotide-free state (Burton et al., 1994; Collins et al., 1997; Nuoffer et al., 1997). Our functional analyses indicate that interactions with Mss4 are required for the inhibitory effect of Rab15 in early endocytosis, suggesting a novel role for Mss4-mediated interactions in early endocytic trafficking.

Materials and Methods

Reagents and Plasmids

General cell culture reagents and chemicals were obtained from Invitrogen Life Technologies and Fisher Chemical, respectively, unless specified otherwise. All restriction enzymes were purchased from New England Biolabs and $Na^{125}I$ was purchased from Amersham Pharmacia Biotechnology. The following antibodies were obtained as indicated: anti-HA monoclonal antibody, 12CA5 (Boehringer Mannheim); anti-human transferrin receptor monoclonal antibody, H68.4 (Zymed). pET15b expressing rat Mss4 and an anti-rat Mss4 polyclonal antibody (Burton et al., 1997) were kindly provided by Pietro De Camilli (Yale). Plasmids encoding Rab5 (Bucci et al., 1994; Stenmark et al., 1994a), Rabex-5 (Horiuchi et al., 1997) Rabaptin-5 (Stenmark et al., 1995), and Rabenosyn-5 (Nielsen et al., 2000) were generous gifts from Marino Zerial (Max Planck Institute for Molecular Cell Biology and Genetics, Germany) and Harold Stenmark (Norwegian Radium Hospital, Norway). A HeLa cell cDNA library pre-transformed into EGY187 (MATα) was kindly provided by Russell Finley Jr. (Wayne State University). The cDNAs for wild type and mutant Rab15 (Q67L, N121I and T22N) containing an amino terminal HA epitope have been described elsewhere (Zuk and Elferink, 2000), and were cloned directly into pCINeo (Invitrogen). Site-directed mutagenesis was performed using the QuikChange™ Mutagenesis Kit (Stratagene) according to the manufacturer's instructions and verified by DNA sequencing (ABI).

Yeast Two-Hybrid Binding Assays

Bait strains were prepared by cloning wild type Rab15 and its respective mutants into pLexA (Clontech); GTP-bound Rab15 (Q67L), nucleotide–free Rab15 (N121I), GDP-bound Rab15 (T22N), Rab15 T22N containing the single mutations K46L or K48Q, and

Rab15 T22N in which the motifs DN (residues 30-31), DFKMK (residues 44-48) and TITK (residues 72-75) were substituted with the corresponding Rab5a sequences KG, AFLTQ and SLAP respectively.

cDNAs encoding Rabaptin-5, Rabex-5, and Rabenosyn-5 were cloned into pB42AD (Clontech). Bait and prey constructs were transformed into RFY206 (MAT a) and EGY187 (MAT α), respectively, using established techniques (Clontech). Expression of the indicated bait and prey constructs was confirmed by SDS PAGE and Western analysis.

Yeast two-hybrid binding assays were performed by mating bait strains with prey strains (Kolonin et al., 2000) as specified in the text. Positive diploids were identified by growth on quadruple synthetic dropout media (Trp/His/Ura/Leu) and LacZ activation. For LacZ activation assays, the appropriate diploids were grown in 5 ml of triple synthetic dropout media (Trp/His/Ura) overnight at 30ºC and subcultured 1:10 in fresh dropout media for 7 hours at 30°C. Cells were pelleted at 1000 x g for 5 minutes at 4 ºC, washed once in 5 ml of Z Buffer (113 mM $Na_2HPO_4 \bullet 7H_2O$, 39 mM $NaH_2PO_4 \bullet H_2O$, 10 mM KCl, 1 mM $MgSO_4 \bullet 7H_2O$, and 35mM ßmercaptoethanol) resuspended in 120-150 ul of Z-Buffer and subjected to 3 cycles of 1 minute freeze/thaws in liquid nitrogen. The lysates were centrifuged at 20,000 x g, 5 min, at 4 ºC and 15 ul of the clarified supernatant incubated with 150 ul of CUG substrate (Molecular Probes) for 30 min at room temperature in darkness. The reactions were terminated with 75 ul of 0.2M Na_2CO_3 and the relative fluorescence measured according to the manufacturer's specifications (Molecular Probes). Each assay was performed in triplicate and repeated at least twice. Relative fluorescence units were normalized to the amount of protein in each sample (Bradford, BioRad) and are reported as a measure of relative ß-Galactosidase activity.

A HeLa cell library was screened by mating EGY187 cells with a RYF206 strain expressing Rab15 T22N as indicated above. Plasmid DNA prepared from positive diploids identified in the library screen, were isolated by transformation into *E.Coli* KC8 to isolate the library construct. Inserts from the resulting cDNAs were PCR amplified using the primers 5'CGTAGTGGAGATGCCTCC-3' and 5'CTGGCAAGGTAGACAAGCCG-3', and analyzed by HaeIII digestion and DNA sequence analysis (ABI). DNA and predicted protein sequences were further analyzed using BLAST searches.

Cell Culture and Transfections

All cells were cultured in DMEM supplemented with penicillin/streptomycin and maintained at 37 ºC with 5% CO2. HeLa media was supplemented with 10% Cosmic Calf

Sera (Hyclone) and penicillin/streptomycin. Over expression studies using T7 recombinant vaccinia virus (vTF73) were performed as previously described (Zuk and Elferink, 1999; Zuk and Elferink, 2000). Transient expression using LipofectAMINE" (Life Technologies) was performed as previously described (Zuk and Elferink, 1999; Zuk and Elferink, 2000).

Biochemical Pull Down Assays

Recombinant rat Mss4 was expressed as a His6 fusion in BL21DE3 pLysS (Stratagene) and purified by NiNTA affinity chromatography (Qiagen). For pull down studies, HeLa cells were transfected with Rab5 or HA-tagged Rab15 using LipofectAMINE" (Life Technologies) as described elsewhere (Zuk and Elferink, 2000). Transfected cells were resuspended in 200 ul of ice cold lysis buffer (10 mM HEPES, pH 7.4, 1.5% IGEPAL (Sigma), 0.1 mM MgCl2, 150 mM NaCl, 10ug/ml each aprotinin, leupeptin and pepstatin A), and cell lysates were clarified at 16,000 x g for 5 min at 4°C. Supernatants were adjusted to 1.0 mM MgCl2, incubated with 1.0 mM GTPγS (Sigma) or 1.0 mM GDPβS (Sigma) for 60 min at 4 °C and the extracts incubated for 2.0 hr at 4 °C with 5 µg of purified His_6-Mss4. Mss4 and its associated proteins were isolated by binding to NiNTA beads in lysis buffer containing 10 mM Imidazole at 4°C for 60 min. Beads were washed three times in cell lysis buffer, three times in 150 mM NaCl, 10 mM HEPES-KOH pH 7.5, 0.1 mM $MgCl_2$ and analyzed by SDS-PAGE followed by Western analysis by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Functional Analysis of Rab15 and Mss4 interactions

^{125}I-Tfn internalization studies, *in vitro* homotypic endosome fusion assays and guanine nucleotide binding studies were performed on transiently transfected HeLa cells as previously described (Zuk and Elferink, 2000). Surface-bound Tfn was assessed as follows. Transiently transfected HeLa cells were depleted of endogenous Tfn for 1 hour at 37°C and chilled at 4°C for 30 min to stop endocytosis. The cells were incubated in IM (DMEM w/20 mM HEPES, pH 7.4 and 20 mg/ml BSA) containing 3 ug/ml ^{125}I-Tfn for 1 hour at 4°C to enable binding of the ^{125}I-Tfn to cell surface associated TfR. The cells were washed in PBS containing 0.1% BSA at 4°C and the total amount of cell associated ^{125}I-Tfn measured with a gamma counter (Packard Instruments). 80-90% of the cell associated ^{125}I-Tfn was routinely removed with three successive acid washes as previously described (Zuk and Elferink, 2000) (**Fig. 4A**). Furthermore, 100-fold unlabeled Tfn blocked the internalization of ^{125}I-Tfn indicating the specificity of ^{125}I-Tfn for the TfR (**Fig. 4B**). All

Table 2: Yeast two-hybrid Assay with Rab15 and selected Rab5 effectors

	β-galactosidase activity (relative fluorescence units)		
pLexA construct	**Rabaptin-5**	**Rabex-5**	**Rabenosyn-5**
Rab15 wt	5824.3 +/- 208.7	4062.2 +/- 50.1	29834.3 +/- 936.6
Rab15 Q67L	8535.4 +/- 279.4	7119.2 +/- 209.7	31798.0 +/- 830.1
Rab15 T22N	4300.7 +/- 64.6	1962.0 +/- 35.1	8708.2 +/- 184.0
Rab15 N121I	3282.3 +/- 140.0	5976.6 +/- 150.1	17000.0 +/- 343.2
Rab5 wt	5273.1 +/- 513.5	1521.4 +/- 31.9	7282.0 +/- 111.5
Rab5 Q79L	2114619.0 +/- 71380.7	5248.2 +/- 224.3	50862.3 +/- 846.8
Rab5 S34N	1606.3 +/- 117.9	131750.0 +/- 2556.0	5913.3 +/- 160.0
Rab5 N133I	227130.0 +/- 4036.6	83173.9 +/- 1537.9	87852.1 +/- 1725.2

numerical results were subjected to an OneWay ANOVA with a Newman Keuls Post-Test to determine statistical significance between selected groups (Prism GraphPad).

Results

Does Rab15 bind Rab5 effectors?

Given the opposing effects of Rab15 and Rab5 on early endocytosis, it is highly likely that the transport steps regulated by these rabs overlap at some point within the endocytic network, possibly in terms of a common effector or target. We first examined the ability of Rab15 to interact with the Rab5 effectors Rabex-5 (Horiuchi et al., 1997), Rabaptin-5 (Stenmark et al., 1995) and Rabenosyn-5 (Nielsen et al., 2000). Rabex-5 is a guanine nucleotide exchange factor for Rab5 originally identified as a component in a complex with Rabaptin-5. Rabaptin-5 increases the exchange activity of Rabex-5 on Rab5, promoting early endosome fusion *in vitro* (Lippe et al., 2001). Rabaptin-5 also interacts with Rab4 through a distinct binding site (Vitale et al., 1998) suggesting a role for this effector in recycling from early endosomes to the cell surface. Rabenosyn-5 preferentially interacts with GTP-bound Rab5 (Rab5 Q79L) and PI3P on early endosomes. Consistent with the stimulatory role of Rab5-GTP in endocytosis, Rabenosyn-5 promotes homotypic endosome and clathrin-coated vesicle fusion *in vitro* (Nielsen et al., 2000). In addition, Rabenosyn-5 interacts directly with Rab4-GTP and promotes transferrin (Tfn) recycling from early sorting endosomes when over expressed in HeLa cells (De Renzis et al., 2002).

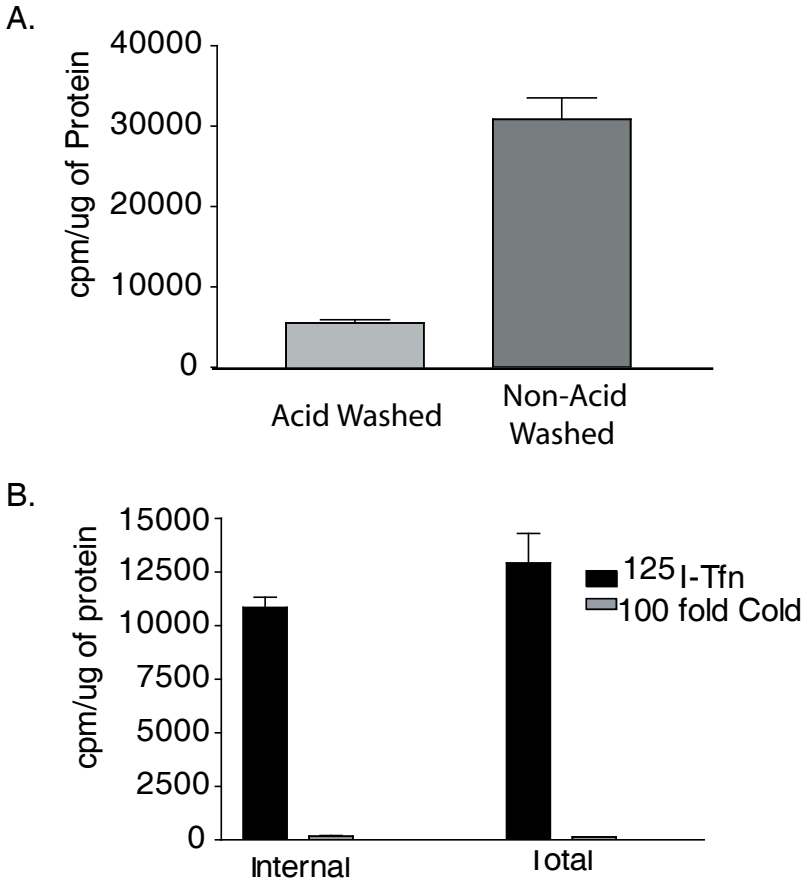

Figure 4: ^{125}I-Tfn Internalization Controls. A, HeLa cells were incubated ^{125}I-Tfn for 1 hour at 4ºC and then washed with either PBS (pH 4.2) or PBS (0.1% BSA). The cells were lysed and ^{125}I-Tfn amounts were quantified using a gamma counter. Values replicate the means of triplicate values ± SEM normalized to protein concentration. These results demonstrate that 80 - 90% of the ^{125}I-Tfn is removed by acid washing B, Cells were incubated in ^{125}I-Tfn or ^{125}I-Tfn with 100-fold unlabeled Tfn for 1 h at 4ºC. The cells were washed and incubated at 37ºC for 15 min to internalize the labeled Tfn. After which the cells were washed with PBS (pH 4.2) and lysates were quantified as described above. 100-fold unlabeled Tfn blocks the internalization of ^{125}I-Tfn demonstrating the specificity of ^{125}I-Tfn.

Rabaptin-5 is a cytosolic protein that was originally identified as an effector for Rab5-GTP using a yeast two-hybrid approach (Stenmark et al., 1995). Thus interactions with shared effectors may functionally couple otherwise distinct rab mediated transport steps within early sorting endosomes (Vitale et al., 1998; De Renzis et al., 2002). To determine if Rab15 binds Rab5 effectors, cDNAs encoding Rabex-5, Rabaptin-5 and Rabenosyn-5 were cloned into the plasmid pB42AD, in frame with the activation domain of the bacterial transcription factor B42, and conditionally expressed from the Gal1 promoter in the presence of galactose. Wild type and mutant Rab15 were expressed in frame with the DNA binding domain of the bacterial transcription factor LexA, and protein-protein interactions were assayed using LacZ activation as the reporter gene. Negligible binding was detected between Rabex-5 and wild type or mutant Rab15. Similarly, we detected no interaction between Rabaptin-5 and wild type or mutant Rab15 (**Table 2**). Western analysis indicates that the absence of any notable interaction between Rab15 and Rabex-5 or Rabaptin-5 in this assay is not related to differences in the relative amounts of the expressed prey and bait proteins (**Fig. 5A and B**). Moreover Rab5 strongly interacted with Rabex-5 and

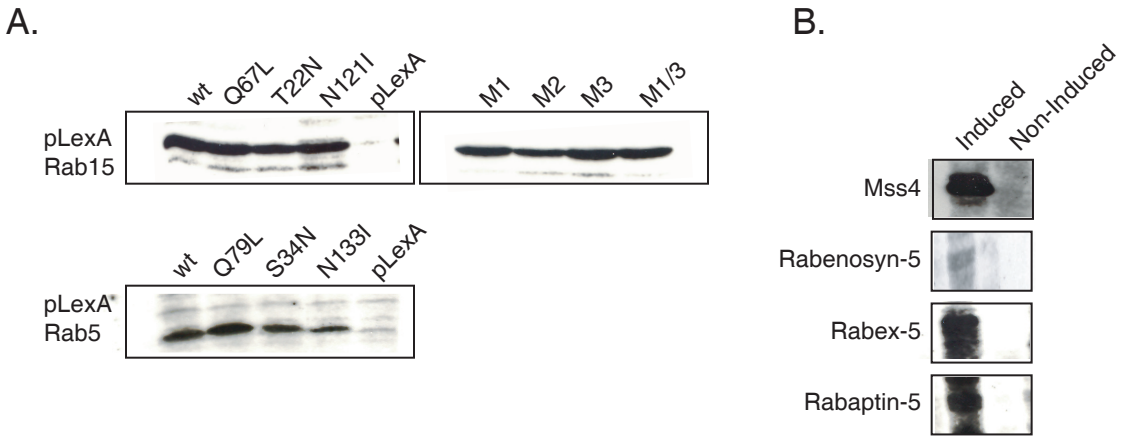

Figure 5: Bait and prey proteins are expressed in yeast A, Haploid yeast strains expressing Rab15 wt and mutants and Rab5 wt and mutants were lysed and were examined using Western analysis. M1, M2, M3 and M1/3 refer to Mss4 binding mutations (see later in text.). B, Haploid prey strains were induced or non-induced by incubation in galactose containing media or glucose containing media respectively. These strains were lysed and examined using Western analysis to verify prey expression.

Rabaptin-5, indicating the specificity of the results. Specifically, Rabex-5 bound GDP-bound (S34N) and the nucleotide deficient mutant Rab5 N133I. Conversely, Rabaptin-5 interacted directly with Rab5-GTP (Q79L) and the nucleotide-free Rab5 mutant, N133I. Interestingly, a weak interaction was detected between Rabenosyn-5 and wild type Rab15 as well as the GTP-bound and nucleotide free Rab15 mutants, Rab15 Q67L and Rab15 N121I respectively. Given the modest binding observed between Rab15 and Rabenosyn-5 in the absence of any discernible nucleotide dependency, the physiological significance of this interaction remains uncertain. Taken together, these data indicate that Rab15 does not directly interact with the Rab5 effectors Rabex-5 and Rabaptin-5.

Mss4 (Mammlian Suppressor of Sec4) Binds to GDP-bound Rab15.

Since Rab15 does not interact with the Rab5 effectors Rabaptin-5 and Rabex-5, we reasoned that the inhibitory effect of Rab15 during early endocytosis is regulated by a unique set of effector molecules. Therefore, we screened a HeLa cell cDNA library using Rab15 T22N as "bait" in a yeast two hybrid system. HeLa cells are known to express Rab15 (Zuk and Elferink, 1999) supporting the premise that Rab15 effectors are represented in this library. Eleven out of the twelve strongly positive clones were identified as Mammalian Suppressor of Sec4 (Mss4). All Mss4 clones isolated from this screen encoded minimally residues 1 to 55. Mss4 and its yeast homologue Dss4p were originally identified in genetic screens for proteins that suppressed the secretory defect of sec4-8 mutants (Burton et al., 1993; Moya et al., 1993). Mss4 specifically binds to and promotes the release of GDP from a subset of rabs including sec4, rabs 1a, 3, 8 10, and 13. Mss4 does not bind rabs 2, 4 or 7; moreover, no interaction is observed between Mss4 and Rab5 (Burton et al., 1994; Burton et al., 1997). While Mss4 and Dss4p facilitate the release of GDP from their target rabs, they lack the ability to promote GTP binding (Collins et al., 1997; Esters et al., 2001). Therefore, Mss4 does not function as a bona fide GEF as previously reported (Yu and Schreiber, 1995), but rather stabilizes rabs in a nucleotide-free state (Nuoffer et al., 1997; Esters et al., 2001). Accordingly, we examined the nucleotide dependency of the interaction between Rab15 and full length Mss4. High levels of ß-Galactosidase activity were observed in strains expressing Mss4 and the inactive Rab15 mutants, Rab15 N121I and to a lesser extent, Rab15 T22N (**Fig**. **6A**). Mss4 failed to bind wild type Rab15 and its GTP-bound mutant Q67L under these conditions. Mss4 did not interact with wild type Rab5 or its guanine nucleotide binding mutants Q79L, S34N and N133I as judged by

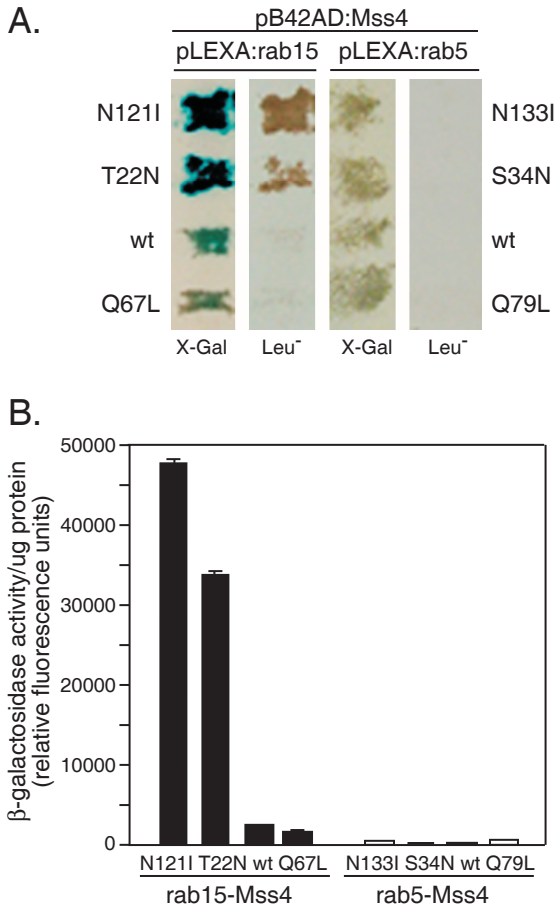

Figure 6: Mss4 preferentially binds constitutively inactive Rab15 mutants, N121I and T22N. A, yeast strains expressing Mss4 fused to the activation domain of B42 (pB42AD: Mss4) with wild type (wt) or the indicated Rab mutants expressed as fusions with the DNA binding domain of LexA (pLEXA) were mated on synthetic media lacking tryptophan and histidine but containing X-gal or synthetic media lacking tryptophan, histidine, and leucine (Leu). Blue colonies on X-gal and growth on Leu plates indicate specific interactions between Mss4 and inactive Rab15 mutants. B, reporter ß-galactosidase activity was determined and represents the means ± S.E. of triplicate experiments and are normalized with respect to protein concentration. Significant differences were observed between experimental and control conditions (one-way ANOVA, $p \leq 0.001$) as described in the text.

