FUNCTIONAL ANALYSIS OF RAB-10 AND ITS INTERACTING PARTNER
EHBP-1 DURING ENDOCYTOSIS IN THE Caenorhabditis elegans INTESTINE

by

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ABSTRACT OF THE DISSERTATION

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The endocytic pathway of eukaryotes is essential for the internalization and trafficking of macromolecules, fluid, membranes, and membrane proteins. One of the most enigmatic aspects of this process is endocytic recycling, the return of macromolecules (often receptors) and fluid from endosomes to the plasma membrane. It has been previously shown that the EH-domain protein RME-1 is a critical regulator of endocytic recycling in worms and mammals. In my studies, the RAB-10 protein was identified as a key regulator of endocytic recycling upstream of RME-1 in polarized epithelial cells of the Caenorhabditis elegans intestine. rab-10 null mutant intestinal cells accumulate abnormally abundant RAB-5-positive early endosomes, some of which are enlarged by more than 10-fold. Conversely most RME-1-positive recycling endosomes are lost in rab-10 mutants. The abnormal early endosomes in rab-10 mutants accumulate
basolaterally recycling transmembrane cargo molecules and basolaterally recycling fluid, consistent with a block in basolateral transport. These results indicate a role for RAB-10 in basolateral recycling upstream of RME-1.

In a yeast two-hybrid screen for RAB-10-interacting proteins, eight candidates with RAB-10-interacting ability were identified. They are EHBP-1, HUM-2, GCK-2, CNT-1, F52E1.13, Y82E9BR.21, ZK1248.10, and F20D1.2. In addition, 5/8 candidates identified interacted with RAB-8 and RAB-10 but not with RAB-5, -7, -11, or -35 in the two-hybrid assay. One of the RAB-10-interacting candidates, EHBP-1, an actin-cyskeleton bundling protein, was revealed to function together with RAB-10 in vivo. Knockdown of EHBP-1 function by RNAi produced a \textit{rab-10}-like phenotype in the intestine. In addition, EHBP-1 was found to be required for endosomal membrane association of RAB-10 and actin association with the enlarged endosomes of \textit{rab-10} mutants. I conclude that RAB-10 and EHBP-1 likely function together in basolateral endocytic recycling pathway of the \textit{C. elegans} intestine.
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DEDICATION

To all my family members
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GCK-2

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CHAPTER 1

Introduction to Endocytosis and Rab Family
INTRODUCTION

Endocytosis is an essential process with many biological functions in animal cells. Endocytosis is required for general cellular functions such as nutrient uptake (e.g. LDL and transferrin and their receptors), the regulation of growth factor receptors in development and homeostasis. Endocytosis also plays important roles in the activities of specialized cells such as antigen presenting by antigen presentation cells, and the recycling of synaptic vesicles in the presynaptic membranes of neurons. Depending on the nature of materials taken up by the cells, endocytosis can be defined as phagocytosis (cell eating of large particles), pinocytosis (cell drinking of extracellular fluids), or receptor-mediated endocytosis (recruitment of extracellular ligands through their membrane receptors). Generally, the endocytic process can be divided into the following steps: internalization at the plasma membrane and endosomal sorting at early endosomes, leading to recycling to the PM through recycling endosomes or lysosomal degradation through late endosomes.

Internalization at the plasma membrane

The pathways of internalization can be defined as clathrin-dependent endocytosis or clathrin-independent endocytosis (Naslavsky et al. 2003). In clathrin-dependent endocytosis, receptors and other plasma membrane proteins with specific tyrosine or dileucine motifs located in the cytoplasmic tails are recognized by the adaptor protein 2 (AP2) complex and directed into clathrin-coated pits where they are internalized (Kirchhausen 1999). These pits will bud off from the plasma membrane to form clathrin-
coated vesicles (CCVs) through the “pinchase” activity provided by the dynamin GTPase. Before CCVs reach the early endosome, their clathrin coats are removed allowing the onset of fusion with early endosomes (Schmid 1992).

The uptake of some cargo molecules is clathrin-independent. Examples of such cargo include uptake of various toxins (e.g. ricin) (Sandvig and van Deurs 1990; van Deurs et al. 1990), fluid uptake in dendritic cells (Garrett et al. 2000), macropinocytosis induced by actin-mediated membrane ruffling (Huet et al. 1980), the uptake of cholesterol and sphingolipids through caveolae (Nichols and Lippincott-Schwartz 2001), and the uptake of integral membrane proteins lacking cytoplasmic signals for AP2/clathrin complex (e.g. IL-2 receptor alpha chain (Tac), major histocompatibility class I (MHC I) and β-integrins) (Powelka et al. 2004; Li et al. 2005).