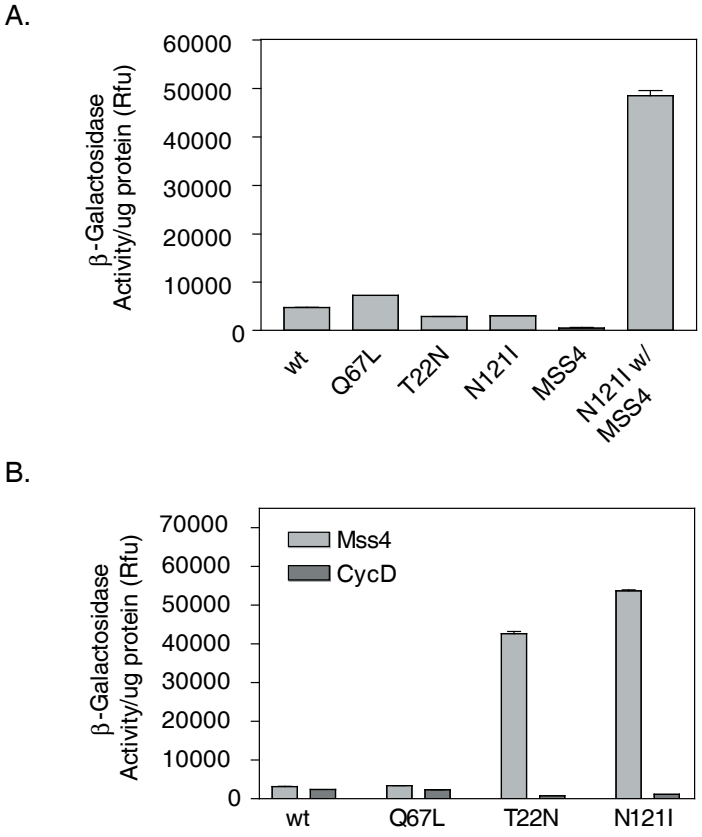

Figure 7: Yeast Two-hybrid interaction controls. A, Bait or prey strains were plated on galactose media to induce expression and then lysates were assayed for ß-Galactosidase acitivy. Values represent means of triplicate values ± SEM. Rab15 yeast strains do not activate the reporter genes in absence of Mss4. B, Rab15 interaction controls with Mss4 and CycD, indicating the specificity of the interaction. Rab15 and Mss4 or CycD diploids were induced with Galactose to drive protein expression and then assayed for ß-galactosidase Activity. Values represent means ±SEM.

control levels of β-galactosidase (**Fig. 6B**) and lack of growth on Leu plates (**Fig. 6A**). No interaction was observed when the diploids were grown on plates containing glucose, indicating that galactose driven expression of Mss4 was essential for the interaction with Rab15 (**Fig. 7A and B**). The absence of ß-galactosidase activity in cells expressing Mss4, wild type or mutant Rab15 alone demonstrates that reporter expression was dependent on a two-hybrid protein-protein interaction (data not shown). These data indicate that Mss4 directly interacts with Rab15; preferentially nucleotide-free Rab15 N121I and the constitutively inactive GDP-bound mutant, Rab15 T22N.

We confirmed the interaction between Rab15 and Mss4 using pulldown assays. Recombinant Mss4 was expressed as a His_6tagged fusion in *E. coli*, purified by affinity chromatography on NiNTA agarose and the purified protein incubated with cell lysates

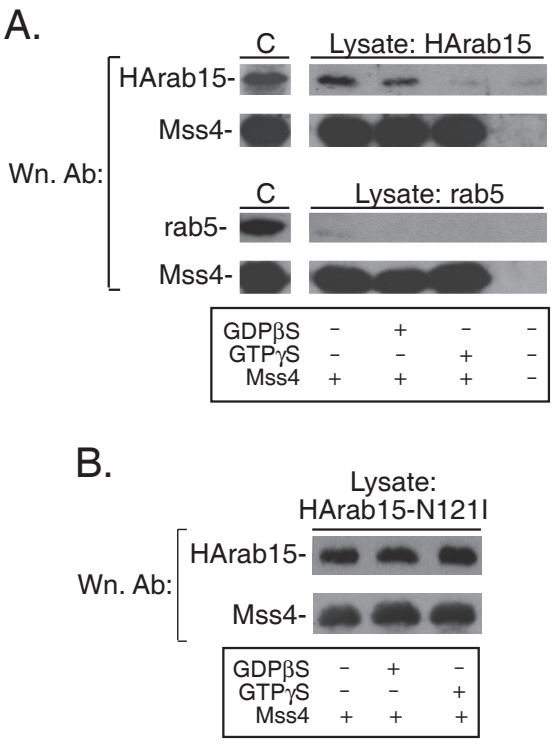

Figure 8: Mss4-Rab15 interactions are guanine nucleotide dependent. A, cell lysates prepared from HeLa cells transiently over expressing wild type HArab15 or Rab5 were incubated with (+) or without (-) GTPγS or GDPβS, prior to incubation with purified, recombinant His6-tagged Mss4. Mss4-Rab complexes were immobilized on NiNTA-agarose beads and analyzed by Western analysis (Wn) using antibodies (Ab) for HArab15, Rab5, and Mss4. Expression of the indicated proteins in cell lysates were confirmed by Western analysis (C). B, Mss4 binding studies using lysates prepared from cells over expressing constitutively inactive HArab15-N121I indicate that Mss4 preferentially binds nucleotide-free HArab15.

prepared from HeLa cells over expressing wild type HARab15. The nucleotide dependence of this interaction was examined by first priming the cell lysates with GDPßS or the non-hydrolyzable GTP analog, GTPγS. Mss4:Rab15 complexes were recovered by binding NiNTA agarose and analyzed by Western analysis. As shown in Fig. 5A, wild type HARab15 binds Mss4 in the absence and presence of GDPβS (**Fig. 8A**). Since wild type HARab15 binds and hydrolyzes GTP when transiently over expressed in Trvb1 (Zuk and Elferink, 2000) and HeLa cells (**Fig. 8A**) the interaction observed between Rab15 and Mss4 in the absence of GDPßS likely reflects binding to GDP-bound and nucleotide-free form of wild type Rab15. Consistent with this hypothesis, priming the lysates with GTPγS prior to the addition of recombinant Mss4, prevents the interaction between Mss4 and Rab15. Moreover a strong interaction occurs between Mss4 and the nucleotide-free Rab15 mutant, N121I in the presence and absence of GTPγS and GDPβS (**Fig. 8B**). No HARab15 immunoreactivity was detected using beads alone or cell lysates expressing wild type Rab5, confirming the specificity of these results (**Fig. 8**). Taken together, these data corroborate our yeast two-hybrid studies and indicate that Mss4 directly interacts with constitutively inactive Rab15 mutants, T22N and N121I.

Identification of Mss4 binding sites on Rab15

Interpreting the effect of over expressing dominant Mss4 mutants on Rab15-mediated endocytosis is confounded by Mss4 binding multiple rabs (Burton et al., 1994). Therefore, we used a yeast two-hybrid approach to identify potential loss off function Rab15 mutants that do not bind Mss4. Comparison of the Rab15 peptide sequence with other Mss4 binding rabs (including rabs 1a, 3a, 8 and 10 and sec4p) reveals three conserved signature motifs that may comprise Mss4 binding sites in these proteins (Lippe et al., 2001). In sec4p and Rab3a, these sites reside within or juxtaposed to the switch I and II regions; regions on the surface of these proteins that change conformation during guanine nucleotide binding and GTP hydrolysis (Yu and Schreiber, 1995; Stroupe and Brunger, 2000). Moreover, these two switch regions are major sites of interaction with regulator and effector molecules in general, including GEFs and GTPase activating proteins. In Rab15, the putative Mss4 binding motifs include DN (m1), DFKMK (m2) and TITK (m3) (comprising amino acid residues 30-31, 44-48 and 72-75 respectively) (Burton et al., 1997) (**Fig. 9A**). To determine if these three regions contribute to the interaction between Rab15 and Mss4, we individually substituted these motifs in Rab15 T22N with the corresponding regions of Rab5, a GTPase known not to bind Mss4 (**see Fig. 9B**). The three Rab15-GDP mutants (m1, m2 and m3) were tested for their ability to bind Mss4 in a yeast two-hybrid binding assay. As shown in Figure 9, binding of Mss4 to Rab15 T22N was abolished by substitution of the DN (m1) and DFKMK (m2) motifs. The motif TITK (m3) is not required for binding Mss4 to Rab15, since comparable levels of β-galactosidase reporter activity were detected relative to strains expressing Rab15 T22N and Mss4 (**Fig. 9C**). β-galactosidase activity was reduced 60-70% in the double mutant m1/m3 relative to yeast lysates prepared from strains co-expressing Mss4 with Rab15-GDP (T22N) or the nucleotide-free form of Rab15 (N121I) confirming the importance of the m1 region for Rab15:Mss4 interactions. These data indicate that the DN (m1) and DFKMK (m2) motifs are required for Mss4 binding to Rab15. Structural analysis of the corresponding m2 motifs in Rab3a and Sec4p suggests that the lysine residues at positions 46 and 48 of Rab15, probably reside on the surface of Rab15, consistent with a role in binding Mss4 (Zhu et al., 2001a). To test this experimentally, we generated two additional mutations in Rab15 T22N in which K46 and K48 were substituted with leucine and glutamine, respectively (i.e. the corresponding residues of Rab5) and assayed Mss4 binding using a yeast two-hybrid assay. β-galactosidase reporter activity was comparable in strains expressing Mss4 with Rab15 T22N or Rab15 K46L. Conversely, binding is

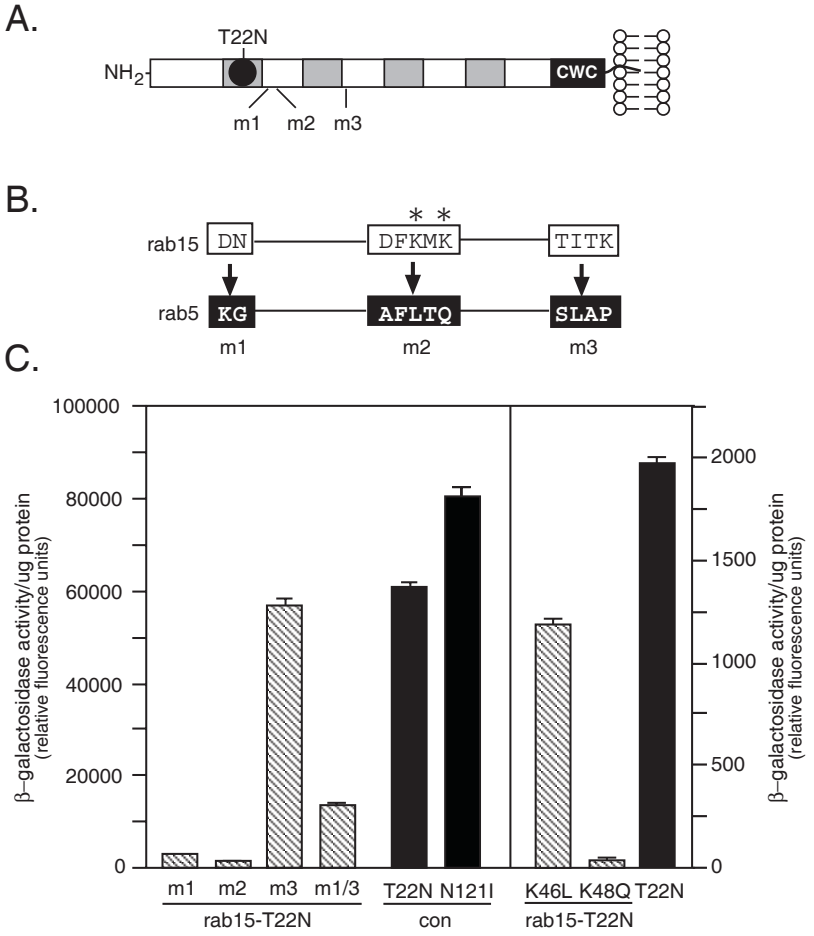

Figure 9: K48Q abolishes the interaction between HArab15 and Mss4. A, The relative positions of the T22N mutation in the first of four GTP-binding motifs (gray boxes) and putative Mss4-interacting motifs m1, m2, and m3 are shown. B, substitution of the indicated Rab15 amino acid residues with the corresponding Rab5 peptide sequence generates the Rab15-T22N mutants m1, m2, and m3. Asterisks indicate the single mutations K46L and K48Q in m2. C, β-galactosidase reporter activity (means ± S.E. of triplicate experiments) produced by interactions between Mss4, T22N, N121I, and the indicated single and double Rab15-T22N mutants. Significant differences were observed between experimental and control conditions (one-way ANOVA, $p \leq 0.001$) as described in the text.

disrupted between Mss4 and Rab15 K48Q indicating that lysine at position 48 is critical for the interaction between Rab15 and Mss4 (**Fig. 9C**).

K48Q counters the inhibitory effect of Rab15 on early endocytosis

We previously demonstrated that wild type HARab15 binds and hydrolyses GTP, and reduces receptor mediated endocytosis when transiently expressed in Trvb1 and BHK cells (Zuk and Elferink, 2000). We suspect that Rab15 mutants may exert their effect on endocytic trafficking by sequestering effectors or by blocking essential rab effector interactions. If Mss4 functions as a chaperone stimulating GDP release and stabilizing Rab15 in a nucleotide-free state, over expression of Rab15 mutants which do not bind Mss4, would be predicted to suppress the inhibitory phenotype of wild type Rab15 on endosomal trafficking and endosome fusion (Zuk and Elferink, 2000). To test this, we compared the internalization of ^{125}I-labeled transferrin (^{125}I-Tfn) in HeLa cells transiently expressing HA epitope tagged forms of Rab15 (Zuk and Elferink, 1999), which bind Mss4 (wild type HARab15, HARab15 K46L and HARab15 m3), with cells expressing the mutant HARab15 K48Q, which does not bind Mss4 (**Fig. 9**). Internalization studies were not performed with HARab15 m1, due to technical limitations associated with poor expression of this mutant. Transfected HeLa cells were depleted of endogenous transferrin (Tfn), incubated with ^{125}I-Tfn for 1 h on ice followed by extensive washing to remove unbound ^{125}I-Tfn. The cells were then incubated at 37°C for 15 min to allow internalization of ^{125}I-Tfn (**Fig. 10**). Expression of wild type HARab15 in HeLa cells results in a 44% reduction in the maximal level of internalized ^{125}I-Tfn relative to mock-transfected cells, consistent with our previous studies (Zuk and Elferink, 2000). A comparable reduction in the level of internalized ^{125}I-Tfn was observed in HeLa's transiently expressing wild type HARab15 harboring the mutations K46L or m3 (38-42% respectively). However, cells transiently expressing HARab15 K48Q internalized significantly more ^{125}I-Tfn (50%) than cells expressing wild type HARab15 or HARab15 m3.

Western analysis indicates that these differences are not a consequence of discernible differences in the relative amount of endogenous Tfn Receptor (TfR) or exogenously expressed wild type and mutant HARab15 (**Fig. 10B**). Similarly, Western analysis confirmed that endogenous levels of Mss4 were not affected by the expression of wild type and mutant forms of HARab15 (**Fig. 11**). Taken together, these data suggest that the mutation K48Q counters the inhibitory effect of wild type Rab15 on receptor-mediated endocytosis. Mss4 has been reported to interact with a specific subset of rabs that regulate

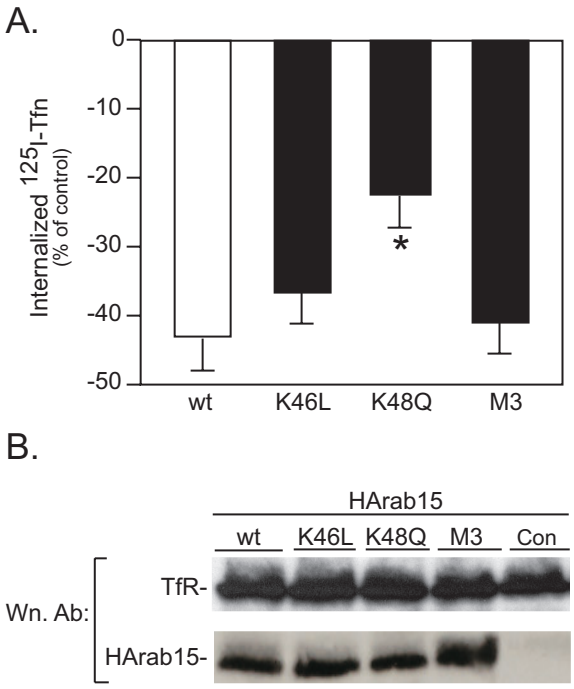

Figure 10: K48Q counters the inhibitory effect of wild type HArab15 on receptor-mediated endocytosis. A, HeLa cells were transiently transfected with wild type (wt) HArab15 and the indicated mutants, depleted of endogenous Tfn and incubated in medium containing ^{125}I-Tfn for 1 h at 4 °C. The cells were extensively washed to remove unbound ligand and incubated at 37 °C for 15 min to promote the internalization of bound ^{125}I-Tfn. Following internalization, the cells were washed to remove residual surface associated ^{125}I-Tfn and counted to quantitate total levels of internalized ^{125}I-Tfn as described previously (Zuk and Elferink 2000). All values represent means of triplicate experiments and are expressed as a percent of mock-transfected cells. B, Western analysis (Wn) using antibodies (Ab) against HArab15 and TfR indicate negligible differences in the relative amounts of transiently expressed HArab15 and endogenous TfR. Con represents mock-transfected cells. Significant differences observed between experimental conditions described in the text were verified using a one-way ANOVA with a Newman-Keuls post hoc test ($p \leq 0.05$) and are indicated by an asterisk.

distinct steps in exocytosis (Burton et al., 1994). To ensure that the observed increase in internalized ^{125}I-Tfn in cells expressing HARab15 K48Q does not result from a corresponding increase in the trafficking of the TfR through exocytic compartments, we directly compared the effect of this mutant with wild type HARab15 on the relative amount of cell surface associated TfR. HeLa cells transiently over expressing wild type HARab15, HARab15 m3, HARab15 K46L or the mutant HARab15 K48Q (which does not bind Mss4), were depleted of endogenous Tfn and incubated on ice for 30 min to reduce endocytic trafficking of the TfR. The cells were subsequently incubated with ^{125}I-Tfn for 1 hour to allow binding of the ligand to surface associated TfR, washed with PBS and the level of cell associated ^{125}I-Tfn determined. Comparable levels of surface bound ^{125}I-Tfn were detected in cells over expressing HARab15, HARab15 m3 and the non Mss4 binding mutant HARab15 K48Q (**Fig. 12**). Interestingly, a two-fold increase in the amount of surface associated ^{125}I-Tfn was observed in cells expressing HARab15 K46L relative to cells expressing wild type HARab15 or the mutants m3 and K48Q (**Fig. 12**). Taken together, these data indicate that interaction between Mss4 and Rab15 specifically affects early endocytosis rather than other aspects of membrane trafficking including exocytosis. We previously reported that wild type HARab15 reduces the level of homotypic early endosome fusion *in vitro* (Zuk and Elferink, 2000). To determine if interactions with Mss4 modulate Rab15 activity at the level of endosome fusion, we compared the effect of over expressing forms of HARab15 that bind Mss4 with the mutant HARab15 K48Q on homotypic early endosome fusion. Endosomal fractions labeled with either bHRP or avidin were mixed with cytosolic fractions prepared from HeLa cells over expressing wild type and mutant HARab15, under conditions that modulate membrane fusion of the labeled endosomal populations. Homotypic endosome fusion is monitored by the immunoisolation of bHRP-avidin complexes, which are assayed for avidin activity. Expression of wild type HARab15 results in 13.3 % reduction in endosome fusion relative to control untransfected cells (**Fig. 13**). Similarly, expression of HARab15 m3 and HARab15 K46L (Rab15 mutants which bind Mss4) results in a 17.0% and 24.9% reduction respectively in endosome fusion

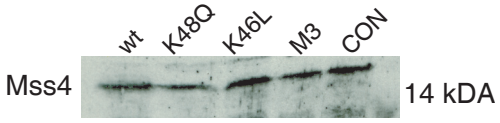

Figure 11: Mss4 expression is unaltered in HeLa cells transfected with HArab15 wt and mutants.

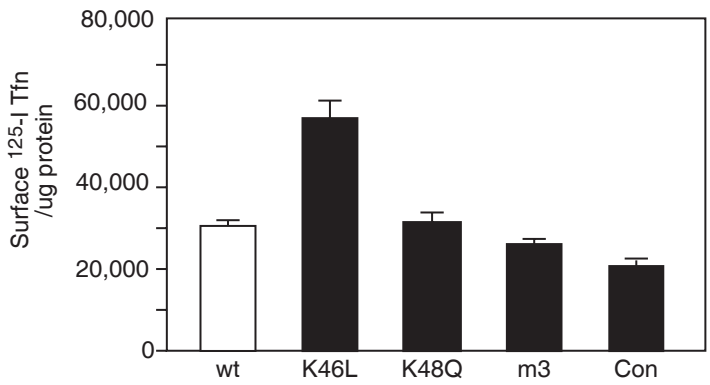

Figure 12: HArab15-K46L increases the relative amount of surface TfR. HeLa cells were ransiently transfected with wild type (wt) HArab15 and indicated mutants, depleted of endogenous transferrin for 1 h at 4 °C, incubated at 4 °C for 30 min to stop endocytosis, and subsequently incubated with ^{125}I-Tfn for 1 h. Cells were washed with PBS and surface-associated radiolabeled Tfn was measured in a gamma counter. Con denotes control, mock-transfected cells. Significant differences were observed between experimental and control conditions (one-way ANOVA, $p \leq 0.001$) as described in the text.

relative to control conditions. In contrast, expression of HARab15 K48Q reverses the inhibitory phenotype of wild type HARab15 resulting in a 5% increase in endosome fusion relative to control cells (**Fig. 13**). Thus, interactions with Mss4 modulate the inhibitory phenotype of wild type Rab15 on early endosome fusion *in vitro*, consistent with our observations on receptor-mediated endocytosis.