**Molecular sorting in early endosomes**

After internalization (clathrin-dependent or clathrin-independent) from the plasma membrane, the internalized cargo reaches the mildly acidified early endosome, with a lumenal pH of about 6.0. In the early endosome, the molecules to be recycled such as fluid, plasma membrane lipids, or certain receptors will be transported to the cell surface either directly (rapid recycling), or indirectly (slow recycling) through recycling endosomes (pH 6.4-6.5). Molecules that are not recycled will enter late endosomes (pH<6.0) either *en route* to the TGN or to lysosome (pH5.5-6.0). In polarized cells, such as epithelial MDCK (Madin-Darby Canine Kidney), molecules internalized from the
apical surface (such as IgA) or from the basolateral surface (such as Transferrin, IgA) will initially go to the apical or basolateral early endosomes respectively, and then both will go to the common recycling endosomes. Transferrin will be recycled back to the basolateral surface directly and IgA will go to an apical recycling endosome (ARE) and finally reach the apical plasma membrane (Brown et al. 2000; Wang et al. 2000).

The mechanism of molecular sorting in early endosomes is still not clear. According to the area-to-volume ratio theory, membrane-bound molecules such as integral membrane proteins are enriched in tubular structures with high-area-to-volume ratio and are recycled with the tubules to the plasma membrane; whereas soluble molecules will be enriched in the vesicular areas with low-area-to-volume ratio remain in these structures as they were captured into late endosomes (Mayor et al. 1993; Maxfield and McGraw 2004).

Recently, it was shown that the molecular sorting in the early endosome is highly regulated. Ubiquitin molecules play crucial roles in regulating endosomal sorting of some growth factor receptors in the multivesicular bodies (MVBs)/late endosomes (Babst et al. 2002; Babst et al. 2002; Katzmann et al. 2002; Katzmann and Wendland 2005; Katzmann 2006; Piper and Katzmann 2006). For example, the binding of EGF (epithelium growth factor) to its receptor activates the intrinsic receptor tyrosine kinase and triggers intracellular MAPK (mitogen-activated protein kinase) signaling cascades to regulate cell proliferation. Later, the activated EGF-EGFR complexes are downregulated through endocytosis followed by sorting into the luminal vesicles of MVBs (late
endosomes), *en route* to the lysosomes for degradation. The E3 ubiquitin ligase Cbl binds to phosphorylated EGFR through its SH2 domain and transfers ubiquitin molecules from the E2 enzyme to the cytoplasmic domain of EGFR through its Ring domain (Joazeiro *et al.* 1999; Joazeiro and Weissman 2000; Donaldson *et al.* 2003; Li *et al.* 2005). Overexpression of wild type Cbl enhances EGFR degradation but not its internalization. In addition, overexpression of a dominant negative Cbl mutant defective in ligase activity does not induce degradation of EGFRs. This work suggests that the ubiquitylation of EGFRs is required for their sorting in MVBs for lysosomal degradation. Non-ubiquitylated EGFRs are recycled to the plasma membrane, leading to prolonged stimulation of downstream MAPK cascades (Thien and Langdon 2001; Thien *et al.* 2001).

**Molecular players controlling endocytic trafficking**

There are four important steps in endocytic trafficking including: vesicle formation, vesicle movement, vesicle docking/tethering, and fusion with target membranes. Each step is highly regulated by various factors. Rab proteins are key regulators of several of these processes.

**Rab proteins**

In 1987, Salminen and Novick published a paper showing that a yeast protein, Sec4p, related to the superfamily of Ras GTPases, is essential for secretory vesicle exocytosis.
(Salminen and Novick 1987). Later, another yeast GTPase protein, Ypt1p, was shown to be necessary for vesicle transport between the endoplasmic reticulum (ER) and cis-Golgi (Botstein et al. 1988; Segev et al. 1988). Now, more than 60 mammalian Rab proteins and 29 nematode Rab proteins have been identified (Pereira-Leal and Seabra 2000; Pereira-Leal and Seabra 2001). Moreover, a variety of Rab-specific effectors have also been found to be associated with Rab proteins to perform their functions. Rab GTPases play important roles in the recruitment of factors related to vesicle formation, membrane budding, tethering, docking, and fusion. Some Rabs can even recruit specific microtubule- or actin- based motor proteins such as kinesin or myosin. (Zerial and McBride 2001).

**The structural basis for rab function**

Rab proteins are members of the superfamily of Ras GTP-binding proteins with molecular weight about 20-25kDa. Similar to Ras, Rab GTPases function between an active, GTP-bound and inactive, GDP-bound status. In the cytosol, the GDP-bound Rab protein binds with a GDP dissociation inhibitor (GDI). GDI delivers the GDP-bound Rab to donor membranes, where GDI is replaced by a GDI displacement factor (GDF). Next, a guanine nucleotide exchange factor (GEF) stimulates exchange of GDP to GTP. Thus, activated Rab-GTP can, in some cases, stimulate budding of transport vesicles and/or recruit effectors required for the motility of vesicles along the cytoskeleton, or that promote docking/fusion with acceptor membranes. After membrane docking/fusion, a GTPase activating protein (GAP) can stimulate the GTPase activity of the Rab leading to
effector dissociation. Finally, GDI extracts Rab-GDP from acceptor membranes and recycles it to donor membranes for another round of transport (Moyer and Balch 2001).