Discussion

Early/sorting endosomes are the nexus for several membrane trafficking pathways (Ceresa and Schmid, 2000; Leof, 2000). Accordingly, trafficking through this compartment is tightly regulated and relies on a fine balance involving multiple rabs functioning to either facilitate or inhibit membrane trafficking at discrete membrane transport steps. Several recent studies indicate that rabs function largely by promoting the recruitment of effectors, which in turn regulate distinct trafficking events (Segev, 2001a). Given the inhibitory phenotype of Rab15 on early endocytosis and the functional relationship between rab-effector interactions, we examined the effector-binding properties of Rab15. Rabaptin-5 and Rabenosyn-5 have been previously reported to bind Rab4 and Rab5 through distinct binding domains (Vitale et al., 1998; De Renzis et al., 2002). The multivalent binding

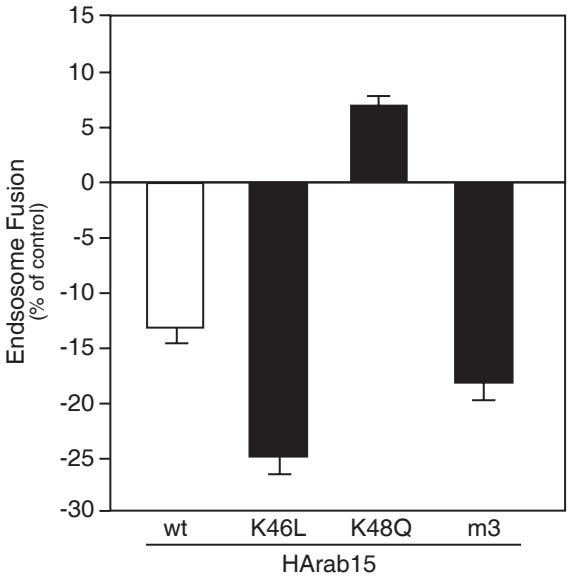

Figure 13: K48Q counters the inhibitory effect of HArab15 on early endosome fusion in vitro. Early/sorting endosomes labeled with biotinylated horseradish peroxidase or avidin were prepared from HeLa cells and incubated with cytosol prepared from untransfected control cells or HeLa cells transiently overexpressing wild type (white bar) or mutant (black bars) HArab15 as indicated. All values represent the means of triplicate experiments and are normalized with respect to protein concentration. Values are expressed as a percentage of base-line fusion observed using cytosols prepared from untransfected HeLa cells ±S.E.

properties of these Rab5 effectors and the opposing effects of Rab15 and Rab5 on early endocytic trafficking in cultured cells, are reconcilable with a model in which Rab15 and Rab5 compete for shared effectors (Zuk and Elferink, 2000). No interaction was detected between Rab15 and Rabaptin-5 or Rabex-5 in a yeast two-hybrid binding assay. Although Rabaptin-5 and Rabex-5 do not bind Rab15 directly, we cannot disregard the possibility that they may functionally interact via additional, unidentified binding partners. For example, the recruitment of Rabaptin-5 with Rab5-GTP on early endosomes is promoted by the guanine nucleotide exchange activity of Rabex-5 (Lippe et al., 2001). Recently however, Rabex-5 was also shown to bind early endosomes and clathrin-coated vesicle membranes independently of Rabaptin-5 and Rab5-GTP, indicating that additional binding partners for Rabex-5 exist (Lippe et al., 2001). Using a yeast two-hybrid binding assay, we detected a potential interaction between Rab15 and Rabenosyn-5. Although it is tempting to speculate on the impact of Rab15 interactions with Rabenosyn-5, the physiological significance of this interaction will require further verification.

This study identified Mss4 as a direct binding partner for Rab15. Mutational analysis indicates that lysine at position 48 (K48Q) is important for the binding of Rab15 to Mss4. Expression of HARab15 K48Q partially reverses the inhibitory phenotype of wild type HARab15 on receptor-mediated endocytosis and homotypic early endosome fusion *in vitro* without altering the relative amount of cell surface associated TfR, indicating

that interactions with Mss4 specifically modulate the inhibitory effect of Rab15 on early endocytosis. Functional analysis of Mss4 and its yeast homologue Dss4p previously demonstrated that these proteins promote GDP removal and stabilization of their target rabs in guanine nucleotide-free states (Burton et al., 1993). Accordingly, our binding studies confirm that Mss4 preferentially interacts with GDP-bound and nucleotide-free Rab15 mutants. In contrast to the GEF Rabex-5, Mss4 does not efficiently promote GTP recruitment (Esters et al., 2001). Therefore, Mss4 appears to function as a chaperone stabilizing its target rabs for subsequent interactions with additional factors that promote GTP binding and rab activation. Indeed, our functional data demonstrating that expression of HARab15 K48Q cannot fully compensate for the inhibitory effect of wild type Rab15 on receptor-mediated endocytosis, suggests that additional factors contribute to Rab15 activation. In this context, Mss4 may facilitate these interactions and increase the relative rate of Rab15 activation.

The three dimensional structure of Mss4 and two of its target GTPases (Rab3a and Sec4p) has been determined; however identification of the residues mediating binding of Mss4 and its yeast homolog Dss4p to their target rabs remains elusive (Zhu et al., 2001a; Zhu et al., 2001b). Structural analysis of Rab3a and Sec4p demonstrated that lysine residues at positions 46 and 48 reside on their surfaces accessible to effector molecules (Stroupe and Brunger, 2000; Zhu et al., 2001b). Our mutational analysis of Rab15 reveals that lysine 48 is essential for the interaction between Rab15 and Mss4. Conversely, mutation of the lysine at position 46 results in minimal loss of Mss4 binding to Rab15. This mirrors the observations in Rab3a where the corresponding mutation K60A did not impact Mss4's ability to promote GDP release from Rab3a *in vitro* (Zhu et al., 2001b). Modeling of the K46L and K48Q mutations on the surface of Rab3a and sec4p does not impart significant conformational changes in the predicted structure of these proteins (unpublished observations, D. Strick and L. Elferink) supporting the premise that the absence of Mss4 binding to HARab15 K48Q is not a result of major perturbations in the overall structure of Rab15.

Interestingly, an increase in the relative amount of TfR was observed on the surface of cells expressing HARab15 K46L. Yet, over expression of HARab15 K46L did not counter the inhibitory effect of wild type Rab15 on receptor-mediated endocytosis or *in vitro* endosome fusion. We reconcile this observation with our earlier studies showing that over expression of constitutively inactive GDP-bound HARab15 T22N in cultured cells promotes recycling of the TfR directly from early/sorting endosomes (Zuk and Elferink,

2000). A similar mechanism may account for the relative increase in surface associated TfR observed in cells expressing HARab15 K46L reported in this study.

Our observation that Mss4 binds Rab15 and regulates its activity during endocytosis is wholly consistent with the emerging concept that exocytosis and endocytic trafficking are functionally linked through shared components. For example the Rab5 effector EEA1 has been shown to bind Syntaxin 6, a SNARE implicated in trafficking between the Trans Golgi Network and early endosomes (Simonsen et al., 1999). In addition to binding Rab5, Rabex-5 and Rabaptin-5 directly interact with Rab33b, a GTPase implicated in retrograde transport from the Golgi to the E.R. (Zheng et al., 1998; Valsdottir et al., 2001). Recently, Rabaptin-5 was reported to form a complex with Rabphilin-3, a protein implicated in the control of exocytosis and endocytosis in the nerve terminal (Burns et al., 1998; Ohya et al., 1998; Schluter et al., 1999; Coppola et al., 2001). Mutational analysis of Rabphilin identified a point mutation (V61A) which disrupted binding to the exocytic GTPase Rab3a, but not Rabaptin-5. Moreover, expression of Rabphilin V61A in cultured cells promoted receptor-mediated endocytosis, implicating a role for rabphilin-Rabaptin-5 interactions in early endocytic events (Zheng et al., 1998). Similarly several genetic and molecular studies support a dual role for the synaptic vesicle protein Synaptotagmin 1 as a calcium responsive trigger that drives neurotransmitter release as well as synaptic vesicle recycling within the nerve terminal (Nonet et al., 1993; Geppert et al., 1994; Jorgensen et al., 1995; Littleton and Bellen, 1995; Reist et al., 1998).

In conclusion, our observation that Mss4 binds Rab15 and modulates its function *in vivo*, is to the best of our knowledge, the first report of an interaction between Mss4 and an endocytic protein. Our studies are consistent with an emerging model in which exocytic and endocytic pathways are functionally linked, expanding the role of Mss4 as a chaperone in early endocytic trafficking. Although our results indicate that Rab15 function involves at least one unique effector interaction, given the opposing effects of Rab15 and Rab5 on endocytic trafficking we anticipate that the transport steps regulated by these GTPases will likely intersect at some point in terms of a shared effector or accessory protein. However, the nature of these interactions remains to be determined and will require the functional analysis of additional interacting partners for Rab15.

CHAPTER 3: RAB15 EFFECTOR PROTEIN: A NOVEL PROTEIN FOR RECEPTOR RECYCLING FROM PERICENTRIOLAR RECYCLING ENDOSOMES[2]

Introduction

Early endosomes comprise two distinct compartments identified primarily through trafficking studies using the Transferrin Receptor (TfR). Internalized TfR resides on Early/ Sorting Endosomes (EEs) and the Pericentriolar Recycling Endosome (RE). In EEs, the TfR is sorted for direct recycling back to the plasma membrane or transported to lysosomes via late endosomes for down regulation. A slower route for receptor recycling occurs from the RE following receptor transit through EEs (Sheff et al., 1999; Maxfield and McGraw, 2004). An emerging model suggests that in addition to sorting desensitized receptors for down-regulation, EEs (and possibly the RE) provide local stores of intracellular receptors for rapid delivery to the cell surface (Szekeres et al., 1998a; Lin et al., 2000).

Rab GTPases are small monomeric GTPases that cycle between a GTP-bound and GDP-bound state that is regulated through molecular interactions with Guanine Nucleotide Exchange Factors and GTPase Activating Proteins. Furthermore, rabs are compartment specific, functioning to recruit effector molecules that regulate vesicle fusion, vesicle budding, receptor sorting and cytoskeletal interactions (Somsel and Wandinger-Ness, 2000; Segev, 2001a). We have previously characterized Rab15 as a GTPase involved in early endocytic trafficking. Unlike other endocytic rabs, Rab15 is novel in that it distributes between EEs and the RE and differentially regulates TfR recycling from these organelles (Zuk and Elferink, 1999; Zuk and Elferink, 2000). Over expression of Rab15 N121I (a mutant deficient in guanine nucleotide binding) stimulates endocytic recycling of TfR from the RE while having no effect on EE recycling. Conversely, Rab15 T22N (GDP-bound) stimulates both the fast and slow recycling of TfR from the EE and the RE respectively (Zuk and Elferink, 2000). The differential effects observed with these Rab15 mutants suggested a role for compartment specific Rab15 effectors in these distinct organelles.

In this chapter, I identified Rab15 Effector Protein (REP15) as a specific binding partner for Rab15. When over expressed in HeLa cells, REP15 colocalized with Rab15

[2] Strick, D.J. and Elferink, L.A. Submitted to Molecular and Cellular Biology as a Manuscript in 2005. Electron Microscopy was done in conjunction with Vsevold Popov (UTMB, Dept of Pathology)

and Rab11 on the RE. Consistent with its localization, REP15 over expression and siRNA mediated depletion of REP15 attenuated the recycling of internalized TfR from the RE and not EEs. Thus our data identify REP15 as a novel component for receptor recycling from the RE, further highlighting that EEs and the RE are mechanistically distinct endosomal compartments.

Materials and Methods

Reagents and Plasmids

Cell culture and general reagents were obtained from Invitrogen (Carlsbad, CA), Fisher Scientific (Hampton, NH) and Sigma-Aldrich St Louis, MO) unless specified otherwise. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Plasmids encoding amino terminal HA-tagged wild type and mutant Rab15 in pCI-Neo and pLexA, and a HeLa cell library pre-transformed into EGY187 (Mat α) have been described elsewhere (Strick et al., 2002). Plasmids encoding Rab11 cDNAs were generous gifts from R. Prekeris (University of Colorado Health Sciences Center, Denver, CO). For yeast two-hybrid analysis, Rab11 cDNA's encoding Q70L and S25N mutations were amplified by PCR using specific primer sets described elsewhere and sub-cloned directly into the EcoR1 site of pLexA. Human REP15 lacking an amino terminal tag was PCR amplified (upper primer: 5'-GAAATGGGGCAGAAAGCATCGCAA-3', lower primer: 5'-GGCTCTAGATCAGAGAATGCTGATATAAAC-3') and subcloned directly in PCR3.1 (Invitrogen, Carlsbad, CA). Human REP15 containing an amino terminal cMyc (MEQKLISEEDL) or HA (MYPYDVPDYA) epitope were PCR amplified using the lower primer described above in combination with the following upper primers; cMyc: 5'GCTGT AGAATGGAACAAAAATTAATCTCAGAAGAAGATCTGGGGCAGAAAGCATCGC AAC-3' and HA: 5'-GATATCATGTACCCTTATGATGTGCCAG-3'. The resulting PCR products were subcloned into PCR3.1 (Invitrogen, Carlsbad, CA). Cell lines - cMycREP15 cells were generated by transfecting HeLa cells with pCR3.1 cMycREP15. Following transfection, the cells were selected in HeLa cell medium containing 400 ug/mL of G418 to generate cell lines expressing cMycREP15.

Cell Culture, Transfections and TfR trafficking Assays

All cell culture, transfections and uptake studies have been described elsewhere (Zuk and Elferink, 1999; Strick et al., 2002). Membrane fractions enriched in early or late

endosomes were prepared by sucrose flotation gradient fractionation as previously described (Prekeris et al., 2000; Zuk and Elferink, 2000; Yan et al., 2004). Cell surface biotinylation assays were performed as described (Schmidt et al., 1997). siRNA depletion experiments were performed using commercial control (D-001210-01-2) and REP15 (M-030132-00) siRNAs (Dharmacon, Lafayette CO) and transfected into cells using Lipofectamine™ 2000 reagent (Invitrogen).

Immunoelectron Microscopy

cMycREP15 cell monolayers were fixed *in situ* in 2.5% formaldehyde and 0.1% glutaraldehyde in 0.05 M cacodylate buffer pH 7.2 (Popov et al., 2000), stained in *en bloc* with 2% uranyl acetate and dehyrdrated in 50% and 75% ethanol and embedded in LR White Resin (Structure Probe). Primary antibodies (9B11 and Rab11) were used at 1:500 and the grids were washed and stained with Goat Anti-Mouse IgG (10nm) (RPN431 Amersham Life Science, Arlington Hts IL) or Goat Anti Rabbit (15 nm) (RPN422, Amersham) and imaged as described (Popov et al., 2000).

Cell Surface Biotinylation

Transfected HeLa cells were rapidly cooled to 4°C, washed twice with ice-cold PBS supplemented with 1mM $CaCl_2$ and 1mM $MgCl_2$ (PBS^{++}) followed by incubation in PBS^{++} containing 0.250 mg/mL of Sulfo-NHS-SS-Biotin (Pierce Biotechnology, Rockford, IL) for 30 min at 4°C. Excess biotin was quenched with 3 ice-cold washes of PBS^{++} containing 50 mM Glycine for 30 min each, followed by 3 washes in PBS^{++} only. Cells were shifted to 37°C for the specified time in DMEM containing 2 mg/mL BSA (IM) (Sigma Aldrich) to promote TfR internalization. Cells were cooled to 4°C to terminate endocytic trafficking, washed for 10 min once with ice-cold Washing Buffer (150 mM NaCl, 1 mM EDTA, 0.2% BSA, 20 mM Tris-HCL, pH 8.6). Cells were stripped for 1 hr at 4°C in freshly prepared MesNa-Wash (Washing buffer containing 20 mM MESNA) to remove residual cell surface biotin. Cells were lyzed in Lysis Buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris pH 7.4, 0.5% IGEPAL, 0.5% Triton-X-100) and 250 μg of protein (BCA Assay, Pierce) incubated with 30 ul of Streptavidin Agarose (Sigma Aldrich) overnight at 4°C. Following incubation, the beads were washed three times with lysis buffer, once with 50 mM Tris-Cl pH 7.5 and biotinylated protein examined by Western analysis. Thirty μg of the cell lysate was routinely examined by Western Analysis to confirm expression of REP15 and to verify that Rab15 over expression did not alter the total amount of TfR. Resulting digitized blots were

quantified using AlphaEase FC v.3.1.2. (AlphaInnotech, San Leandro CA) and normalized to the band intensity of surface biotinylated cells at 4°C representing total surface TfR at the time of biotinylation. The results represent means ± S.E. of triplicate experiments. Results were subjected to a One Way ANOVA with a Newman-Keuls test to determine statistical significance between experimental groups (Prism 3.0 GraphPad).

TfR Recycling Assays

Surface biotinylated cells were incubated at 37°C for 10 min in DMEM with 2 mg/mL BSA to promote TfR uptake into EEs. Cells were placed at 4°C, washed for 1 hr in MESNa buffer (50 mM MESNa 150 mM NaCl, 1 mM EDTA, 0.2% BSA, 20 mM Tris, pH 8.6) and then multiple ice-cold washes in PBS^{++}. Cells were shifted to 37°C for 20 min to promote TfR recycling from EEs and TfR transport to the RE. Cells were shifted to 4°C and MesNa washed as described above to cleave biotin from recycled TfR. One set of plates remained at 4°C as a measure of total TfR internalized into the RE. The second set was incubated at 37°C for 10-30 min to allow TfR recycling from the RE. Cell surface biotin was removed with a third MESNa wash and lysates were examined for internalized TfR as described above (Schmidt et al., 1997). Values were expressed as % TfR recycled from the RE and represent means ± S.E. for quadruplicate experiments.

Tfn-ELISA Assays

Tfn-depleted cells were incubated in DMEM with 5 µg/mL of Biotinylated Tfn (B-Tfn, Pierce) for 1 hr at 16°C to load EEs. Non internalized ligand was removed by 3 alternating washes with PBS (pH 4.2) and PBS (2 mg/mL BSA). The cells were incubated at 37°C for 0-10 min in DMEM with a 100-fold excess of unlabeled Tfn, to promote ligand recycling from the EEs. In studies measuring Tfn recycling from the RE, the cells were subjected to a 10 min chase at 37°C followed washes with PBS (pH 4.2) and PBS (2 mg/mL BSA). The cells were incubated at 37°C for the indicated times to promote recycling from the RE. Recycled B-Tfn in the chase media was bound to Tfn antibody coated plates (Bethyl Laboratories, Montgomery, TX). Bound B-Tfn was detected using Streptavidin HRP and Quantablue™ Fluorogenic Peroxidase Substrate (Pierce, Rockfield, IL) and measured in a Spectromax Gemini Fluorescent microplate reader (Molecular Devices). Values were normalized to protein concentration and expressed as relative fluorescent units (Rfu) ± S.E.

Antibodies

HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals, Indianapolis, IN), monoclonal human TfR H68.4, polyclonal Rab11 antibodies (Zymed Laboratories, San Francisco, CA), cMyc monoclonal 9B11 antibody (Cell Signaling Technology, Beverly, MA) were purchased as indicated. Alexa594-labeled Transferrin, goat anti mouse and rabbit secondary antibodies coupled to Alexa488 or Alexa594 were purchased from Molecular Probes (Eugene, OR). Rabbit polyclonal antiserum for Rab15 was generated against the synthetic peptide (NH2-CQAHRKELDGLRTC-COOH) (Covance, Denver, PA). To prepare an antibody against REP15, Human REP15 (residues 3-236) was cloned directly into pGEX-4T (Clontech) expressed as a GST-fusion and purified by affinity chromatography using glutathione agarose beads (Sigma). When thrombin cleaved, recombinant REP15 devoid of GST was insoluble; therefore insoluble REP15 was used as an antigen to prepare a rabbit polyclonal antisera (Covance, Denver PA). The resulting REP15 anti-sera was purified by initial passage over GST immobilized on AminoLink™ Coupling Gel and the flow through subsequently purified against GST-REP15 immobilized to AminoLink™ Coupling Gel (Pierce,). Antibody specificity was tested by demonstrating that REP15 antibody specifically recognized GST-REP15 as well as REP15 transiently expressed in HeLa cells. No Immunoreactivity was detected with GST nor with untransfected HeLa cells demonstrating the specificity of the antibody.

Confocal Microscopy and Colocalization Studies

For confocal analysis HeLa cells were seeded onto Matrigel™ (BD Biosciences) coated cover glasses (Fisher Scientific) and transfected with the appropriate plasmid DNA for 48 hr. Cells were fixed in 4% paraformaldehyde (Ted Pella Inc. Redding, CA) in PBS for 10 min and blocked and permeabilized with 10% goat sera (Hyclone), 0.02% Saponin and 1% BSA in PBS and incubated subsequently the appropriate antibody in PBS supplemented with 1% BSA, 0.02% saponin overnight at 4˚C. Cells were washed 3 times with PBS and incubated with the appropriate secondary antibody coupled to $Alexa^{488}$ or $Alexa^{594}$ in PBS containing 1% BSA, 0.02% saponin for 1 h at room temperature. The cover glasses were mounted onto glass slides using Fluorsave™ Reagent (Calbiochem, San Diego, USA). Images were generated using an Olympus BX50 epifluorescent microscope equipped with an Olympus Leeds Confocal Microscopy system with Argon and Krypton lasers with excitations at 488 and 568 nm, respectively. Image generation was done with

a Plan-Apo 100X/1.35 oil immersion objective and Fluoview 2.0 imaging Software. To avoid unbiased selection, the two channels were imaged separately and not merged until acquisition was complete. Before acquisition, PMT and the laser power adjustments were optimized for each channel to avoid saturation of a particular channel. Images were processed using Adobe Photoshop 6.0 (Adobe) , were saved as TIFF files and imported into Metamorph 4.6 (Universal Imaging Corp., West Chester, PA) and analyzed using the Measure Colocalization Application. The percentage of overlap was calculated as an area of overlap expressed and normalized to the total area of a given cell. The final values represent the mean ± S.E. for 6 - 10 randomly selected cells from three independent experiments.

Membrane Fractionation Studies

For membrane fractionation studies, Post Nuclear Supernatants (PNS) were prepared from cells transiently expressing HA or cMyc tagged REP15. Cells were harvested in 10 mM HEPES pH 7.4 and 1 mM EDTA supplemented with protease inhibitors and lyzed by 10 passes with a ball bearing homogenizer (0.1 um bore) followed by centrifugation at 1000 x g, 4˚C for 10 min.

Results

REP15, a Novel Binding Partner for Rab15-GTP

To understand how Rab15 differentially regulates receptor trafficking through EEs and the RE, it is essential to identify functional binding partners for this novel GTPase. Using a yeast two-hybrid approach, I recently reported that Mammalian Suppressor of Sec4 (Mss4) directly interacted with GDP-bound and nucleotide-free mutants of Rab15. Moreover, interactions with Mss4 modulated the inhibitory effect of wild type Rab15 on TfR trafficking through early endosomes in HeLa cells and on EE fusion *in vitro* (Chapter 2). To identify potential downstream effectors for GTP-Rab15, I performed additional yeast two-hybrid screens of a HeLa cell library using GTP-bound, Rab15 Q67L as bait. I isolated two clones that shared identical sequences encoding an open reading frame of 233 amino acids (**Fig. 14A**). BLAST searches of the human and mouse genomes indicated that it shared 100% and 73.8% identity respectively with amino acids 3-236 of a hypothetical protein of unknown function (accession numbers XP_370686 and NP_079896, respectively). Detailed analyses of the predicted amino acid sequence using the

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ATGGGGCAGAAAGCATCGCAACAGTTGGCTCTGAAGGACAGCAAAGAGGTGCCCGTCGTC
 M  G  Q  K  A  S  Q  Q  L  A  L  K  D  S  K  E  V  P  V  V   20
TGTGAGGTGGTCAGTGAAGCTATAGTCCATGCAGCTCAGAAACTGAAGGAGTACCTTGGA
 C  E  V  V  S  E  A  I  V  H  A  A  Q  K  L  K  E  Y  L  G   40
TTTGAATATCCTCCAAGTAAACTCTGCCCAGCTGCAAATACTCTGAATGAGATCTTCTTA
 F  E  Y  P  P  S  K  L  C  P  A  A  N  T  L  N  E  I  F  L   60
ATCCATTTCATCACTTTCTGCCAAGAAAAGGGAGTTGATGAGTGGCTGACCACCACCAAG
 I  H  F  I  T  F  C  Q  E  K  G  V  D  E  W  L  T  T  T  K   80
ATGACCAAGCACCAAGCCTTCCTGTTTGGTGCAGACTGGATTTGGACCTTTTGGGGATCC
 M  T  K  H  Q  A  F  L  F  G  A  D  W  I  W  T  F  W  G  S  100
AACAAGCAAATAAAGCTTCAGCTCGCAGTACAGACTCTGCAGATGTCTTCACCTCCTCCT
 N  K  Q  I  K  L  Q  L  A  V  Q  T  L  Q  M  S  S  P  P  P  120
GTGGAATCTAAGCCTTGTGACCTTTCCAATCCAGAATCAAGGGTAGAGGAGTCTTCCTGG
 V  E  S  K  P  C  D  L  S  N  P  E  S  R  V  E  E  S  S  W  140
AAGAAAAGTAGATTTGATAAGCTGGAAGAATTCTGTAACTTAATAGGAGAGGATTGCCTG
 K  K  S  R  F  D  K  L  E  E  F  C  N  L  I  G  E  D  C  L  160
GGTCTGTTTATCATCTTTGGTATGCCAGGAAAGCCTAAAGACATCAGGGGAGTTGTCCTG
 G  L  F  I  I  F  G  M  P  G  K  P  K  D  I  R  G  V  V  L  180
GACAGTGTCAAAAGTCAGATGGTGAGGAGCCATCTGCCAGGAGGGAAGGCTGTGGCTCAG
 D  S  V  K  S  Q  M  V  R  S  H  L  P  G  G  K  A  V  A  Q  200
TTTGTCCTGGAAACTGAAGATTGTGTGTTCATCAAAGAGCTGCTCAGAAATTGTCTGAGT
 F  V  L  E  T  E  D  C  V  F  I  K  E  L  L  R  N  C  L  S  220
AAGAAAGACGGGCTGAGAGAGGTGGGCAAGGTTTATATCAGCATTCTCTGA
 K  K  D  G  L  R  E  V  G  K  V  Y  I  S  I  L  *          236
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B.

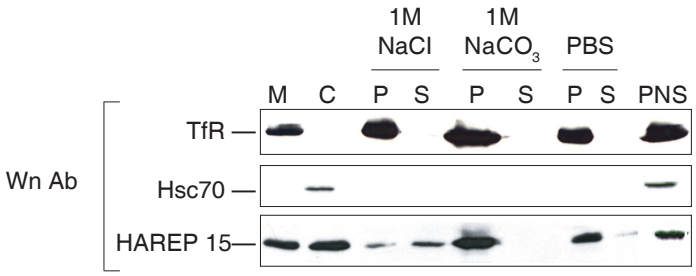


Figure 14: REP15 binds to membranes. A, Predicted cDNA sequence of REP15. B, Postnuclear supernatants (PNS) prepared from HeLa cells transiently over expressing HA-tagged REP15 were fractionated into high speed membrane (M) and cytosol (C) fractions and analyzed by Western analysis (Wn) for TfR, Heat shock cytosolic 70 protein (Hsc70) and REP15 (REP15). Membrane pellets were washed with 1 M NaCl, 1 M $NaCO_3$ pH 11.0 or PBS and the supernatants (S) and washed membranes (P) were analyzed by Western analysis.

SMART database detected no putative functional domains and no similarity with any other rab effector identified to date. Accordingly, I named the predicted protein Rab15 effector protein or REP15 (Accession no. AY662682).

As a binding partner for Rab15, I predicted that REP15 would also be enriched in membrane fractions. However, since REP15 does not contain any apparent transmembrane or lipid modification motifs for membrane association, we examined its putative membrane binding properties using biochemical criteria. A post nuclear supernatant was prepared from HeLa cells transiently expressing HA-tagged REP15, fractionated into membrane and cytosolic fractions by centrifugation, and analyzed by Western analysis (**Fig. 14B**). When over expressed in HeLa cells, REP15 distributes between membrane and cytosolic fractions akin to our earlier reports of Rab15 (Nagata et al., 1992; Zuk and Elferink, 1999; Zuk and Elferink, 2000). Subsequent biochemical analysis of the membrane fraction revealed that REP15 was partially extracted from salt washed membranes (NaCl and PBS) but not from membranes washed with a high pH buffer (**Fig. 14B**). These data suggest that the membrane binding properties of REP15 likely involve protein-protein interactions rather than binding via neutral phospholipids.

REP15 specifically binds GTP-bound Rab15

We next examined the guanine nucleotide-dependence of REP15 binding to Rab15 using a yeast two-hybrid binding assay. High levels of β-galactosidase activity were detected in strains co-expressing REP15 with GTP-bound forms of Rab15 (wild type or the GTP-bound mutant Q67L), but not the GDP-bound (T22N) or nucleotide-free (N121I) mutants of Rab15 (**Fig. 15A**). Western analysis confirmed that Rab15 was expressed at comparable levels in the diploid strains, demonstrating the specificity of the results (**Fig. 15A**). I verified the guanine nucleotide-dependence of Rab15 binding to REP15 by performing pull-down studies. We previously reported that endogenous Rab15 is not readily detected in BHK, CHO, Trvb-1 and HeLa cells using our existing Rab15 antibodies (Zuk and Elferink, 1999; Zuk and Elferink 2000). Thus, we performed binding studies using HeLa cell lysates transiently over expressing wild type, HA-tagged Rab15 (HARab15) and full-length REP15 expressed as a recombinant GST-fusion protein. Prior to incubation with recombinant REP15, cell lysates were incubated in the absence or presence of GDPβS or GTPγS to lock wild type Rab15 into a GDP or GTP-bound state respectively. The lysates were incubated with GST-REP15 immobilized on glutathione agarose beads and the resulting REP15-HARab15 complexes were isolated and examined by Western analysis

(**Fig. 15B**). REP15 bound efficiently to wild type Rab15 in the presence of GTPγS. Conversely, no REP15 binding was detected with wild type Rab15 in the presence or absence of GDPβS or with beads containing GST only. Taken together, these data indicate that REP15 binds guanine nucleotide-dependently to GTP-bound forms of Rab15.

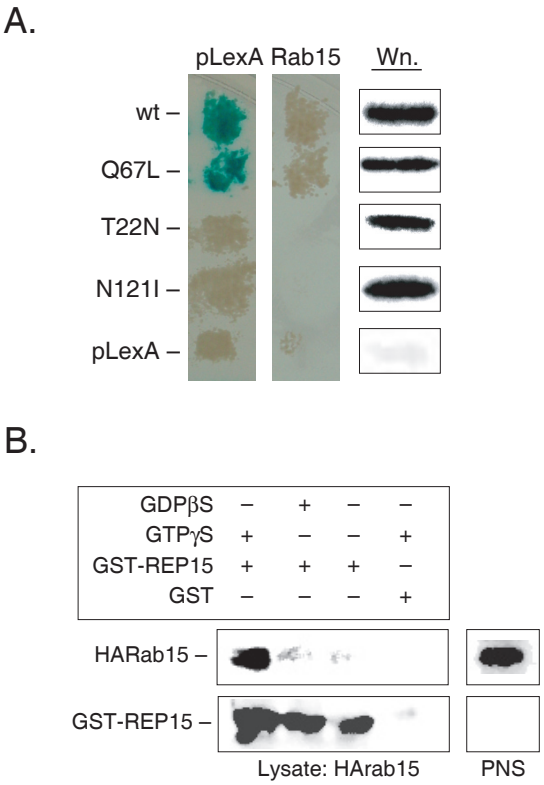


Figure 15: A, REP15 was coexpressed with wild type (wt) Rab15 or the indicated mutants in a yeast two-hybrid assay. Blue colonies on X-Gal or growth on Leu- plates indicate binding between REP15 and Rab15. Western Analysis (Wn) confirmed comparable levels of Rab15 expression in all yeast strains. B, A Postnuclear supernatant (PNS) prepared from HeLa cells expressing wild type HA-tagged Rab15 (HARab15) were incubated in the absence (-) or presence (+) of GTPγS or GDPβS and used in a pull down assay with GST-REP15.

REP15 localizes with Rab15-GTP on endosomal membranes

To determine the subcellular localization of REP15, HeLa cells were cotransfected with REP15 containing an amino terminal cMyc epitope and wild type or GTP-bound Rab15. Their colocalization was examined by confocal microscopy (**Fig. 16A**). Punctate Rab15 staining was observed in the periphery of the cytoplasm as well as the perinuclear region of the cell consistent with the distribution of Rab15 between EEs and REs respectively. Conversely, strong REP15 staining was detected principally in the perinuclear region of transfected cells coinciding partially with Rab15 staining. Quantitative analysis of the confocal micrographs revealed that 54 ± 5.2 % and 56 ± 4.4 % of REP15 colocalized with wild type Rab15 and Rab15 Q67L, respectively. To ensure that the presence of an amino terminal epitope did not interfere with the subcellular localization of REP15, we prepared a polyclonal antibody against recombinant REP15 and confirmed its specificity by Western analysis. As shown in **Fig. 16B**, anti-REP15 reacted only with GST-REP15

and not with GST only. Anti-REP15 did not detect endogenous REP15 protein in whole cell lysates prepared from in HeLa cells by Western analysis but did detect cMycREP15 transiently over expressed in HeLa cells (**Fig. 16B**). Therefore, to confirm the intracellular distribution of untagged REP15, confocal microscopy was performed and quantified using HeLa cells coexpressing untagged REP15 with wild type or GTP-bound Rab15 Q67L. Consistent with our studies using cMycREP15, untagged REP15 colocalized with wild type Rab15 and GTP-bound Rab15 Q67L in the perinuclear region of transfected cells (63 ± 3.4 % and 70 ± 4.4 %, respectively) (**Fig. 16C**). These data indicated that in HeLa cells, REP15 colocalized with a perinuclear pool of Rab15-GTP and that the presence of an amino-terminal epitope did not interfere with its subcellular localization.

To confirm the endosomal localization of transiently expressed cMycREP15 in HeLa cells, fractions enriched in EE/RE membranes or LE membranes, were isolated by sucrose gradient fractionation and the fractions analyzed by Western analysis. cMycREP15 was detected in early endosomal membranes enriched in Early Endosomal Antigen 1, the Transferrin Receptor (TfR) and the endosomal tSNARE Syntaxin 13 which are known markers for EEs (Trischler et al., 1999), (**Fig. 16D**). Conversely, no REP15 immunoreactivity was detected with down regulated TfR in fractions enriched for LE membranes. Moreover, no immunoreactivity of the early endosome markers EEA1 and Syntaxin 13 were detected in the LE fractions, thus demonstrating that REP15 associates with EE membranes, consistent with a role as a binding partner for Rab15-GTP.

We previously reported that Rab15 distributed between peripheral EEs and the RE (Zuk and Elferink, 1999) where it functioned to differentially regulate TfR transport (Zuk and Elferink, 2000). Our data showing REP15 colocalization with Rab15 in a perinuclear region in HeLa cells suggested that REP15 may be a compartment specific effector for Rab15 at the RE. Consistent with this, no significant overlap in REP15 staining was observed with endogenous EEA1 and Rab4, specific markers for EEs **(Fig. 17)** (Bottger et al., 1996; Simonsen et al., 1998). Conversely, REP15 staining overlapped strongly with endogenous Rab11 (56 ± 7.1 %), an established marker for the RE (Ullrich et al., 1996) (**Fig. 18A**).

To verify the association of REP15 with the RE, we examined the colocalization of REP15 with internalized Tfn, under chase conditions that promoted Tfn transport from EEs to the RE. HeLa cells stably expressing cMycREP15 were incubated with Alexa^{594}-labeled Tfn (Alexa-Tfn) by incubation at 16°C for 1 h to promote ligand uptake into EEs. The cells

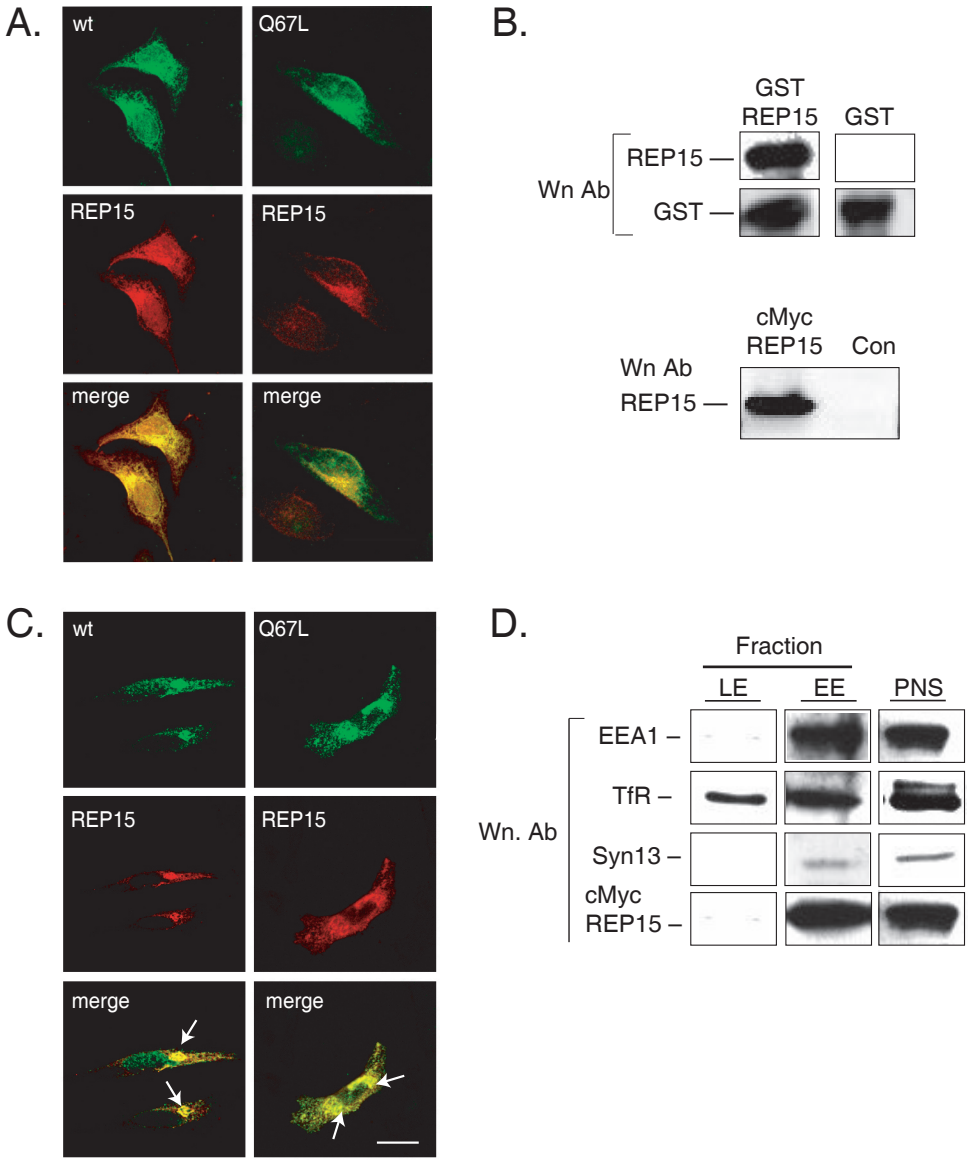


Figure 16: REP15 localizes to EE membranes. A, HeLa cells transiently coexpressing cMycREP15 with wild type (wt) or GTP-bound (Q67L) Rab15 were analyzed by confocal microscopy. B, Western analysis (Wn) of recombinant GST or GST-REP15 using anti-REP15 (REP15) or anti-GST antibodies (Ab). Cell lysates prepared from mock-transfected (Con) or HeLa cells transiently over expressing cMycREP15 were examined by Western analysis (Wn) using the affinity purified anti-REP15 antibody (Ab). C, HeLa cells transiently coexpressing REP15 with wild type Rab15 (wt) or Rab15-Q67L (Q67L) were examined by immunofluorescent confocal microscopy. Arrows indicate areas of colocalization (yellow) in the merged (merge) image. Scale 10 um D, Fractions enriched in Late Endosomes (LE) and Early Endosomes (EE) were prepared by sucrose flotation gradient centrifugation from a postnuclear supernatant (PNS), TCA precipitated and analyzed by Western analysis (Wn) as indicated.

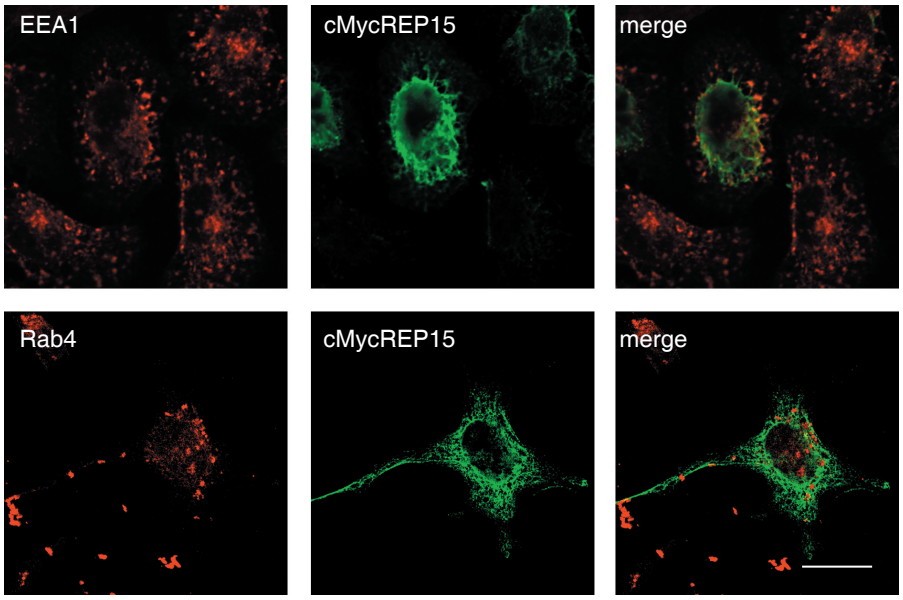


Figure 17: REP15 does not colocalize with early endosomal markers. HeLa cells transiently expressing cMycREP15 were examined by confocal microscopy using antibodies against endogenous EEA1 or Rab4. Merged images (merge) are shown. Scale 10 um.

were washed, then chased for 1 or 30 min at 37°C to promote TfR recycling from EEs or transport to the RE and then analyzed for colocalization with EEA1, Rab11 and REP15. Following a 1 min chase, high amounts of Alexa-Tfn colocalized with EEA1 in EEs (57.9 ±13.6%); lower levels of ligand colocalized with Rab11 and REP15 (11.1 ± 3.0 % and 23.8 ± 3.8 % respectively). Conversely, a 30 min chase at 37°C resulted in decreased colocalization with EEA1 and a concomitant increase in Alexa-Tfn costaining with REP15 and Rab11 (54.4 ± 1.8 % and 68.1 ± 4.3 % respectively) consistent with ligand transport to the RE **(Fig. 18B)**. Immuno-precipitation studies of homogenized membrane fragments using a Rab11 antibody confirmed that REP15 coprecipitated with Rab11 and the TfR but not with the early endosomal marker EEA1 (**Fig. 18C**) indicating that REP15 localized to Rab11 positive organelles. To verify that REP15 localized to the RE we performed immuno-electron microscopy on cMycREP15 expressing cells. Due to technical problems, we were unable to perform costainings on Rab11 and REP15. Rab11 and REP15 localized to vesicles ranging in size from 100 - 150 nm (**Fig. 18D**), consistent with the size of the RE (Ullrich et al., 1996; Prekeris et al., 1998). Additionally, REP15 localized to membrane

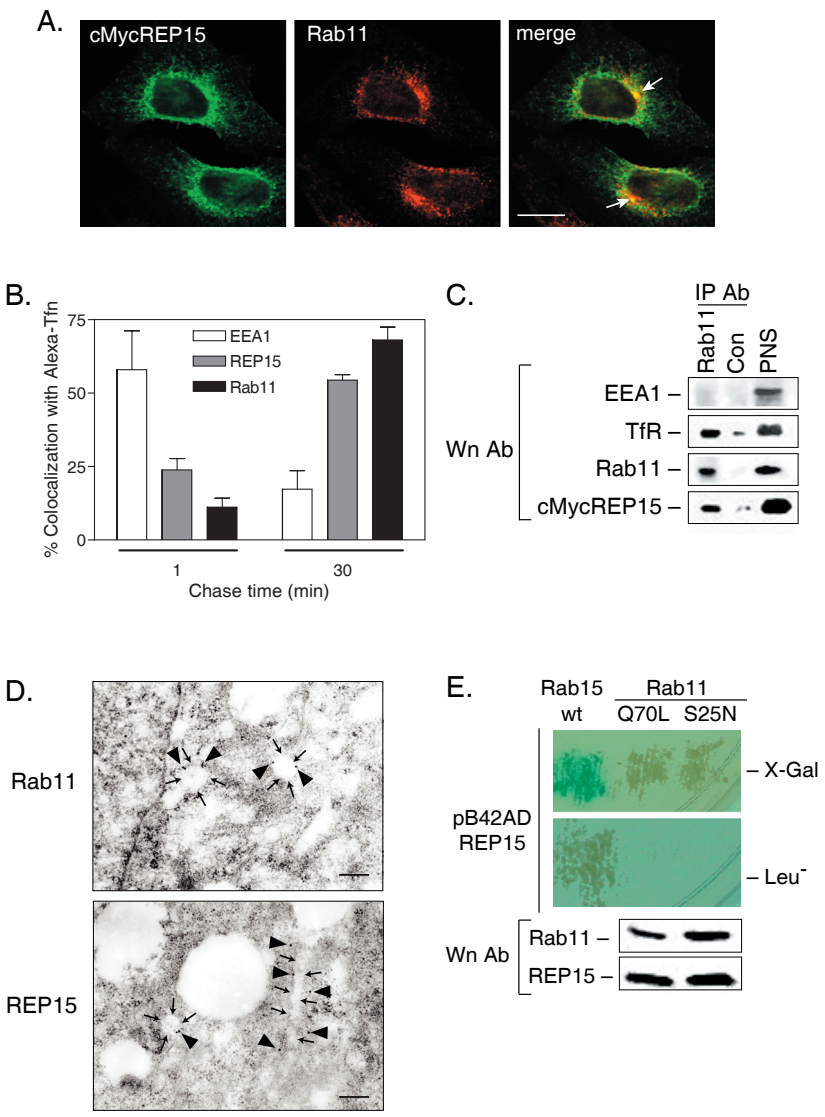


Figure 18: REP15 binds to the Rab11 positive RE. A, HeLa cells were stained for transiently expressed REP15 and endogenous Rab11 (Rab11). Arrows indicate colocalization (yellow) in the merged image. Scale 10 uM. B, Duplicate sets of HeLa cells expressing REP15 were loaded with Alexa-Tfn in EEs by incubation at 16°C for 1 hr to label EE's. Cells were pulse chased at 37°C for 1 min and 30 min and colocalization of Alexa-Tfn and EEA1, Rab11, and REP15 quantified by confocal microscopy. C, A PNS prepared from HeLa cells transiently expressing cMycREP15 was immunoprecipitated (IP) under non-denaturing conditions with antibodies (Ab) against Rab11 or with control antisera (Con) and examined by Western analysis (Wn) as indicated. D, REP15 was detected on vesicular and tubular membranes. HeLa's expressing cMycREP15 were prepared for immunoEM and stained for Rab11 (Top, 15 nm gold particles) or REP15 (bottom, 10 nm gold particles). Rab11 labeling (large arrows) was detected on vesicles (small arrowheads). REP15 labeling was detected in vesicles and in some cases, tubular membranes. Scale, 250 nm. E, REP15 did not interact with GTP-bound (Q70L) or GDP-bound (S25N) mutants of Rab11 in yeast two-hybrid assay. Western analysis (Wn) confirmed comparable levels of REP15 and Rab11 expression in the yeast strains.

tubules reminiscent of tubulated recycling endosomes (Prekeris et al., 1998).