Rab proteins include three highly conserved guanine nucleotide-binding motifs (G1 to G3) and three conserved phosphate/magnesium-binding motifs (PM1 to PM3). In addition, there are two regions, called switch I and switch II, that respond to GAP-stimulated hydrolysis of GTP and undergo dramatic conformational changes. Such a conformational change results in inactivation of the Rab and reduced affinity for effectors. Finally, there are hypervariable regions formed discontinuously in the N-terminus, central region, and C-terminus, which coalesce into a deep pocket to form a Rab complementary-determining region (Rab CDR). For the most part of Rab CDRs are not conserved between different members of the Rab family. It is believed that individual GTP-bound Rabs can bind with varying effectors through specific interaction with the hypervariable Rab CDR regions.

**Regulation of intracellular transport**

It has been reported that Rab proteins have several functions, with some Rabs associated with vesicle budding, motility, tethering/docking, and/or fusion. In the early endocytic pathway, Rab5 regulates the clathrin-coated-vesicle (CCV)-mediated transport from the plasma membrane to the early endosome, and homotypic early endosome fusion (Rubino et al. 2000).
The first Rab5 effector found is called Rabaptin-5. Rabaptin-5 can form a complex with Rabex-5, a GEF which catalyses GDP-GTP exchange on Rab5. Once Rab5 is activated by Rabex-5, the GTP-bound Rab5 can recruit the phosphatidylinositol-3-kinases (PI(3)K) and increase the local concentration of phosphatidylinositol-3-phosphate (PI(3)P) in the vesicle membrane. The enrichment of PI(3)P is necessary for the recruitment of early endosome antigen 1 (EEA1) through the FYVE fingers in the EEA1 protein. Thus, through EEA1 protein, the GTP-bound Rab5 can enhance vesicle tethering/docking to the acceptor membranes and subsequently the SNARE-SNAP-NSF-dependent membrane fusion (Christoforidis et al. 1999; McBride et al. 1999).

Recently, it has been shown that Rab proteins are not restricted to membrane fusion, but also function in movement of vesicles between organelles along cytoskeletal filaments such as microtubules and actin microfilaments. In the microtubule-dependent transport, it has been shown that Rab5 regulates the motility of early endosomes along microtubules (Nielsen et al. 1999).

The existence of endosomal sub-domains

Now you may ask how Rab proteins can regulate various biochemical reactions and how these processes are coordinated? If Rab proteins and their effectors are randomly recruited and distributed in the membranes of donor, transport vesicle, or acceptor? There is more and more evidence to support the idea that Rab proteins and their related effectors are not randomly distributed in membrane but are enriched in specific domains.
with different lipid compositions. For example, as mentioned above, Rab5 protein and its effectors may be able to form a specific “Rab5 domain” enriched in PI (3) P and EEA1 in early endosomes. This “Rab5 domain” may be maintained through protein-protein interactions. On the membrane, Rab5, EEA1, Rabaptin-5, and Rabex-5 may form oligomeric complexes with NSF necessary for membrane fusion (Christoforidis et al. 1999; McBride et al. 1999). In addition, a scaffold, such as the actin cytoskeleton may be also helpful to stabilize the local membrane composition of Rab proteins and their effectors. By using fluorescent protein (GFP)-tagged Rab5, Rab4, and Rab11 proteins, immunofluorescence and video microscopy studies, Zerial and colleagues showed that these three proteins were localized within subdomains on endosomal membranes. Three kinds of endosome were identified with different expression profiles: one primarily containing Rab5 corresponding to the early endosomes, a second containing Rab4/Rab5 corresponding to the sorting endosomes, and a third containing Rab4/Rab11 corresponding to the recycling endosomes (Wilson et al. 2000).

**Differences in endocytic trafficking between yeast and metazoans**

Many factors involved in secretion and endocytosis are highly conserved between yeast and higher organism such as nematode and mammalian cells (Gruenberg 2001). In yeast and animal cells, most of the mechanisms in secretion and endocytosis are similar except in the steps of internalization and recycling. Clathrin-dependent internalization is prevalent in mammalian cells. On the contrary, actin activation is required in plasma membrane internalization by yeast (D'Hondt et al. 2000). For the recycling process, most
membrane proteins will be recycled back to the plasma surface in metazoans but may not be in yeast. It has been shown that most membrane proteins will be degraded in vacuoles (lysosomes) or be sent to the TGN through the pre-vacuolar compartments (late endosomes) to join the secretion pathway (Shaw et al. 2001).