The majority of Rab-GTP effectors identified to date interact specifically with their cognate Rab GTPase. However, some effectors have been shown to functionally interact with multiple Rabs involved in sequential steps in membrane transport. The effectors Rabaptin-5 and Rabenosyn-5 interact with GTP-bound Rab4 and Rab5 on EEs (Vitale et al., 1998; De Renzis et al., 2002). Whether these effectors functionally link Rab5-stimulated receptor uptake into EEs with Rab4-mediated receptor recycling from this compartment is currently unclear. The guanine nucleotide exchange chaperone Mammalian Suppressor of Sec4 (Mss4) binds multiple Rab GTPases involved in exocytosis, including Sec4, Ypt1, Rab1a, 3a, 8 and 10 (Burton et al., 1994; Burton et al., 1997). In addition, Mss4 directly regulates the inhibitory effect of Rab15 on receptor trafficking through EEs (Chapter 2). Our data demonstrating the coincident staining of REP15 with Rab11 and Rab15 on the RE raises the possibility that REP15 is a shared effector for these GTPases. In this context, REP15 may interact with Rab15 and Rab11 through distinct binding sites or alternatively, these interactions may be mutually exclusive events. To distinguish between these possibilities, we examined the Rab11 binding properties of REP15 using a yeast two-hybrid assay. No interaction was observed between REP15 and constitutively active, GTP-bound Rab11 (Q70L) or the GDP-bound mutant Rab11 S25N (**Fig. 18E**). Western analysis confirmed comparable levels of REP15 and Rab11 expression in the diploid strains, confirming the specificity of the results. Taken together, these data indicate that REP15 is an effector for Rab15-GTP on the RE.

REP15 regulates TfR recycling through the RE

Since REP15 colocalized with Rab15 on the RE, we examined the effect of REP15 over expression on TfR internalization and recycling from this organelle. To measure receptor internalization, cell surface TfR was biotinylated with NHS-SS-Biotin at 4°C, that is cleaved by washing with the cell impermeable reducing agent MESNa (Schmidt et al., 1997) (**Fig. 19A and B**). In control cells, the maximum level of internalized receptor was 26.3 ± 6.9% at 15 min and 19.5 ± 7.1% at 30 min, consistent with TfR recycling from EEs and the RE respectively (**Fig. 19C**). However, REP15 over expression caused a 52.6 ± 9.2% and 83.7 ± 7.6% increase in internalized TfR at 15 and 30 min respectively. No significant difference was observed between the internalization rate constants for TfR in REP15 expressing and control cells (**Fig. 19D**) indicating that the increase in internal TfR in these cells is not due to differences in TfR internalization.

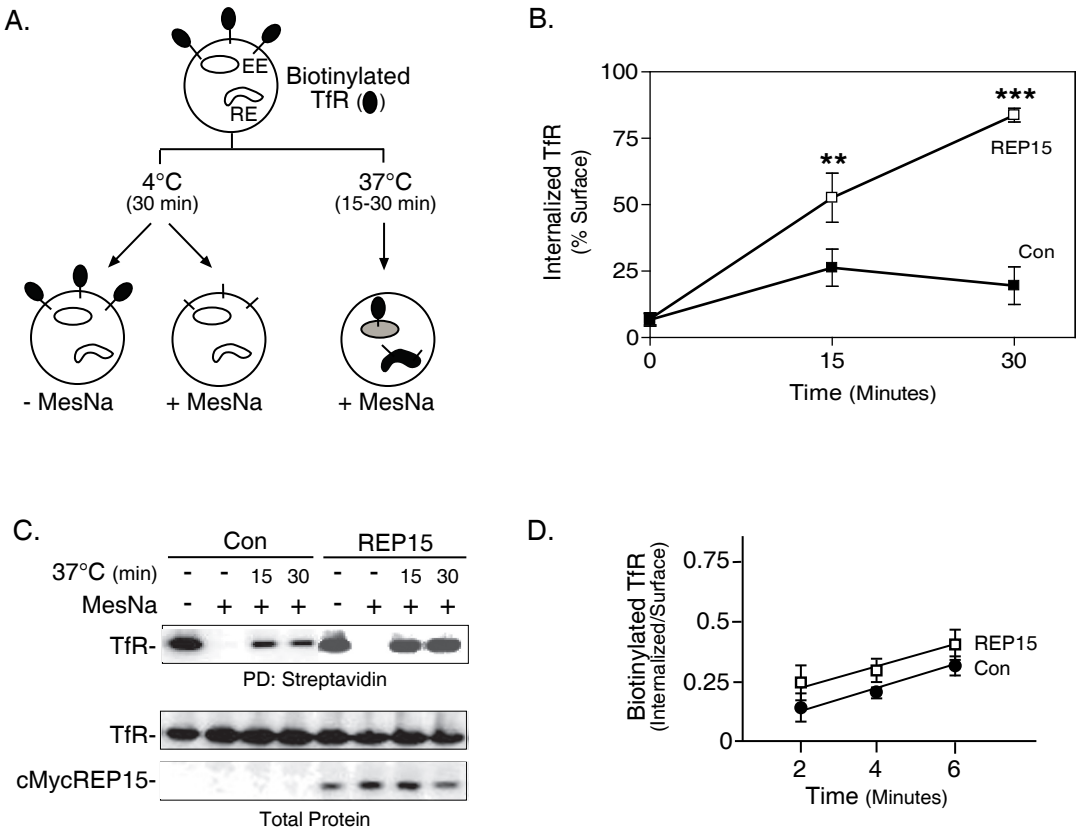


Figure 19: Internalized TfR accumulates in cells over expressing REP15. A, Schematic diagram of the cell surface biotinylation assay for TfR internalization. B, Representative example of TfR internalization in HeLa cells transiently expressing cMycREP15 (REP15) or mock-transfected cells (Con). Biotinylated cell were incubated at 4°C (-) block endocytosis or at 37°C for 15 or 30 min to allow TfR uptake. MESNa (+ or -) washes were used to remove the cell surface biotin. Total protein in lysates and internalized TfR following Streptavidin pull downs (PDs) were examined by Western analysis as indicated. C, TfR endocytosis was quantified using surface biotinylation assays. Values represent the mean ± S.E. of endocytosed TfR from 3 independent assays and are expressed as a % of the total (surface) TfR. ** (ANOVA, $p<0.01$) and *** (ANOVA, $p<0.001$) indicate statistical differences between experimental and control sets. D, Surface biotinylated cells over expressing REP15 or mock transfected cells (Con) were incubated at 37°C for 2, 4 or 6 min and the amount of internalized TfR quantified as described above. The ratio of internalized TfR versus surface ± S.E. were plotted against time and rate constants for TfR internalization (Ke) in REP15 expressing and control cells calculated as a linear regression coefficients (Ke of 0.045 ± 0.002 min-1 and 0.049 ± 0.006 min^{-1} respectively).

To determine if the REP15-mediated increase in TfR in the RE was due to increased receptor transport or defective recycling, we performed a modified cell surface biotinylation assay (**Fig. 20A**). Mock transfected control cells and cells transiently expressing REP15 were incubated at 37°C for 10 min to allow internalization of surface biotinylated receptor into the EE. Surface biotin was removed with a primary MesNa wash at 4°C and the cells shifted to 37°C for 20 min to promote receptor recycling from the EE as well as receptor transport to the RE. Following a second MesNa wash the cells were shifted to 37°C for increasing periods of time to promote receptor recycling from the RE. One set of cells were incubated at 4°C as a control for the maximal level of internalized receptor in the RE. After a third MesNa wash to remove surface associated biotin, the cells were lysed and the biotinylated TfR was detected by Streptavidin pull down and Western analysis. The amount of recycled TfR was calculated at the percent TfR recycled from the RE. In control cells, 70.6 ± 13.8% of the internalized TfR recycled to the cell surface by 30 mins (**Fig. 20B**). Conversely, only 14.7 ± 6.8 % of the internalized TfR recycled to the cell surface in cMycREP15 expressing cells indicating that REP15 over expression reduces TfR exit from the RE.

In order to verify that inhibition of TfR recycling is not due to faulty EE recycling, we performed studies that measured recycling from the EE. In these assays, cells were labeled with biotinylated Tfn (B-Tfn) at 16°C for 1 h, washed and chased for 5 and 10 min at 37°C. The amount of B-Tfn recycled into the media from EEs was quantified using a B-Tfn ELISA (see *Materials and Methods*). We detected comparable levels of B-Tfn in the chase media from REP15 expressing and untransfected, control HeLa cells confirming that REP15 does not regulate TfR recycling from EEs (**Fig. 20C**).

To confirm the effect of REP15 on TfR recycling from the RE, we used siRNAs to deplete REP15. The specificity of the REP15 siRNA was verified using HeLa cells stably expressing cMycREP15. Western analysis confirmed that transfection with REP15 siRNA depleted 80% - 90% of cMycREP15 with no effect on the level of endogenous TfR, β-Actin, EEA1 and the endocytic GTPases Rab11 and Rab5. Comparable levels of REP15, TfR, β-Actin, EEA1, Rab11 and Rab5 were detected in untransfected cells and cells transfected with control siRNA, confirming the specificity of the REP15 siRNA (**Fig. 21A**). We next examined the effect of depleting REP15 on TfR recycling from the RE. HeLa cells transfected with control or REP15 siRNA were incubated with B-Tfn for 1 h at 16°C, washed and chased for increasing time periods at 37°C and the amount of

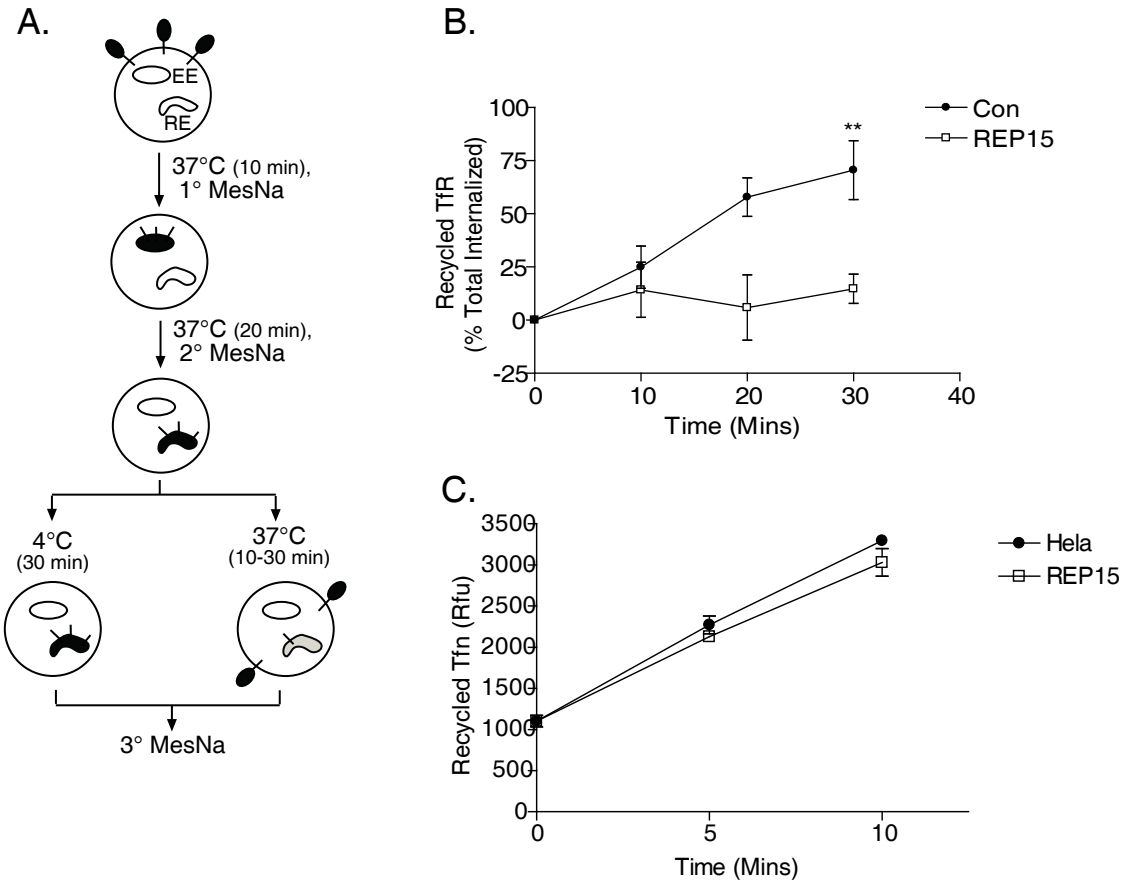


Figure 20. REP15 over expression delayed TfR recycling from the RE and not rapid TfR recycling from EEs. A, Schematic of the TfR recycling assay from the RE. B, The relative amount of TfR recycled from the RE in HeLa cells over expressing REP15 (REP15) and Mock-transfected cells (Con) was quantified as described previously in Fig 19. All values represent the mean ± S.E. of four independent assays and are expressed as a % of TfR recycled from the RE. Statistical differences (ANOVA $p < 0.01$**) between experimental and control sets were observed. C, Comparable amounts of B-Tfn were recycled from EEs in control cells and HeLa's over expressing REP15. B-Tfn recycling from EEs was quantified using a fluorescent ELISA assay (see Methods). Values indicate mean recycled B-Tfn (Relative Fluorescent Units) ± S.E.

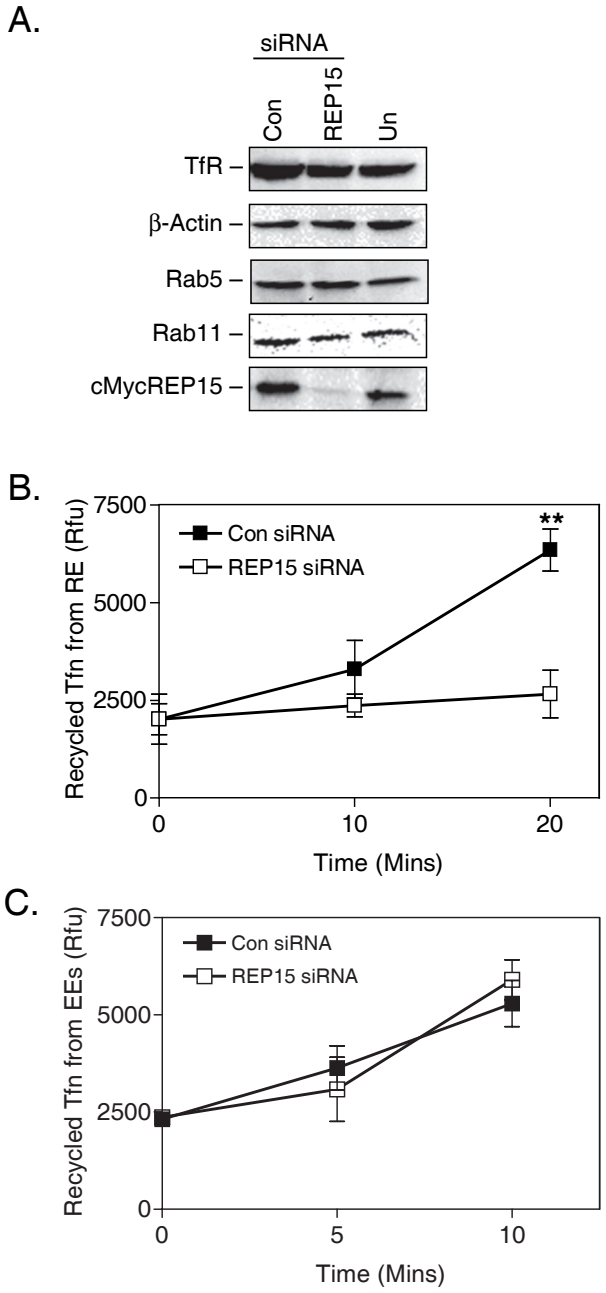
recycled B-Tfn in the media measured using a B-Tfn ELISA (see *Materials and Methods*). Tfn recycling from the RE was reduced by ~4-fold at 20 min in REP15-depleted cells, consistent with our over expression studies (**Fig. 21B**). Control studies confirmed that siRNA-mediated depletion of REP15 had no effect on Tfn recycling from EEs (**Fig. 21C**). Together, these data identify REP15 as a novel Rab15 effector important for TfR recycling from the RE.

Discussion

In this study, we present functional evidence that REP15 is a novel protein important for TfR recycling from the RE. When over expressed in HeLa cells, REP15 is compartment specific, colocalizing with Rab15 and Rab11 on the RE but not with Rab15, Rab4, or EEA1 on EEs. Consistent with its localization, siRNA-mediated depletion of REP15 resulted in retention of the TfR in the RE, without affecting receptor transport to the RE or fast receptor recycling from EEs. TfR recycling from the RE was also defective in cells over expressing wild type REP15. Over expression and siRNA silencing of the EH domain (EHD) protein has also been reported to inhibit TfR endocytosis (Guilherme et al., 2004). Similarly, EGFR down regulation was disrupted in HeLa cells over expressing full length Hrs, and in cells transfected with siRNA specific for Hrs (Bache et al., 2003). Thus the inhibition in TfR recycling detected in cells over expressing REP15, could be due to the titration of partner proteins involved in this endocytic step. Our data showing that REP15 over expression did not alter the delivery of internalized TfR to SEs or the ERC, or the fast mode of TfR recycling from the SE further supports our contention that REP15 specifically regulates TfR recycling from the ERC.

The inhibitory effect of REP15 over expression and siRNA-mediated knockdown on TfR recycling from the RE is wholly consistent with our previous reports on Rab15 function. In these studies, Rab15 was shown to localize to EEs and the RE in a variety of cell types and differentially regulate TfR transport through these endosomal compartments (Zuk and Elferink, 1999; Zuk and Elferink, 2000). For example over expression of Rab15 N121I (a mutant deficient in guanine nucleotide binding), stimulated the slow recycling of TfR from the RE without affecting receptor recycling from EEs (Zuk and Elferink, 2000). Conversely, over expression of Rab15 Q67L (GTP-bound) specifically reduced endocytic trafficking through EEs primarily at the level of EE fusion (Zuk and Elferink, 2000). Inconsistencies exist because over expression of Rab15 Q67L has no effect on

Figure 21. REP15 depletion by siRNA reduces TfR recycling from the RE. A, Untransfected (Un) HeLa cells stably expressing cMycREP15 and cells transfected with REP15 or control (Con) siRNA duplexes were examined by Western analysis as indicated. B, HeLa expressing cMycREP15 and parental HeLa (Con) cells were transfected with control or REP15 siRNA's for 72 hours and used to measure TfR recycling from the RE using a Tfn ELISA assay. All values are the mean of triplicate values and are expressed Relative Fluorescent Units (Rfu). Statistical differences between experimental and control sets were observed (ANOVA, $p<0.01$**). C, Control and REP15 siRNAs were transfected into HeLa cells as described in B. TfR recycling from the EEs was measured using a Tfn ELISA as the described in Fig 20C. No difference in TfR recycling from the EEs was detected following REP15 depletion.



recycling from the RE, while its binding partner REP15 strongly inhibits TfR recycling from the RE. I believe that the over expression of Rab15 Q67L does not allow us to truly measure Rab15's affects on TfR recycling from the RE. Rab15 Q67L expression inhibits internalization of the TfR to the EE, which indirectly affects the measurement of recycling from the RE. Thus, identification of an effector that binds to GTP-bound Rab15 and inhibits recycling of TfR from the RE, is entirely consistent with Rab15 being an inhibitory rab.

Recently, over expression of Rab11 Q70L has been shown to cause retention of the β_2-Adrenergic receptor as well as the TfR in the RE. In these studies the Rab11 Q70L induced accumulation of the β_2-Adrenergic receptor correlated with impaired trafficking to lysosomes (Moore et al., 2004). Similarly, interactions between Rab11 and RCP were reported to divert sorting of TfR from the degradative pathway to receptor recycling via the RE (Peden et al., 2004) implying a role for Rab11 (and its effectors) in receptor sorting at the RE. While REP15 did not interact directly with Rab11, we cannot exclude the possibility that REP15 interacts indirectly with Rab11 via downstream binding partners, to link what appear to be distinct Rab11 and REP15/Rab15 recycling pathways. Whether the REP15 functions to sort receptors for recycling or the generation of recycling vesicles remains unclear. In conclusion, REP15 is a novel regulator of TfR recycling from the RE, highlighting the mechanistic differences between receptor recycling from EEs and the RE.

CHAPTER 4: A NOVEL NEURO-SPECIFIC RAB15 BINDING PROTEIN

Introduction

Receptor internalization and recycling in the nervous system are important regulatory control mechanisms for synaptic transmission, long-term depression and long-term potentiation (Park et al., 2004). AMPA receptors are stored in the endosomal recycling compartments (EE and the RE) for rapid plasma membrane reinsertion upon stimulation with agonist or NMDA receptor activation (Lin et al., 2000; Man et al., 2000; Lee et al., 2001; Sheng and Lee, 2001; Park et al., 2004; Gerges et al., 2004). Thus AMPA receptor recycling represents an important regulatory mechanism for synaptic transmission and learning and memory (Sheng and Lee, 2001; Park et al., 2004). Recent studies have also demonstrated that the rapid recycling of the muscarinic acetylcholine receptor enables the cell to rapidly reinsert the receptors in the plasma membrane in response to signaling mechanisms (Szekeres et al., 1998a; Szekeres et al., 1998b). Together, these data indicate that the endocytic trafficking system is a key regulatory mechanism governing synaptic transmission and hence learning and memory.