**Using *C. elegans* to decipher endocytosis**

*C. elegans* has already become one of the most important models in the study of synaptic trafficking (Harris et al. 2001). Recently, more endocytic assay systems for genetic screens have been established in various *C. elegans* tissues such as oocytes (Grant and Hirsh 1999) coelomocytes (Fares and Greenwald 2001), and the intestine. Through genetic screens in *C. elegans*, we have previously identified new endocytic genes such as *rme-1* (Grant et al. 2001), *rme-2* (Grant and Hirsh 1999), *rme-6* (Sato et al. 2005), and *rme-8* (Zhang et al. 2001) involved in the recycling, internalization, and late endosomal process of endocytic processes. In my thesis, I study and characterize several other endocytic genes in the intestine of *C. elegans*, called *gum-1*, which is the *C. elegans rab-10* gene, and one of its interacting partners, called *ehbp-1* gene.
CHAPTER 2

RAB-10 Is Required for Endocytic Recycling in the

*Caenorhabditis elegans* Intestine

This chapter was published as presented here in *Molecular Biology of the Cell* (Chen et al. 2006). My contributions to this paper were writing of the paper, and results except the genetic mapping, and sequencing analysis of *rab-10* mutants.
INTRODUCTION

Endocytosis and endocytic trafficking controls the uptake and sorting of extracellular macromolecules as well as the components of the cell membrane itself, counterbalancing secretion and allowing a complex interplay between cells and their environment that is important for a myriad of cellular activities (Brodsky et al. 2001; Maxfield and McGraw 2004). The steps involved in the uptake and trafficking of cargo within the endosomal system have been well described, but many of the components mediating these steps at the molecular level remain to be identified (Brodsky et al. 2001; Maxfield and McGraw 2004). Many receptors and their associated ligands cluster into clathrin-coated pits, whereas other types of cargo utilize clathrin-independent uptake mechanisms (Nichols 2003; Gesbert et al. 2004). Plasma membrane invaginations pinch off into vesicles, uncoat, and then fuse with one another and with early endosomes. In early endosomes some ligand-receptor complexes dissociate because of the reduced pH of the endosomal lumen (Mukherjee et al. 1997). Many receptors then recycle to the plasma membrane (PM) either directly or indirectly via recycling endosomes (Mukherjee et al. 1997; Maxfield and McGraw 2004). Many ligands do not recycle but instead are transported from early to late endosomes and eventually to lysosomes for degradation (Mukherjee et al. 1997). In polarized epithelial cells such as cultured Madin-Darby canine kidney (MDCK) cells, an additional layer of complexity in the endocytic pathway contributes to formation and/or maintenance of the specialized apical and basolateral domains (Nelson and Yeaman 2001; Mostov et al. 2003; Hoekstra et al. 2004). Both the apical and basolateral membranes deliver cargo to early endosomes. Basolaterally derived and
apical cargo can reach medially located “common endosomes,” which can sort cargo from either source to the basolateral plasma membranes or to apical recycling endosomes (ARE), which can then ultimately send cargo to the apical plasma membranes (Brown et al. 2000; Wang et al. 2000; Hoekstra et al. 2004). One group of master regulators of trafficking that has come under intense scrutiny are the Rab proteins, members of the Ras superfamily of small GTPases (Zerial and McBride 2001). The human genome encodes 60 Rab proteins, whereas the Caenorhabditis elegans genome encodes 29 predicted members (Pereira-Leal and Seabra 2001). Each step in membrane transport is thought to require at least one such Rab protein (Pfeffer 1994; Zerial and McBride 2001). Rabs act to recruit effector proteins to membranes and have been proposed to regulate transport in several ways including promoting vesicle formation and recruitment of cargo into budding transport carriers, promoting molecular motor-based movement of vesicles toward target membranes, and regulating fusion of vesicles with target membranes (Zerial and McBride 2001). Similar to Ras, Rab GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. GDP-bound Rab proteins are primarily found in the cytoplasm bound to GDP dissociation inhibitor (GDI) (Seabra and Wasmeier 2004). GDI protein delivers GDP-bound Rab proteins to donor membranes where GDI is replaced by a GDI displacement factor (GDF) (Pfeffer and Aivazian 2004). Next, a guanine nucleotide exchange factor (GEF) stimulates replacement of GDP with GTP. Thus, activated Rab-GTP can recruit effectors and promote transport. After membrane docking and fusion, a GTPase-activating protein (GAP) can stimulate the GTPase activity of the Rab protein, leading to GTP hydrolysis and dissociation of the Rab from its effectors. Finally, GDI extracts Rab-GDP from the membrane and recycles it to the donor
compartment for another round of transport (Seabra and Wasmeier 2004). A number of Rab proteins have been implicated in the endocytic recycling pathway including Rab4, Rab11, and Rab15 in the clathrin pathway, and Rab22 and the more distantly related Arf6 in the recycling of cargo internalized independently of clathrin (van der Sluijs et al. 1992; Ullrich et al. 1996; Sheff et al. 1999; Zuk and Elferink 2000; Kauppi et al. 2002; Naslavsky et al. 2004; Weigert et al. 2004). In nonpolarized cells Rab4 is primarily associated with early endosomes and is thought to contribute to direct transport from early endosomes to the plasma membrane as well as early endosome to recycling endosome transport (Sheff et al. 1999). In MDCK cells Rab4 has also been reported to associate with early endosomes and a later compartment named the common endosome, where it promotes apical delivery of transcytotic cargo (Mohrmann et al. 2002). Rab11 is primarily associated with recycling endosomes and has been reported to act after Rab4 in nonpolarized cells, mediating recycling endosome to plasma membrane transport of clathrin-dependent and clathrin-independent recycling cargo (Sheff et al. 1999; Weigert et al. 2004). In polarized cells Rab11 is primarily associated with the ARE and is thought to function in the transport of cargo from the ARE to the plasma membranes (Casanova et al. 1999; Sheff et al. 1999; Brown et al. 2000). Arf6 and Rab22 are thought to specifically regulate the recycling of cargo that does not depend on clathrin for its internalization and act sequentially in such transport (Weigert et al. 2004). In polarized cells Arf6 has been proposed to mediate apical uptake of clathrin-dependent cargo and was not found to mediate recycling (Altschuler et al. 1999). Recently, we and others have established in vivo endocytic assay systems for genetic analysis of trafficking in several C. elegans tissues such as oocytes (Grant and Hirsh 1999), coelomocytes (Fares
and Greenwald 2001), and the intestine (Grant et al. 2001). Through such genetic analysis in *C. elegans*, we have identified several new key endocytic regulators such as *rme-1* (Grant et al. 2001), *rme-6* (Sato et al. 2005), and *rme-8* (Zhang et al. 2001) that are conserved from worm to human but lack clear homologues in yeast, the traditional genetic system for analysis of trafficking. In our current studies we have focused on the endocytic recycling pathways of polarized epithelial cells. In particular we have focused on the *C. elegans* intestine, a polarized epithelial tube one cell layer thick (Leung et al. 1999). The apical microvillar surface faces the lumen and is responsible for nutrient uptake from the environment (Figure 1). The basolateral surface faces the pseudocoelom (body cavity) and is responsible for the exchange of molecules between the intestine and the rest of the body (Figure 1). Several endocytic tracers such as the lipophilic dye FM4-64 and fluid-phase markers such as rhodamine-dextran, Texas Red-BSA, or GFP secreted by muscle cells have been used in the studies of *C. elegans* intestinal endocytosis (Grant et al. 2001). When such tracers are taken up by the intestine lumen (apical surface), they all accumulate in the gut granules (lipofuscin-positive lysosomes) (Grant et al. 2001). On the contrary, when these tracers are applied to the pseudocoelom by microinjection (or by expression in the case of GFP) and taken up from the basolateral plasma membrane of the intestine, only the FM 4–64 dye reaches the lysosomes while the fluid-phase markers are instead recycled back to the pseudocoelom (Grant et al. 2001). *rme-1* mutants display endocytic recycling defects in several tissues (Grant et al. 2001). These defects include strongly reduced uptake of yolk proteins by oocytes, because of poor recycling of yolk receptors, reduced uptake of fluid-phase markers by coelomocytes, and the accumulation of gigantic fluid-filled recycling endosomes in the intestinal cells, due to defective
recycling of pseudocoelomic fluid (Grant et al. 2001). The accumulation of large fluid-filled endosomes in the worm intestine is a hallmark phenotype that can be used to identify mutants with basolateral recycling defects. Although it is sometimes difficult to image fluid phase markers in recycling endosomes in normal cells, perhaps because cargo is diluted within thin membrane tubules, the recycling of endocytosed fluid has been amply demonstrated in cultured mammalian cells (Tooze and Hollinshead 1991; Apodaca et al. 1994; Barroso and Sztul 1994). At least 50% of internalized fluid recycles back into the culture medium, presumably in the same transport carriers as recycling receptors (Besterman et al. 1981; Bomsel et al. 1989; Gagescu et al. 2000). When recycling is blocked by pharmacological means in either MDCK cells (Apodaca et al. 1994) or HepG2 cells (van Weert et al. 2000) gigantic endosomal structures similar to those described in C. elegans rme-1 mutant intestinal cells are formed. Here we report molecular cloning and functional characterization of *gum-1* (*gut morphology abnormal-1*), a mutant that displays a rme-1-like mutant phenotype in the worm intestine. We show that *gum-1* is required for basolateral endocytic recycling in the *C. elegans* intestine and that *gum-1* is the *C. elegans* rab-10 gene. We provide evidence that *C. elegans* RAB-10 is physically associated with early endosomes and Golgi and propose that RAB-10 functions upstream of RME-1 in the basolateral transport of fluid and other recycling cargo.
RESULTS