Rab15 was initially identified in a screen for Rab GTPases in rat brain (Elferink et al., 1992). Analysis of the expression profile revealed that Rab15 is highly expressed in the cortex, hippocampus, thalamus, hypothalamus, midbrain, striatum, cerebellum, brainstem and spinal cord (Elferink et al., 1992). To date, the functional aspects of Rab15 on endocytic trafficking have been exclusively studied using non-neuronal cell lines such as HeLa, CHO and BHK cells (Zuk and Elferink, 1999; Zuk and Elferink, 2000). It is not clear if Rab15 is a general regulator of receptor endocytosis or if Rab15 regulates the trafficking of a specific subset of receptors. To address these issues I used yeast two-hybrid approach to identify and isolate Rab15 binding proteins from a human brain cDNA library[3]. In this chapter I describe the identification of the neural specific protein, Rab15 Binding Protein (RBP15) as a binding partner for Rab15-GTP. Over expression of RBP15 in HeLa cells results in enlarged endocytic structures indicating that RBP15 may regulate endosome fusion or protein recycling from EEs.

[3] This work was done in collaboration with Jagath Junutula Ph.D., Genetech and Ping Wu M.D. Ph.D. at UTMB

Materials and Methods

Reagents and Plasmids

General cell culture reagents and chemicals were obtained from Invitrogen Life Technologies and Fisher Chemical, respectively, unless specified otherwise. All restriction enzymes were purchased from New England Biolabs. NeuN and GFAP monoclonals were obtained from Calbiochem. The cDNAs for wild type and mutant Rab15 (Q67L, N121I and T22N) containing an amino terminal HA epitope have been described elsewhere (Zuk and Elferink, 2000) and were cloned directly into pEGFP-C3 (Clontech). pCR3.1-RBP15 was generated by subcloning full length RBP15 into pCR3.1 (Invitrogen) according to the manufacturers' instructions. pGEX 4T RBP15 was made by subcloning the cDNA insert from the yeast two-hybrid construct into pGEX 4T.

To prepare an antibody against RBP15, Human RBP15 was cloned directly into pGEX-4T (Clontech), expressed as a GST-fusion and purified by affinity chromatography using glutathione agarose beads (Sigma). Recombinant GST-RBP15 was used as the antigen to prepare rabbit polyclonal antisera (Covance, Denver PA). The resulting RBP15 antisera was purified by initial passage over GST immobilized on AminoLink™ Coupling Gel and the flow through subsequently purified against GST-RBP15 immobilized to AminoLink™ Coupling Gel (Pierce).

Yeast Two-Hybrid Binding Assays

Bait strains were prepared by cloning wild type Rab15 and its respective mutants into pGBKT7 (Clontech); GTP-bound Rab15 (Q67L), nucleotide–free Rab15 (N121I), GDP-bound Rab15 (T22N), For LacZ activation assays, the appropriate diploids were grown in 5 ml of the appropriate dropout media overnight at 30ºC and subcultured 1:10 in fresh dropout media for 7 h at 30°C. Cells were pelleted at 1000 x g for 5 min at 4 ºC, washed once in 5 ml of Z Buffer (113 mM $Na_2HPO_4 \cdot 7H_2O$, 39 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, and 35mM ßmercaptoethanol) resuspended in 120-150 ul of Z-Buffer and subjected to 3 cycles of 1 minute freeze/thaws in liquid nitrogen. The lysates were centrifuged at 20,000 x g, 5 min, at 4 ºC and 15 ul of the clarified supernatant incubated with 150 ul of CUG substrate (Molecular Probes) for 30 min at room temperature in darkness. The reactions were terminated with 75 ul of 0.2M Na_2CO_3 and the relative fluorescence measured according to the manufacturer's specifications (Molecular Probes). Each assay was performed in triplicate and repeated at least twice. Relative fluorescence

units were normalized to the amount of protein in each sample (Bradford, BioRad) and are reported as a measure of relative ßgalactosidase activity.

Cell Culture, Transfections and Confocal microscopy

All cells were cultured in DMEM supplemented with penicillin/streptomycin and maintained at 37 ºC with 5% CO_2. HeLa media was supplemented with 10% Cosmic Calf Sera (Hyclone). Transient expression using LipofectAMINE™ (Life Technologies) was performed as previously described (Zuk and Elferink, 1999; Zuk and Elferink, 2000).

For confocal analysis, HeLa cells were seeded onto Matrigel™ (BD Biosciences) coated cover glasses (Fisher Scientific) and transfected with the appropriate plasmid DNA for 48 hr. Cells were fixed in 4% paraformaldehyde (Ted Pella Inc. Redding, CA) in PBS for 10 min and blocked and permeabilized with 10% Goat Sera (Hyclone), 0.02% Saponin and 1% BSA in PBS and incubated subsequently with the appropriate antibody in PBS supplemented with 1% BSA, 0.02% Saponin overnight at 4˚C. Cells were washed 3 times with PBS and incubated with the appropriate secondary antibody coupled to Alexa^{488} or Alexa^{594} in PBS containing 1% BSA, 0.02% Saponin for 1 h at room temperature. The cover glasses were mounted onto glass slides using Fluorsave™ Reagent (Calbiochem, San Diego, USA). Images were generated using an Olympus BX50 epifluorescent microscope equipped with an Olympus Leeds Confocal Microscopy system with Argon and Krypton lasers with excitations at 488 and 568 nm, respectively. Image generation was done with a Plan-Apo 100X/1.35 oil immersion objective and Fluoview 2.0 imaging Software. To avoid unbiased selection, the two channels were imaged separately and not merged until acquisition was complete. Before acquisition, PMT and the laser power adjustments were optimized for each channel to avoid saturation of a particular channel. Images were processed using Adobe Photoshop 6.0 (Adobe).

Preparation and staining of brain and spinal cord sections were performed as follows. Tissues were fixed in 4% paraformaldehyde for 4 h then sucrose infiltrated in 5% Sucrose 1 h, 10% sucrose for 7 h, and 30% sucrose overnight. Tissues were mounted in OCT compound (Tissue Tek) and 40 um cryosections (Leica Cryostat) were sliced and mounted onto gelatin coated slides. Slides were stored at -80°C. This sections were blocked and permeabilized in 2.5% BSA/PBS, 5% Goat Serum and 0.25% Triton-X-100 for 30 mins and then incubated with primary antibody in 5% BSA, in PBS overnight at 4°C. Sections were incubated in the appropriate secondary for 1 h at room temp and subsequently mounted in Fluoromount G (Southern Biotech).

Membrane Fractionation and GST-Pulldowns

Rat brains were lysed in a 40 mL dounce homogenizer with 10 strokes in a buffer with 10 mM HEPES, pH 7.4 and 1 mM EDTA pH. 8.0 plus protease inhibitors. The resulting lysates was centrifuged at 4°C for 10 min and 1000 x g. The resulting PNS was centrifuged at 100,000 x g to separate membrane and cytosol fractions.

Recombinant GST-RBP15 was expressed in BLR-DE3 cells (Stratagene) and purified using Glutathione Agarose (Sigma). For pull down studies, HeLa cells were transfected with HA-tagged Rab15 using LipofectAMINE™ (Life Technologies) as described elsewhere (Zuk and Elferink, 2000). Transfected cells were resuspended in 200 ul of ice cold lysis buffer (10 mM HEPES, pH 7.4, 1.5% IGEPAL (Sigma), 0.1 mM $MgCl_2$, 150 mM NaCl, 10 ug/ml each aprotinin, leupeptin and pepstatin A), and cell lysates were clarified at 1000 x g for 10 min at 4°C. Supernatants were adjusted to 1.0 mM $MgCl_2$, incubated with 1.0 mM GTPγS (Sigma) or 1.0 mM GDPβS (Sigma) for 60 min at 4 °C and the extracts incubated for 2.0 hr at 4 °C with 50 ul of GST-RBP15 coated beads. Beads were washed three times in cell lysis buffer, three times in 150 mM NaCl, 10 mM HEPES-KOH pH 7.5, 0.1 mM $MgCl_2$ and analyzed by SDS-PAGE followed by Western analysis using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Results

RBP15 binds to GTP-bound Rab15

The enriched expression of Rab15 in neural tissue implies a role for Rab15 in endocytic trafficking in neurons (Zuk and Elferink, 1999; Zuk and Elferink, 2000). To address this issue I used the mutant Rab15 Q67L to screen a human brain cDNA library using the yeast two-hybrid system. One clone encoding an open reading frame of 204 amino acids was identified from this screen (**Fig. 22**). The human and mouse genomes contained an open reading frame that shared 100.0% and 95.6% identity, respectively, with a hypothetical protein of unknown function (Accession #'s NM_033201.1 and XP_489514.1, respectively). The protein was named Rab15 Binding Protein or RBP15.

Yeast two-hybrid analysis demonstrated that RBP15 binds to active forms of Rab15. High levels of β-galactosidase activity were detected in yeast strains coexpressing Rab15 wt and Rab15 Q67L (GTP-bound) while low levels of β-galactosidase activity were detected in strains expressing GDP-bound and nucleotide free mutants of Rab15

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ATGGAATTAAAGCAATCTTTGTCCACCCATCTGGAAGCCGAGAAGCCTCTGAGGCGCTAT
 M  E  L  K  Q  S  L  S  T  H  L  E  A  E  K  P  L  R  R  Y
GGGGCGGTGGAGGAGACGGCTTGGAAAACGGAGAGACTGGGGAGAAATCAGCTGGACATC
 G  A  V  E  E  T  A  W  K  T  E  R  L  G  R  N  Q  L  D  I
ATCTCCATGGCGGAGACAACCATGATGCCAGAGGAGATTGAGCTGGAGATGGCAAAAATT
 I  S  M  A  E  T  T  M  M  P  E  E  I  E  L  E  M  A  K  I
CAGCGTCTCCGGGAAGTCTTGGTCCGCCGGGAGTCTGAGCTCAGGTTCATGATGGATGAC
 Q  R  L  R  E  V  L  V  R  R  E  S  E  L  R  F  M  M  D  D
ATCCAGCTCTGCAAGGACATCATGGACTTGAAGCAGGAGCTGCAGAACTTGGTCGCCATC
 I  Q  L  C  K  D  I  M  D  L  K  Q  E  L  Q  N  L  V  A  I
CCAGAAAAAGAAAAAACCAAACTGCAGAAGCAGAGAGAGGATGAGCTAATCCAGAAGATC
 P  E  K  E  K  T  K  L  Q  K  Q  R  E  D  E  L  I  Q  K  I
CACAAACTGGTGCAGAAGAGAGACTTCCTGGTGGACGATGCGGAGGTCGAGCGGTTAAGG
 H  K  L  V  Q  K  R  D  F  L  V  D  D  A  E  V  E  R  L  R
GAGCAAGAAGAAGACAAGGAAATGGCTGATTTCCTGAGAATCAAGTTAAAACCTCTAGAC
 E  Q  E  E  D  K  E  M  A  D  F  L  R  I  K  L  K  P  L  D
AAAGTAACCAAATCTCCAGCCAGCTCCCGGGCAGAGAAGAAAGCAGAGCCCCCACCTAGC
 K  V  T  K  S  P  A  S  S  R  A  E  K  K  A  E  P  P  P  S
AAGCCCACGGTGGCCAAGACGGGGCTGGCATTGATCAAGGATTGTTGCGGGGCCACCCAG
 K  P  T  V  A  K  T  G  L  A  L  I  K  D  C  C  G  A  T  Q
TGCAACATCATGTAG
 C  N  I  M  *
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Figure 22: Putative cDNA Sequence of RBP15.

(**Fig. 23A**). Furthermore, RBP15 does not bind to active forms of rabs 1, 3A, 14, 15, 16, or 37 demonstrating the specificity of interaction with Rab15 (**Fig. 23B**) (Kusano et al., 1992; Johannes et al., 1994; Wilson et al., 1994; Zuk and Elferink, 1999; Masuda et al., 2000; Junutula et al., 2004a). In order to verify the yeast two-hybrid interaction we performed GST-pulldown assays. HeLa cell lysates over expressing HA-tagged Rab15 were preloaded with GTPγS or GDPβS. The cell lysates were subsequently incubated with Glutathione Agarose coated with recombinant GST-RBP15 and the GST-RBP15:Rab15 complexes were isolated and examined using Western analysis (**Fig. 23C**). Rab15 was detected in lysates preloaded with GTPγS, but not GDPβS, thus verifying the guanine-nucleotide dependence of the interaction. Furthermore, no interaction was detected with HARab15 lysates not preloaded with GTPγS or GDPβS or with beads coated with GST only, indicating the specificity of the interaction for Rab15-GTP. These results indicate that RBP15 is a binding protein for GTP-bound Rab15.

RBP15 is expressed in Neural Cells

I next examined the expression profile of RBP15. Using GST-RBP15 as the antigen, I produced a polyclonal antibody against recombinant RBP15. The polyclonal antiserum

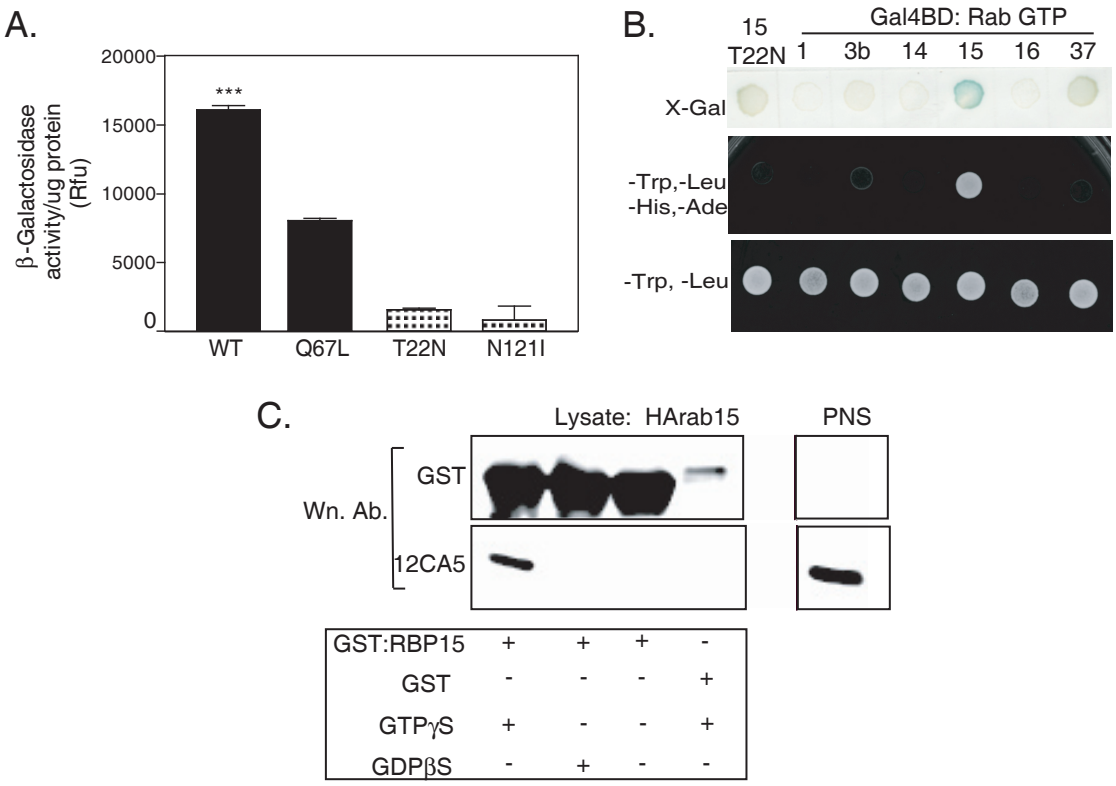


Figure 23: RBP15 binds to GTP-bound Rab15. A, Yeast strains expressing Gal4-RBP15 and Rab15 wt and mutants (pLexA Rab15) were analyzed for ß-Galactosidase activity. ß-Galactosidase activity was measured in relative fluorescent units ± S.E. (*** denotes $p<0.001$ ANOVA) B, Gal4AD-RBP15 expressed in a yeast two hybrid assay with the indicated BD-Rab GTPase. The presence of growth on –Trp-Leu-His-Ade plates or blue color on X-gal indicates an interaction. Growth on –Trp-Leu media demonstrate differences are not due to lack of growth of the strains. C, A post nuclear supernatant (PNS) was prepared from HeLa cells transiently over expressing wild type HArab15 were incubated with (+) or (-) GTPγS or GDPßS and subsequently incubated with GST-RBP15 or GST alone immobilized on glutathione agarose. GST-RBP15: Rab15 complexes were resolved by SDS-PAGE and examined using Western analysis (Wn.) for the indicated antibodies.

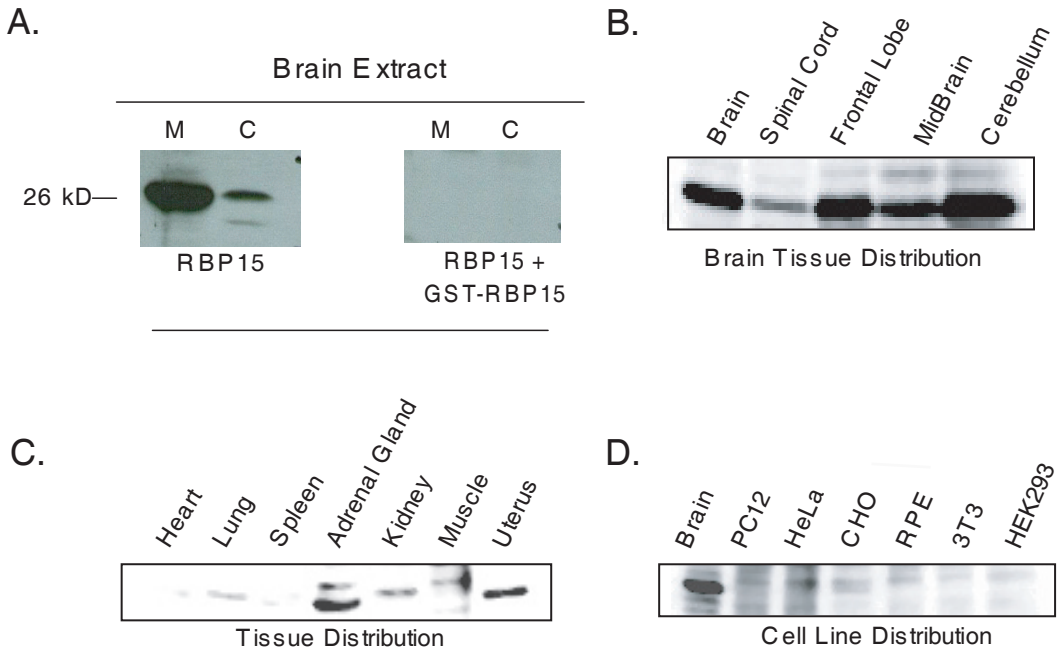


Figure 24: RBP15 is expressed in Neural Tissue. A. Brain membrane (M) and cytosol (C) fractions were examined using Western analysis and the RBP15 antibody. Second pane, Immunodepletion of the antibody prior to analysis results in loss of the RBP15 signal. B. Crude brain fractions examined using Western analysis for RBP15. C. Tissue Distribution of RBP15 using Western analysis. D. Expression of RBP15 in cell lines using Western analysis

was affinity purified using an affinity column containing immobilized GST-RBP15 antigen. To verify the specificity of the antibody, I prepared membrane and cytosolic fractions from rat brain. The fractions were examined for the presence of RBP15 using Western analysis. As predicted we detected a single band around 26 kDa in size that corresponded to the predicted size of RBP15 (**Fig. 24A**). In control studies, RBP15 antibody was depleted by incubation with GST-RBP15 immobilized on glutathione agarose. When used for Western analysis, RBP15 was not detected confirming the specificity of the antibody for RBP15.

Western analysis demonstrated that RBP15 is primarily expressed in brain and spinal cord tissue. Western analysis of crude dissections of a rat brain demonstrated that RBP15 is expressed in sections enriched in the frontal lobe, midbrain and cerebellum. (**Fig. 24B**). To determine the expression pattern of RBP15, I performed Western analyses using lysates prepared from non-neuronal tissues. The analysis demonstrated that the heart, lung,

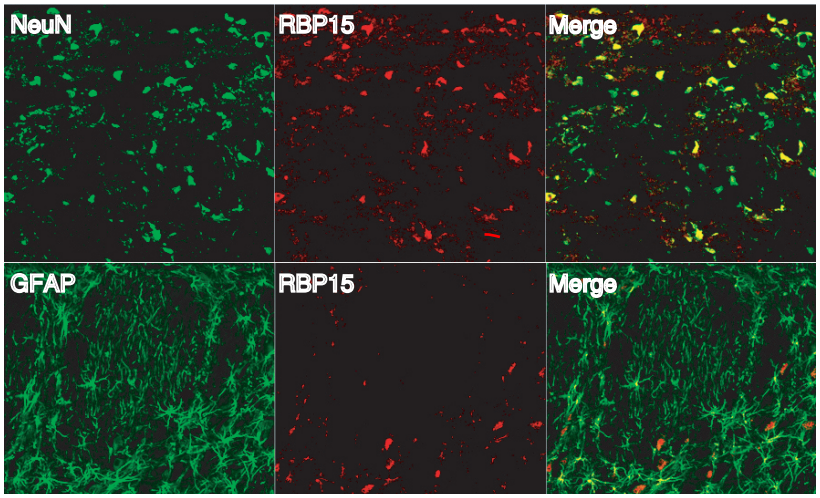


Figure 25: RBP15 is expressed in neurons. Rat brain tissue sections were stained for RBP15 and neuronal or astrocyte markers and examined using confocal microscopy. Sections were costained for RBP15 and for NeuN (neuronal marker) or GFAP (astrocyte marker). Coexpression is indicated by yellow in the merged image.

spleen, adrenal gland, kidney, muscle, and uterus expressed low levels of RBP15 that could reflect innervation into these tissues (**Fig. 24C**). To identify a potential cell line to perform functional analysis of RBP15, I examined RBP15 expression in several immortalized cell lines. RBP15 expression was not detected in PC12, HeLa, CHO, mouse 3T3 fibroblast, retinal pigment epithelium and HEK 293 cell lines (**Fig. 24D**). Therefore like Rab15, RBP15 is enriched in neural tissues.