*gum-1* mutants display *rme-1*-like endocytosis defects in the intestine

*rme-1* mutants display endocytic trafficking defects in several *C. elegans* tissues. These defects include the accumulation of large vacuoles in the intestinal cells, strongly reduced uptake of yolk proteins by oocytes due to poor yolk receptor recycling, and reduced uptake of fluid-phase markers by coelomocytes (Grant *et al.* 2001). RME-1 is thought to function specifically in endocytic recycling, and the vacuoles that accumulate in *rme-1* mutant intestinal cells are thought to be grossly enlarged basolateral recycling endosomes (Grant *et al.* 2001). To identify additional proteins that function in endocytic recycling, we screened for new mutants with *rme-1*-like intestinal phenotypes. One such mutant, *gum-1(q373)* (Gut morphology abnormal-1) was isolated. An additional allele of *gum-1*, *dx2*, was isolated in a genetic complementation screen with *q373*. In both of these *gum-1* mutants, large, transparent vacuoles accumulate in the worm intestine similar to those observed in *rme-1* mutant worms (Figure 2, A-C). To determine if the abnormal vacuoles in *gum-1* mutant intestines are enlarged basolateral endosomes like those that form in *rme-1* mutants, we crossed the *gum-1* mutants into the background of a transgene that directs secretion of GFP from body-wall muscle cells into body cavity, from which the secreted GFP is normally endocytosed by the basolateral intestine and then recycled into the body cavity (Fares and Greenwald 2001; Grant *et al.* 2001). We found that the large abnormal vacuoles in *gum-1* mutant intestinal cells accumulated GFP from the body cavity, indicating a defect in basolateral recycling similar to that previously found in *rme-1*.
l mutants (Figure 2, D-F). Also similar to rme-1 mutants, it was found that uptake of the fluid-phase marker Texas-red BSA or the membrane intercalating dye FM 4-64, administered to the apical intestinal membrane by feeding, was normal, and neither marker accumulated in the abnormal vacuoles of gum-1 mutants (Eric Lambie, unpublished data). Taken together these results indicate that gum-1 mutants, like rme-1 mutants, have severe defects in basolateral endocytic recycling by the intestine.

Next we sought to determine if transmembrane cargo also accumulated in the abnormal endocytic compartments of gum-1 and rme-1 mutants. To test this, we expressed three transmembrane proteins as GFP fusions in the C. elegans intestine and compared their steady-state localization in wild-type animals and with that in gum-1 or rme-1 mutant animals. Equivalent GFP-fusions for these three proteins have previously been shown to be functional and traffic normally in mammalian cells and/or C. elegans. First we expressed the human transferrin receptor (hTfR-GFP), a marker for clathrin-dependent uptake and rme-1-dependent recycling in mammalian cells (Yamashiro et al. 1984; Burack et al. 2000; Lin et al. 2001). We expressed the α-chain of the human IL-2 receptor TAC (hTAC-GFP), a marker for clathrin-independent endocytosis and rme-1-dependent recycling in mammalian cells (Caplan et al. 2002; Naslavsky et al. 2004). Finally we examined C. elegans LMP-1 (LMP-1-GFP), a worm homologue of mammalian CD63/LAMP, that is found in lysosomes of coelomocyte cells (Treusch et al. 2004), but that labels other endocytic compartments in the intestine (Hermann et al. 2005). All three of these markers primarily labeled basolateral membranes in wild-type worm intestine, with apparent localization to the plasma membranes and plasma
membrane proximal small endosomal vesicles and tubules (Figure 2, G, J, and M). In
addition to this pattern, LMP-1-GFP also labeled large round vesicular structures near the
basolateral membranes (Figure 2M).

None of these markers accumulated appreciably on apical membranes or in the auto-
fluorescent lysosomes of the worm intestine. We found that the abnormal vacuoles of
gum-1 and rme-1 mutants showed visible accumulation of all three of these
transmembrane cargo markers: hTAC-GFP (Figure 2, G-I), hTfR-GFP (Figure 2, J-L),
and LMP-1-GFP (Figure 2, M-O). Interestingly, hTAC-GFP labeled the enlarged
endosomes more strongly than did hTfR-GFP or LMP-1-GFP. These results suggest that
all three of these proteins normally transit through endosomes regulated by GUM-1 and
RME-1. The strong accumulation of hTAC-GFP in the enlarged endosomes of gum-1
and rme-1 mutants suggests that hTAC, like fluid internalized from the body cavity,
requires GUM-1 and RME-1 for export from these endosomes. The relatively weak
labeling of the enlarged endosomes of gum-1 and rme-1 mutants by the other cargo
proteins may indicate that they transit these endosomes but are less dependent on GUM-1
or RME-1 for endosome exit.