The nervous system is comprised of two main cellular subtypes: neurons and glial support cells. In order to determine the cell type specificity of RBP15, brain and spinal cord sections were costained for RBP15 and neuronal nuclear antibody NeuN or the glial/astrocyte marker glial fibrillary acidic protein (GFAP) (Debus et al., 1983; Mullen et al., 1992). RBP15 localized to cells that costained with NeuN, indicating that RBP15 is specifically detected in neurons. Furthermore, no costaining was observed on sections stained with RBP15 and GFAP indicating that RBP15 is not expressed in glial/astrocyte cells (**Fig. 25**). In order to determine the profile of RBP15 expression in the spinal cord, sections were stained with the RBP15 antibody. RBP15 is detected strongly in large motor neurons of the ventral horn, as well as the afferent terminals leading from the dorsal root ganglion (**Fig. 26**). Dorsal root ganglion sections also stained positive for RBP15 indicating

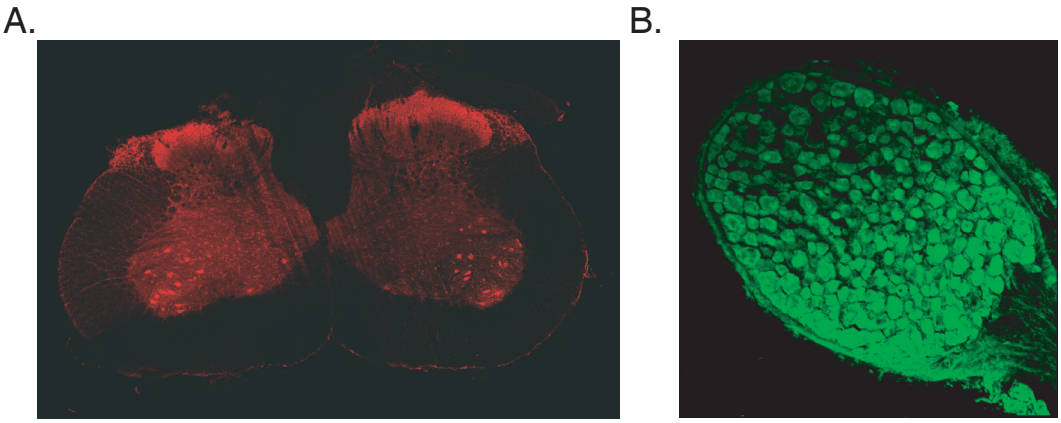


Figure 26: RBP15 is expressed in the spinal cord and dorsal root ganglion. spinal cord and dorsal root ganglion sections were stained for RBP15 and examined using confocal microscopy. Resulting images were assembled in Photoshop 6.0.

that RBP15 could be involved in sensory neuron processing from the dorsal horn as well as from motor neurons at the ventral horn.

RBP15 localizes to vesicular structures in HeLa cells

RBP15 is highly expressed in neurons in the brain, spinal cord and dorsal root ganglion. I first wanted to verify whether Rab15 and RBP15 colocalize. Because the antibodies for RBP15 and Rab15 were generated in the same species, I was unable to costain tissue sections for RBP15 and Rab15. As an alternative approach I used HeLa cells cotransfected with RBP15 and GFP-Rab15. Cotransfected HeLa cells showed distinct colocalization in large endocytic structures in the cell periphery reminiscent of EEs (**Fig. 27A**). In order to determine if RBP15 localized to an endosomal compartment such as EEs, I costained sections for EEA1 and RBP15. Confocal microscopy showed that RBP15 and EEA1 localized to adjacent endocytic structures, which might be microdomains on the same endosome or separate adjacent endosomes (**Fig. 27A**). Over expression of RBP15 resulted in enlarged in enlarged vesicular structures (**Fig. 27B**). These results indicate that RBP15 colocalizes with GFP-Rab15 in HeLa cells and may localize to distinct microdomains adjacent to EEA1 positive endosomes. Furthermore, RBP15 over expression results in enlarged vesicular structures indicating that RBP15 may regulate some type of endosome fusion event.

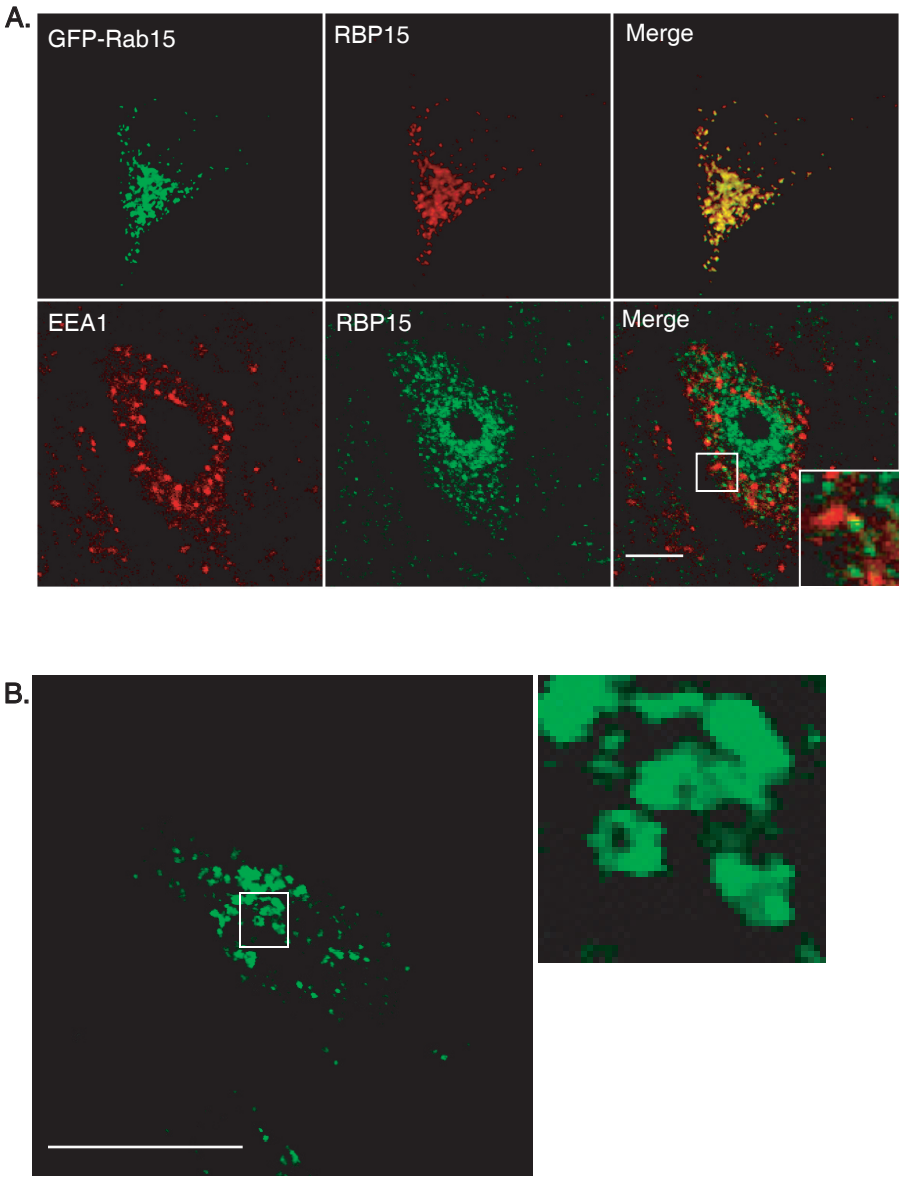


Figure 27: RBP15 colocalizes with Rab15 and enlarges endosomes in HeLa cells. A. Upper panel GFP-Rab15 and RBP15 were co-expressed in HeLa cells and imaged using confocal microscopy. Yellow denotes areas of colocalization. Lower panel, spinal cord sections were stained for EEA1 and RBP15 and imaged using confocal microscopy. B. HeLa cells were transfected with RBP15 and imaged using confocal microscopy. Arrows denote enlarged endosomes. Scale 10um

Discussion

This chapter details the initial characterization of RBP15 localization and function as a potential effector for the small GTPase Rab15 in neural tissue. Binding of RBP15 to Rab15 is GTP dependent, indicating that RBP15 is a potential effector for Rab15. Furthermore, GFP-Rab15 and RBP15 colocalize on vesicles within the cytoplasm, reminiscent of early endosomes. RBP15 is specifically expressed in neurons in the brain, spinal cord and dorsal root ganglion and morphologically, RBP15 is expressed in large motorneurons in the spinal cord and the afferent terminals leading to the dorsal root ganglion indicating that RBP15 may be involved both receiving and sending sensory information. These results demonstrate that RBP15 is a Rab15 binding protein that is neural specific.

RBP15's exact subcellular distribution remains to be determined. Costaining of both RBP15 and Rab15 was not possible because the antibodies are from the same species. Therefore, I examined colocalization of GFP-Rab15 and RBP15 using an over expression approach. I determined that GFP-Rab15 and RBP15 colocalize in HeLa cells providing evidence that these proteins localize to similar types of vesicles. Conversely, colocalization of RBP15 with EEA1 was not observed in the spinal cord. However, RBP15 and EEA1 localized to adjacent structures indicating that RBP15 and EEA1 might be localized to distinct microdomains reminiscent of Rabs 4, 5 and 11 on the EE (Sonnichsen et al., 2000; De Renzis et al., 2002). Another possibility is that RBP15 may localize to and regulate endocytic trafficking through the clathrin-independent pathway. RBP15 over expression also enlarges endosome-like structures reminiscent of over expression of Rab5 Q79L. RBP15 may play a role in regulating fusion of endocytic vesicles with the EE, functioning in parallel with Rab5. Alternatively, RBP15 may function in the receptor recycling pathway from the EE and that the enlarged endosomes are due to a failure to generate recycling vesicles from the EE. Future studies will entail defining whether RBP15 regulates general endocytosis or the endocytic trafficking of a specific receptor. Once a receptor is defined, and then further mechanistic studies will be able to determine the mechanistic function of RBP15 in neurons.

CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

General Conclusions

The identification of downstream rab effector molecules has provided insight into how rabs function to regulate membrane trafficking steps, particularly in endocytosis. Over expression of GTP-bound forms of Rab5 resulted in increased internalization of the TfR and increased homotypic endosome fusion in cells (Gorvel et al., 1991; Kinsella and Maltese, 1991; Bucci et al., 1992; Barbieri et al., 1994; Barbieri et al., 1996). While the Rab5-regulated endosome fusion machinery is well characterized, identification of new Rab5 binding protein have implicated Rab5 in a variety of biological process such as cellular signaling and nuclear transcription (Stenmark et al., 1995; Simonsen et al., 1998; Christoforidis et al., 1999a). For example, recent characterization of RN-TRE and RinI as proteins that regulate the guanine nucleotide bound state of Rab5 in response to EGF signaling, affirms previous studies that GTP hydrolysis of Rab5 is essential for EGF-R internalization (Barbieri et al., 2000; Lanzetti et al., 2000; Tall et al., 2001). Moreover, identification of the APPL1 and APPL2 proteins which require Rab5 binding to function, directly link Rab5 to transcriptional events in the nucleus (Miaczynska et al., 2004). These results demonstrate that endocytosis and cellular signaling are functionally linked and that endocytosis may be a causative link in cancer.

Rab15 is the first inhibitory rab GTPase identified in the endocytic pathway (Zuk and Elferink, 1999; Zuk and Elferink, 2000). Arf6 and Rab3a are two other inhibitory GTPases that function in membrane trafficking pathways involved in synaptic vesicle fusion and clathrin-independent endocytosis, respectively (Johannes et al., 1994; Geppert et al., 1997; Donaldson, 2003; Naslavsky et al., 2003). Over expression of Arf6-GTP inhibits the fusion of vesicles derived from the clathrin-independent pathway with the classical EEA1 positive EE (Naslavsky et al., 2003; Donaldson, 2003). Rab3a inhibits the fusion of secretory vesicles with the presynaptic membrane; causing an accumulation of secretory vesicles (Johannes et al., 1994b; Geppert et al., 1997). Rab15 is unique in that it inhibits homotypic endosome fusion and internalization of both the fluid phase marker HRP as well as the internalization of TfR to the EEs (Zuk and Elferink, 1999; Zuk and Elferink, 2000). Furthermore, Rab15 distributes between two endocytic compartments, the EE and the

RE, and differentially regulates TfR trafficking in these compartments. Over expression of inactive mutants of Rab15 (T22N and N121I) differentially modulate TfR recycling through these compartments. Over expression of Rab15 T22N (GDP-bound) stimulates recycling of TfR from both EEs and the RE. Conversely, over expression of Rab15 N121I (nucleotide-free) stimulates recycling of TfR from the RE only while having no effect at the EE (Zuk and Elferink, 2000). These results indicate that Rab15 may modulate internalization and recycling at EEs and the RE by binding to compartment specific binding proteins. Because previous Rab15 studies used an over expression approach to determine Rab15 localization and function, it was unclear whether the endogenous protein would localize to both peripheral EEs and the RE and affect trafficking at both of these compartments. This body of work builds on the lab's previous studies, by identifying Rab15 binding proteins that exert Rab15-mediated events at both the EE as well as the RE. This dissertation is the functional characterization of three different Rab15 binding molecules: Mammalian suppressor of Sec4 (Mss4); Rab15 Effector Protein (REP15); and Rab15 Binding Protein (RBP15). I have shown that Mss4 functions during early endocytosis of the TfR and that interactions between Rab15 and Mss4 are necessary for Rab15's inhibitory effect on early endocytic trafficking at the level of both TfR internalization and homotypic endosome fusion (Chapter 2) (Strick et al., 2002). Using mutants of Rab15 that were not able to physically associate with Mss4 (Rab15 K48Q) resulted in a 50% reversal of the inhibitory phenotype when compared to controls. Furthermore, Rab15 K48Q reversed the inhibitory phenotype of Rab15 on homotypic endosome fusion resulting in a 5% stimulatory increase compared with controls. These studies are also the first studies that identify Mss4 as a component of the endocytic pathway, demonstrating that endocytosis and exocytosis are functionally linked by shared effector molecules. In addition, studies have demonstrated that Mss4 is up-regulated in pancreatic cancer as well as up-regulated upon antidepressant treatment in Rats indicating that Mss4 is an essential molecule for both cellular signaling as well as synaptic transmission (Muller-Pillasch et al., 1997; Andriamampandry et al., 2002). Further studies will have to be performed in order to determine if Rab15 protein levels or activity is altered in these disorders.

REP15 (Rab15 Effector Protein) binds to GTP-bound Rab15 and localizes specifically to the RE and not to peripheral EEs (Chapter 3). Over expression of REP15 had no affect on internalization of the TfR, however the TfR is retained in the RE over long internalization periods. REP15 over expression and REP15 siRNA-mediated knockdown

demonstrate that REP15 is required for TfR recycling from the RE and not peripheral EEs. While over expression and siRNA mediated knockdown of REP15 resulted in an inhibition of recycling of the TfR specifically from the RE, more studies will need to be performed to detail the exact function of REP15. REP15 could function to stabilize membrane domains enriched in the molecular components necessary for vesicle generation or REP15 could bind directly to a receptor and mediate the sorting of receptors into distinct domains. Alternatively, REP15 could regulate the generation o,f RE-derived vesicles. REP15 could also function to regulate receptor down regulation to the LE by regulating sorting of receptors to the LE. Recent evidence links Rab11 and RCP (a Rab11 effector) to sorting of the β-Adrenergic receptor and the TfR to the degradative pathway (Moore et al., 2004; Peden et al., 2004). Further studies will determine if REP15 functions as a receptor sorting molecule or if REP15 actively participates in vesicle biogenesis from the RE.

RBP15 (Rab15 Binding Protein), is a novel neural specific protein that binds to GTP-bound Rab15 (Chapter 4). RBP15 is enriched in neuronal tissues and is specifically expressed in neurons and not glial cells. RBP15 colocalizes with Rab15 when exogenously co-expressed in HeLa cells. While we know that RBP15 and Rab15 colocalize and function in neuronal tissues we have little data on how RBP15 actually functions. RBP15 is expressed in the large motor neurons of the brain and spinal cord as well as neurons in the dorsal root ganglion and is also localized to afferent fibers leading to the DRG indicating that it may play a role in transmitting sensory information as well as regulating motor control of the extremities. We also have preliminary data that RBP15 over expression in HeLa cells enlarges the endocytic compartment most likely due to increased endosome fusion or faulty recycling mechanisms. Further studies need to be performed to identify the putative endosomal compartment that RBP15 is localized to.

The goal of this dissertation was to identify and characterize specific binding molecules for Rab15 in order to further delineate Rab15's function in early endocytic trafficking. This dissertation characterizes three unique effector/binding proteins for Rab15, demonstrating that these proteins are important regulators of endocytic trafficking. Furthermore, I demonstrated that these effectors differentially exert Rab15-mediated events at EEs and the RE and thus further verify that Rab15 functions at both of these organelles. In conclusion, this dissertation demonstrates that Rab15 is a bi-functional molecule that performs unique functions at both the EE and the RE using a combination of general and compartment specific effector proteins.

Future Directions

Do Rab15 and Its Effectors Regulate Endocytosis of Different Receptors?

Several endocytic trafficking proteins are receptor specific, functioning in internalization of a specific type or subset of receptors (Lanzetti et al., 2000; Tall et al., 2001; Hayes et al., 2002). All previous Rab15 functional studies were characterized using TfR as a model receptor since the endocytic trafficking pathway for the TfR is well characterized (Zuk and Elferink, 1999; Zuk and Elferink, 2000; Strick et al., 2002). Furthermore, TfR endocytosis is biomedically significant because of iron storage diseases as well as the potential use for coupling potential drugs and gene therapy delivery systems for delivery across the cell membrane as well as the blood brain barrier (Li and Qian, 2002). Rab15 and its effectors could function as general regulators of endocytosis or as specific regulators of the internalization of a specific subset of receptors. Further studies need to be performed to determine if Rab15, Mss4, REP15 or RBP15 functionally affect internalization and recycling of signaling receptors such as EGF-R and the TGFβ-R and whether Rab15 or its effectors modulates cellular signaling.

Identification of Alternate Rab15 effectors

While this dissertation focused on the characterization of three Rab15 binding proteins, multiple proteins may exist to regulate and carry out the inhibitory effect of Rab15 on receptor internalization and recycling. While the yeast two-hybrid system has many advantages and worked quite efficiently for this project, many disadvantages do exist. First, a yeast two-hybrid screen is as only as good as the library. If a protein of interest is under represented in the library, then it may not be detected in a yeast two-hybrid screen. Second, the yeast two-hybrid does not allow for isolation of high molecular weight complexes that may exist in the endocytic system. Further studies using a recombinant baculovirus expressed Rab15 protein immobilized on an affinity column may be able to detect unique interacting proteins as well as interactions with high molecular weight complexes from bovine brain lysates.

Some rab effectors bind to multiple rab proteins, as well as accessory proteins. For instance Rabaptin-5, Rabenosyn-5 and Rabip4 bind to both Rab5 and Rab4 and function as shared effectors for internalization/endosome fusion and direct recycling, respectively (Vitale et al., 1998; De Renzis et al., 2002; Fouraux et al., 2004). Furthermore, Rabaptin-5 and Rabex-5 also bind to GM130 and Rab33b in the secretory pathway, linking endocytic

trafficking to exocytosis (Valsdottir et al., 2001). Arfophilins 1 and 2 are shared effector for the Arf5 and Arf6 as well as Rab11 (Hickson et al., 2003). Further studies screening for REP15 and RBP15 binding proteins using the yeast two-hybrid system would facilitate identification of potential downstream effectors, enabling us to further understand how Rab15 and its binding proteins control endocytic mechanisms. Moreover, identification of novel Rab15 binding proteins will determine if Rab15 functions in a parallel pathway to Rab5 and Rab11 regulated pathways, or if these pathways converge at the level of a shared protein.

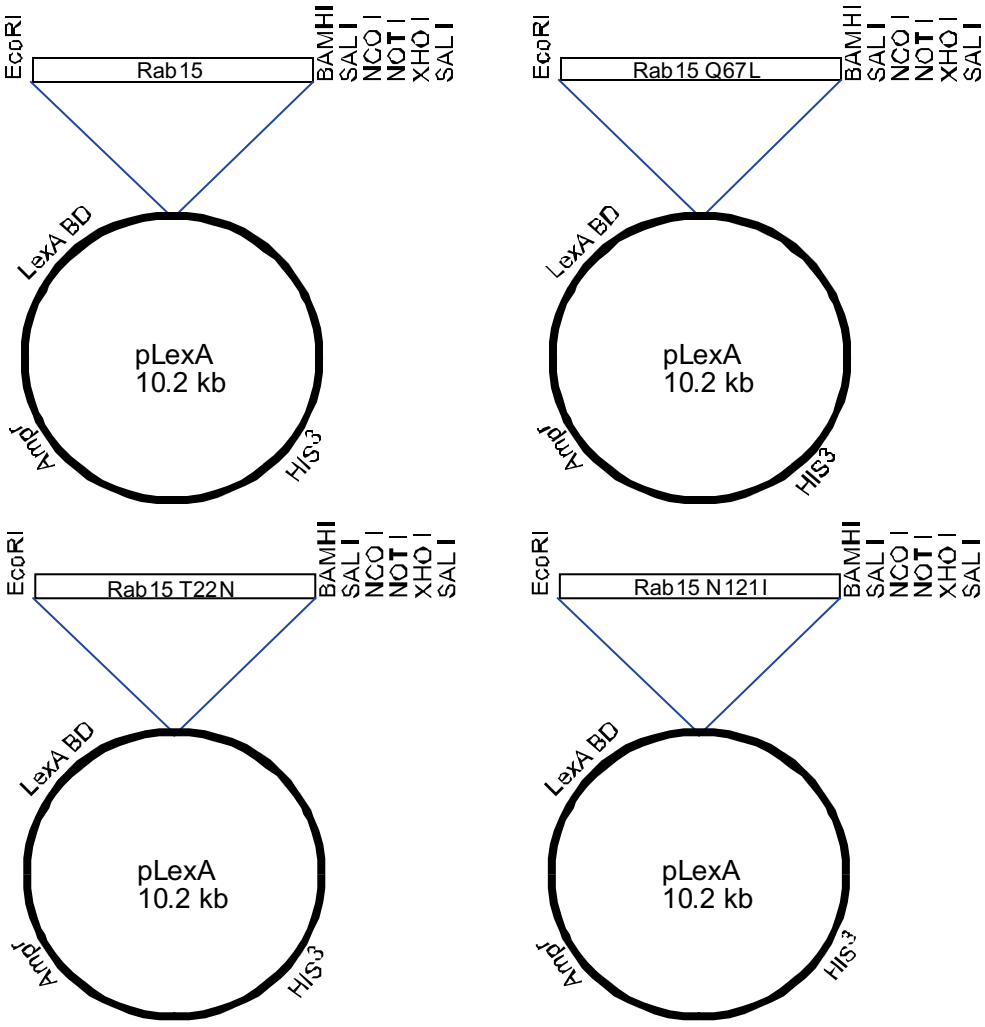
Rab15 function in Neurons

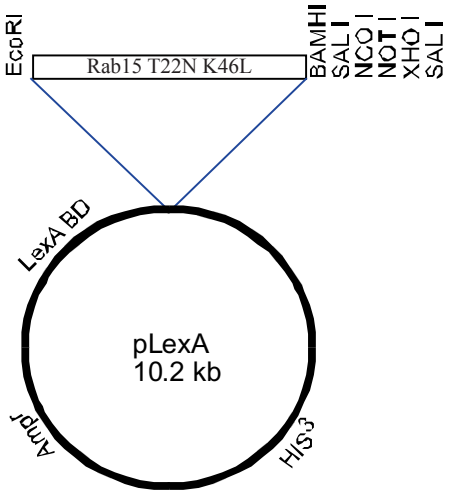
Rab15 is highly expressed in nervous tissue. Furthermore, RBP15 binds to GTP-bound Rab15 and is specifically expressed in neurons. Several studies have demonstrated that the recycling of AMPA receptors and acetylcholine receptors modulates specific downstream signaling events in response to activation of the respective receptor. Upon stimulation, the overall recycling of AMPA receptors is increased from the RE thus enhancing synaptic efficacy (Park et al., 2004). Furthermore, rapid recycling of the muscarinic acetylcholine receptor recycling enables a cell to maintain the cell surface receptor level at 65% of normal levels but a 100% cellular response measured by Ca^{2+} concentration (Szekeres et al., 1998a; Szekeres et al., 1998b). Furthermore, the recycling of acetylcholine receptors is regulated in a Rab11 dependent process (Volpicelli et al., 2002). All of the previous Rab15 studies have been performed using non-neuronal cells. Perhaps Rab15 regulates important changes in receptor level that modulate AMPA receptor location and activity. Studies could be performed to determine the effect of Rab15 and RBP15 on muscarinic acetylcholine receptor and AMPA receptor trafficking.