In one major respect however, gum-1 mutants do not resemble rme-1 mutants. rme-1
mutants display endocytic defects in multiple tissues of C. elegans such as oocytes and
coelemocytes. As far as we could determine using standard assays, gum-1 mutants do
not show endocytic trafficking defects in oocytes or coelomocytes, suggesting that GUM-
1 is required for trafficking in a more restricted set of tissues than RME-1. We assayed
oocyte endocytosis of the YP170-GFP yolk protein marker (Grant and Hirsh 1999), and
coelemocyte endocytosis of GFP secreted by muscle cells (Fares and Greenwald 2001;
Fares and Greenwald 2001) and found no defects in *gum-1* mutants at steady state (Figure
2, P-R and S-U). *rme-1* mutants show strong defects in both of these assays (Grant *et al.*
2001). Similar to *rme-1* mutants, basolateral secretion of YP170-GFP by the intestine
and secretion of GFP by muscle cells appeared normal in *gum-1* mutants, suggesting that
*gum-1* is not required for secretion. We also note that intestinal vacuoles of *gum-1*
mutants are generally larger than those of *rme-1* mutants, and the changes in intracellular
distribution of some transmembrane cargo proteins appears slightly different between the
two mutants (Figure 2, K, L, N, and O).

**gum-1 but not rme-1 mutations result in a dramatic increase in RAB-5-positive early endosome number with a concomitant loss in RME-1-positive recycling endosomes**

All of these phenotypes suggest that *gum-1* mutants are defective in basolateral recycling
in the worm intestine. To better understand which step in trafficking is impaired in *gum-
1* mutants, we crossed a set of intestinally expressed GFP-tagged transgenes, encoding
endosomal marker proteins, into *gum-1* mutant backgrounds and assayed the effects on
endosome morphology. We also performed a parallel analysis in an *rme-1* null mutant
background for comparison. As we have previously reported (Hermann *et al.* 2005),
wild-type intestinal cells contain abundant, GFP-RAB-5-positive early endosomes that
appear as small punctuate structures near the basolateral and apical membranes and some
larger structures in the medial cytoplasm (Figure 3A). With a GFP-RAB-7 marker we
see similar small puncta near the plasma membranes and abundant larger ring-like structures deeper in the cytoplasm (Figure 3C). These results are consistent with the idea that the small puncta are early endosomes, which are known to label for RAB-5 and RAB-7 in other systems (Zerial and McBride 2001), whereas the larger ring-like vesicles are late endosomes, known to be RAB-5 negative and RAB-7 positive in other systems. We also examined markers for intestinal recycling endosomes, GFP-RAB-11 and GFP-RME-1. In wild-type animals GFP-RAB-11 strongly labels abundant small puncta concentrated near the apical plasma membranes and less frequent puncta scattered in the cytoplasm (Figure 3E), suggesting that RAB-11 marks apical recycling endosomes, as does its ortholog Rab11 in MDCK cells (Casanova et al. 1999). RAB-11 has also been localized to the TGN in some systems and has been implicated in the secretory pathway (Chen et al. 1998). GFP-RME-1 strongly labels tubulo-vesicular endosomes very near the basolateral plasma membranes (Figure 3G), thought to be basolateral recycling endosomes. GFP-RME-1 also more weakly labels structures very near the apical plasma membranes (Figure 3I) that could be apical recycling endosomes.

In *gum-1* mutants, the GFP-RAB-5- and GFP-RAB-7-positive endosomes accumulated in abnormally high numbers (Figure 3, B, D, and K), but the number of endosomes labeled by GFP-RAB-11 was not affected (Figure 3, F and K). We performed quantitative image analysis on confocal micrographs from these GFP-tagged strains (Figure 3K) and calculated a twofold increase in RAB-5-positive endosome number in *gum-1* mutants compared with wild-type animals. We believe that this is an underestimate of the increased number of labeled endosomes, because the endosomes became so abundant in
the mutant background that it became difficult to resolve individual puncta. In addition, the size of GFP-RAB-5-labeled endosomes were also increased in *gum-1* mutants (Figure 3L). Higher percentages of endosomes with larger sizes (>0.75 µm²) were found in *gum-1* mutants than in control animals. Many, but not all, of the gigantic vacuoles present in *gum-1* mutant intestinal cells were positive for GFP-RAB-5 and GFP-RAB-7 (Figure 3, B and D; Figure 4, A-F, arrows) but not GFP-RAB-11 or GFP-RME-1 (unpublished data), suggesting that many of these structures are grossly enlarged early endosomes. In addition, the tubulovesicular basolateral recycling endosomes normally labeled by GFP-RME-1 were lost almost completely in *gum-1* mutants (Figure 3, G-J). GFP-RME-1 appears mostly diffuse in the cytoplasm in *gum-1* mutants, with rare basolateral puncta remaining (Figure 3, G and H). However, apical GFP-RME-1 appears normal or even enhanced in *gum-1* mutants (Figure 3, I and J). Autofluorescent lysosome morphology was unchanged in *gum-1* mutants but displayed enhanced autofluorescent intensity (personal observation). The diffusion of RME-1 in *gum-1* mutants could indicate a defect in recruiting RME-1 to basolateral recycling endosomes, or it could indicate a loss of basolateral recycling endosomes altogether (and thus a loss of RME-1-binding sites). The second model, in which early endosome to basolateral recycling endosome transport is blocked, might better explain the observed increase in early endosomes number in *gum-1* mutants, because *rme-1* null mutants do not accumulate increased numbers of early endosomes (Figure 3K; and Figure 45, A-D). In addition, the morphology and number of endosomes labeled by GFP-RAB-10 or GFP-RAB-11 were not affected in *rme-1(b1045)* mutant (Figure 45, E-F and G-H). Taken together these results indicate that RAB-10 and RME-1 function in two consecutive steps; that is RAB-10 functions in
trafficking from early to recycling endosomes and RME-1 functions in trafficking from recycling endosomes to the basolateral plasma membrane.