In summary, we know that Rab15 is a bi-functional GTPase, regulating trafficking through the EE as well as receptor recycling through the RE. Rab15 binds to specific effectors that differentially function to regulate Rab15-mediated trafficking events at these compartments. However, more studies need to be accomplished to determine a more precise role for Rab15. These studies would be enhanced by studies that isolate and identify alternate effector proteins for Rab15 as well as binding partners for REP15 and RBP15. Furthermore, studies need to be performed to determine Rab15's exact role in the nervous system. These studies will enable us to further understand the role of regulatory molecules in endocytic trafficking and their effect on human health and disease.

APPENDIX A

PLASMID/CONSTRUCT MAPS

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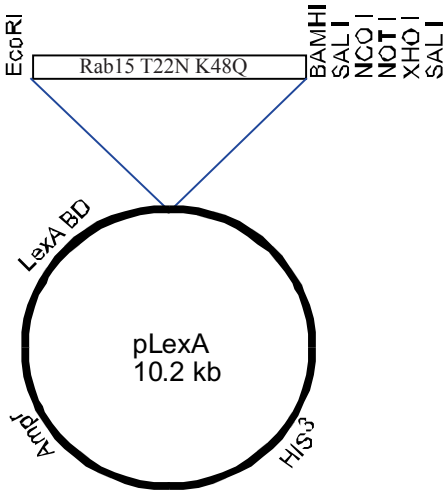
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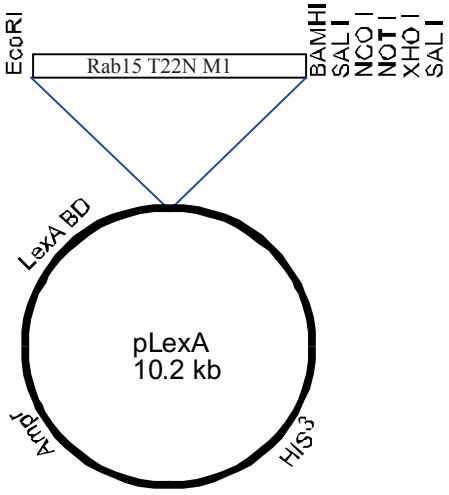
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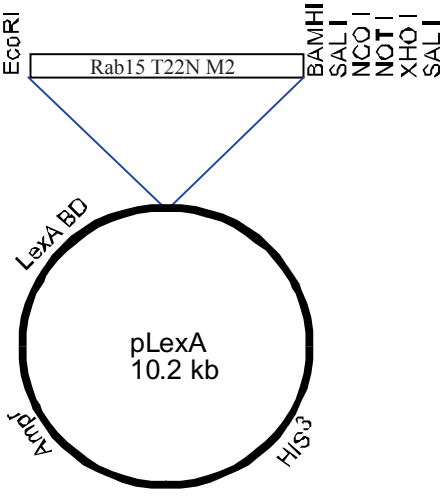
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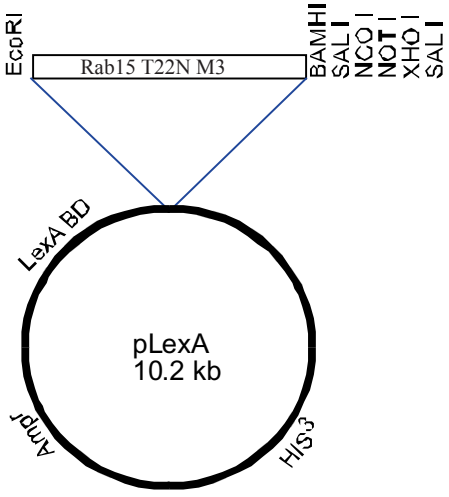
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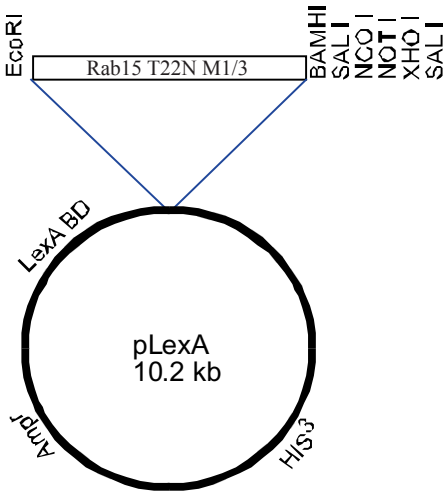
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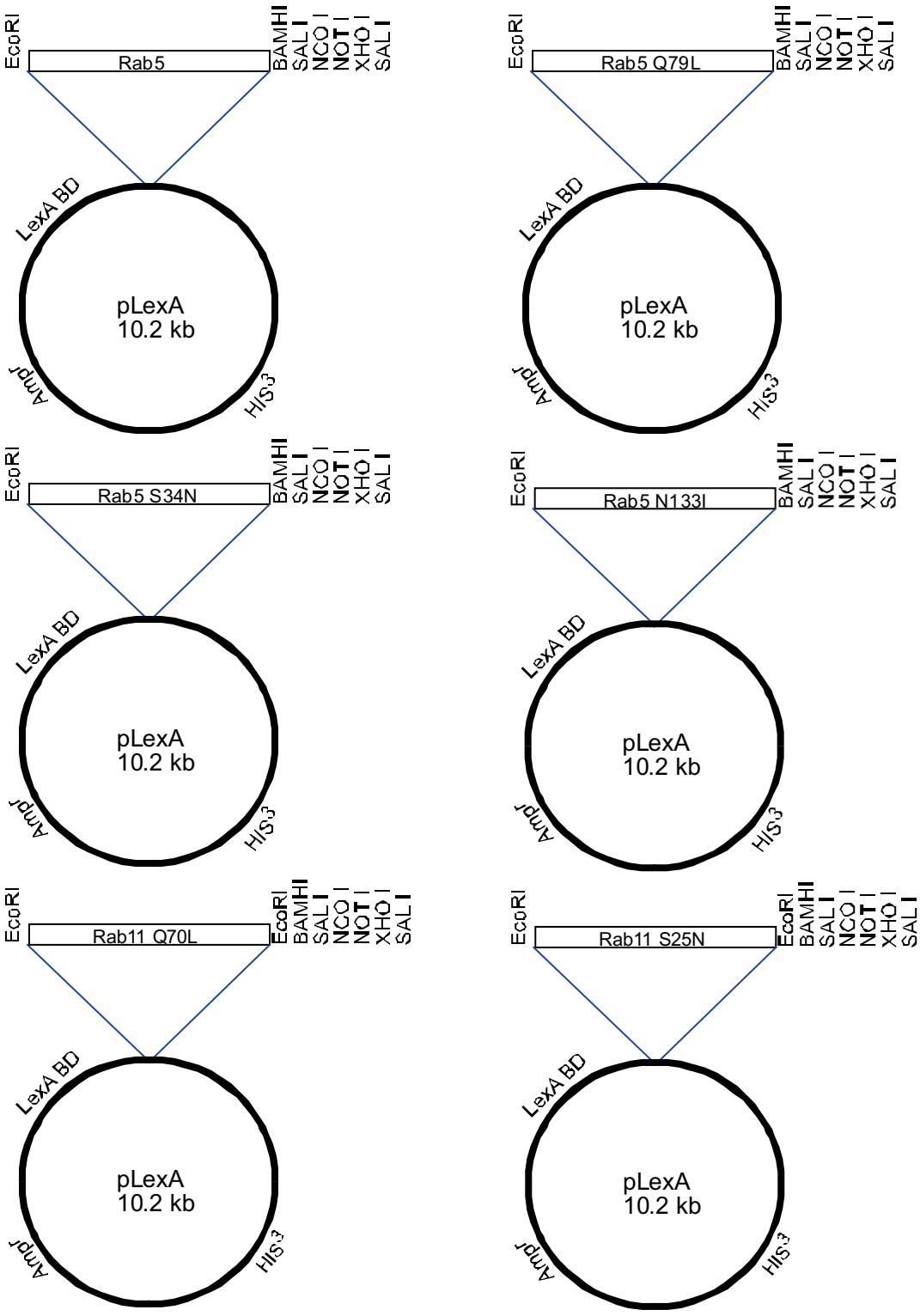
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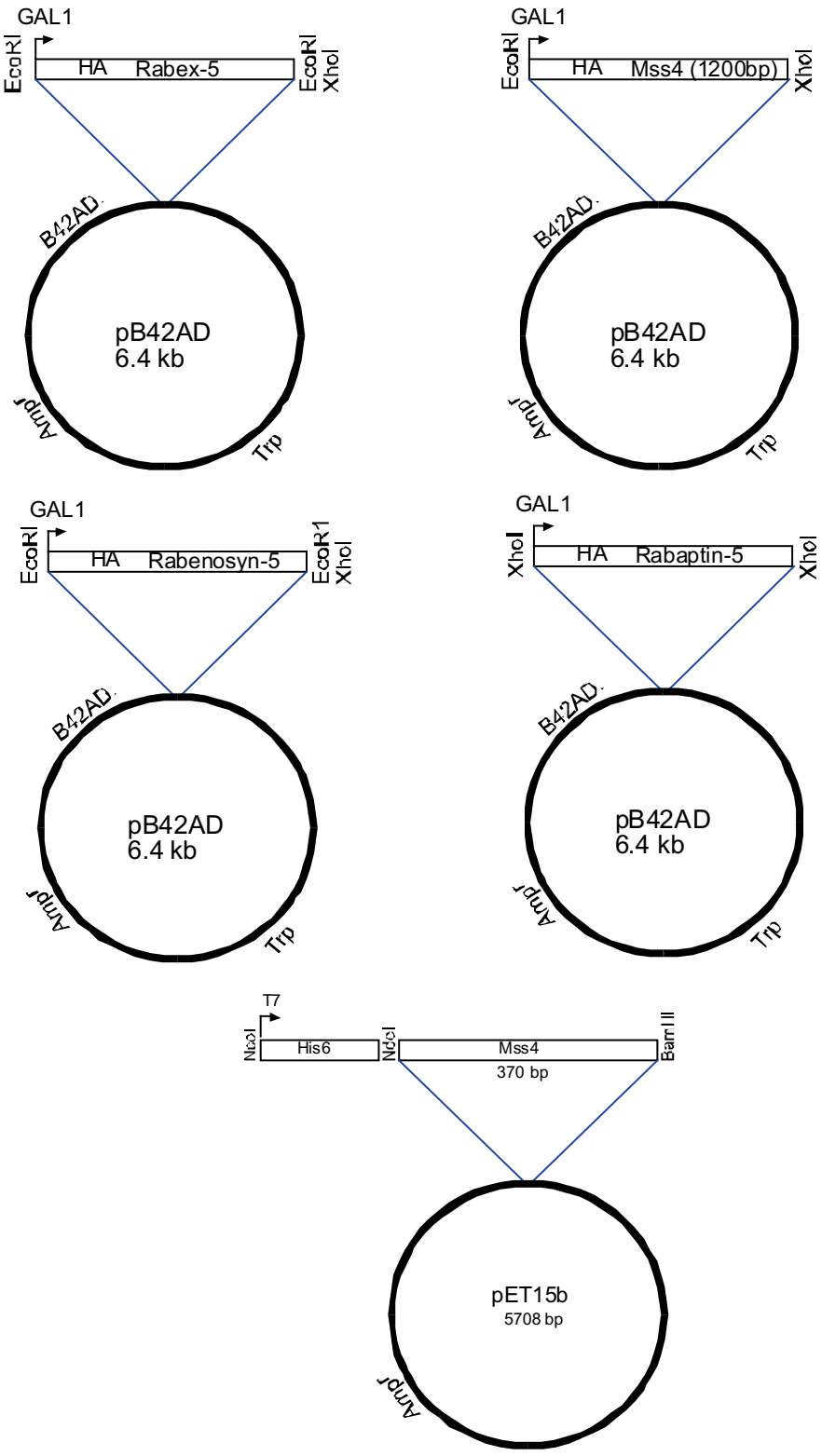
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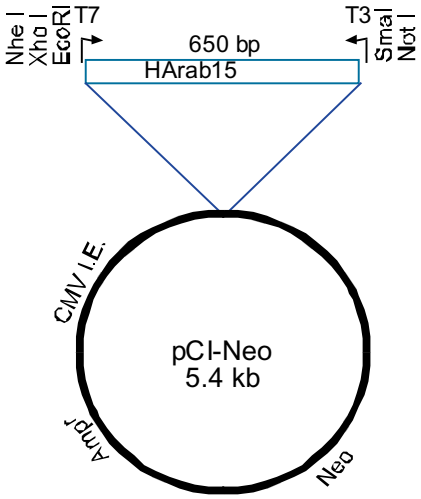
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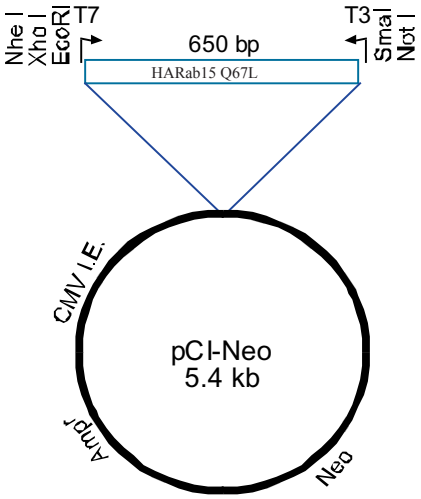
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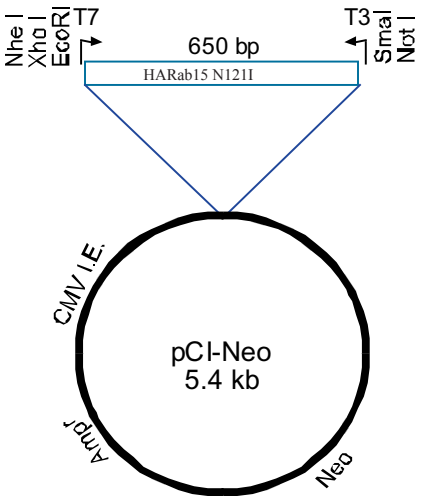
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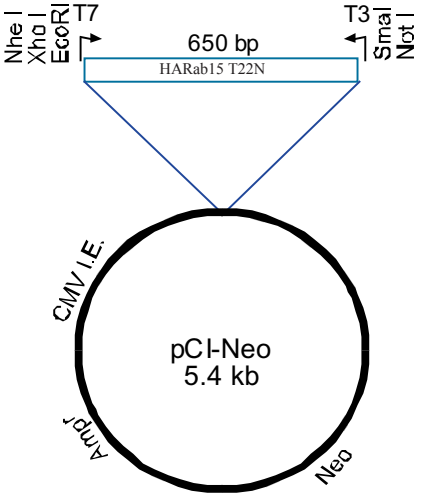
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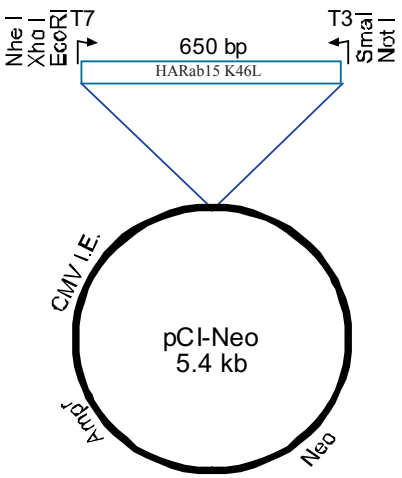
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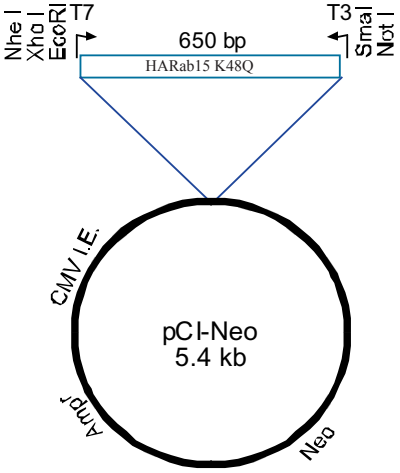
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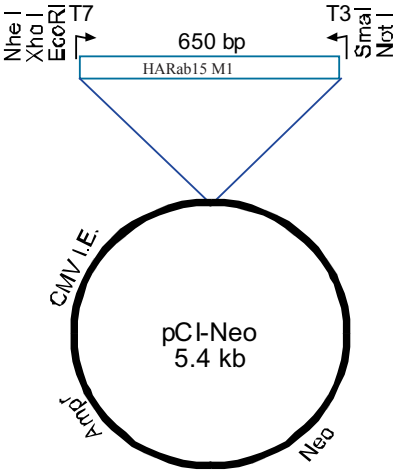
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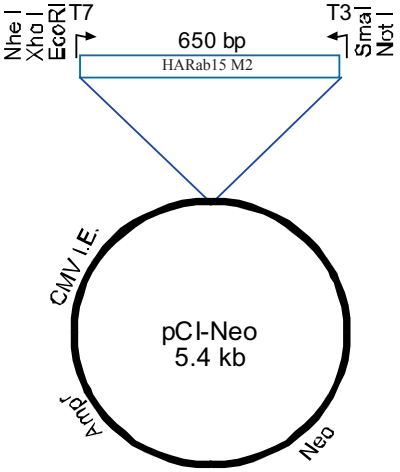
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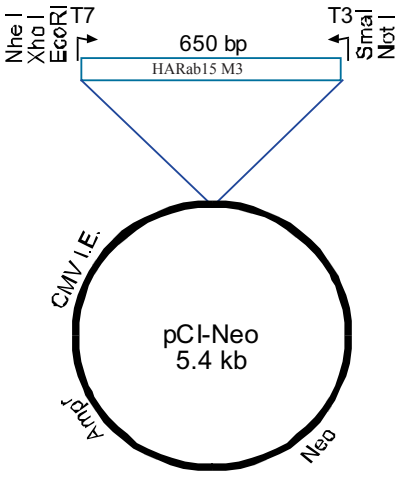
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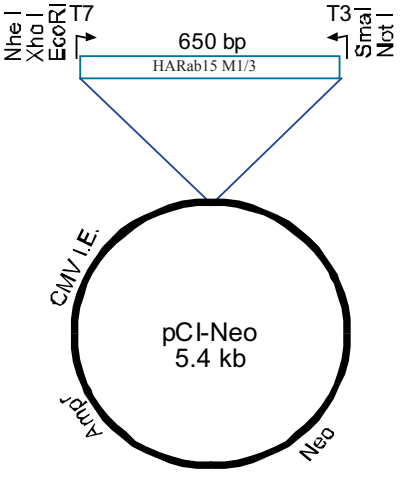
Nhe I
Xho I
EcoRI
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SmaI
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CMV I.E.
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Ampr
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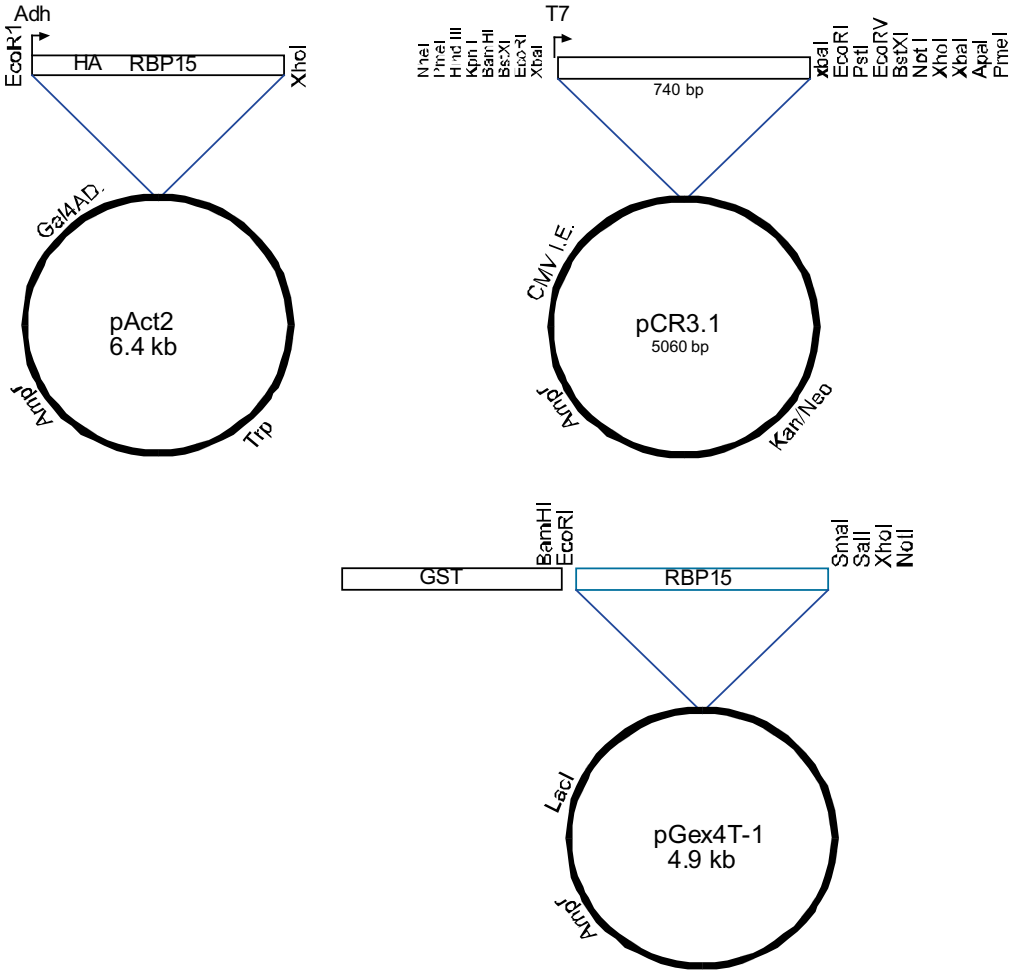
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4.9 kb
AmpR

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VITA

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David gained significant teaching experience while at Wayne State University, serving as Teaching Assistant for a senior level Genetics course and has helped to mentor numerous rotation students in the Elferink Lab.

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