**gum-1 encodes the C. elegans RAB-10 protein**

Using standard methods we mapped *gum-1* close to the right of *bli-4* on chromosome I (see *Materials and Methods, Chapter 5*). To determine which of the genes in this region corresponded to *gum-1*, we microinjected *gum-1* mutants with wild-type *C. elegans* genomic DNA and assayed for rescue of the intestinal phenotype. We found that transgenic *gum-1* mutants carrying cosmid clone T23H2 were fully rescued. We were able to narrow the rescuing activity to a 4-kb region containing only one predicted gene, T23H2.5, the *C. elegans* *rab-10* gene, the apparent ortholog of human Rab10. We then confirmed that *gum-1* is *rab-10* by RNAi phenocopy of the intestinal vacuole phenotype and by identifying specific sequence changes in the *rab-10* gene amplified from *gum-1* mutant genomic DNA (see *Materials and Methods, Chapter 5*). We identified a nonsense mutation in the *dx2* allele resulting in a premature stop codon predicted to truncate the protein at amino acid 21. Thus *dx2* is a predicted null allele of *rab-10*. We identified a missense mutation in the *q373* allele resulting in a predicted amino acid change (L26R) in the conserved GTP-binding domain of RAB-10 that would be predicted to interfere with GTP binding, an essential feature of any Rab protein (Figure 5). Taken together these results showed that *gum-1* is *rab-10* and showed that the phenotypes we described above for *gum-1(dx2)* represent the null phenotype for *rab-10* in *C. elegans*. Hereafter we refer to this gene as *rab-10* to reflect its molecular nature.
RAB-10 is broadly expressed in C. elegans

To determine when and where rab-10 is normally expressed in C. elegans, we created transgenic animals expressing either GFP only, or a GFP-rab-10 fusion gene, driven by 2.9 kb of rab-10 upstream sequences (the predicted promoter region). We found that either of these constructs expressed almost ubiquitously. We observed expression in the intestine, hypodermis, seam cells, body-wall muscles and many neurons, oviduct sheath cell and spermatheca, coelomocytes, and pharynx and nerve ring (Figure 6, A-G). The GFP-RAB-10 fusion protein appeared punctate in most tissues. In the intestine GFP-RAB-10 localized to distinct cytoplasmic puncta resembling endosomes that ranged in size from 0.5 to 1.0 μm (Figure 6A, arrowheads). We found that the intestinal phenotype of rab-10(dx2) and rab-10(q373) was completely rescued by the GFP-RAB-10 fusion protein, indicating that the expression pattern and subcellular localization of the reporters very likely reflect those of the endogenous protein.

The rab-10-mutant phenotype can be rescued by GFP-Ce-RAB-10 or GFP-human Rab10 fusion proteins expressed under the control of an intestine-specific promoter

We also sought to determine if RAB-10 functions cell autonomously in the intestine and if the human Rab10 protein (hRab10) can substitute for worm RAB-10 in vivo. Toward this end we created transgenic worms expressing GFP-tagged C. elegans RAB-10 or human Rab10 driven by an intestine-specific promoter derived from the vha-6 gene (Oka et al. 2001). We then assayed the rescuing ability and subcellular localization of GFP-
Ce-RAB-10 and GFP-hRab10 fusion proteins after crossing them into a \textit{rab-10(dx2)} null mutant background. Rescue was assayed quantitatively by counting the number of abnormal intestinal vacuoles. GFP alone expressed in the intestine of \textit{rab-10(dx2)} mutant worms was used a baseline control. We found that the \textit{rab-10} mutant intestinal phenotype was fully rescued by intestine-specific expression of GFP-Ce-RAB-10 or GFP-hRab10, indicating that RAB-10 functions autonomously in the intestine and that human Rab10 is a true ortholog of \textit{C. elegans} RAB-10 (Figure 7, A, B, and E).

In addition, we tested the importance of the predicted GTP binding and GTP hydrolysis activities of Ce-RAB-10 for in vivo function. Taking advantage of the well-studied biochemistry of ras/rab family GTPases, we engineered specific mutations into RAB-10 in the context of the \textit{vha-6} promoter-driven GFP-RAB-10 construct. We engineered point mutations into the construct that would be predicted to lock RAB-10 into the GDP-bound conformation (T23N) or the GTP-bound conformation (Q68L) and assayed the ability of the two mutant forms of RAB-10 to rescue a \textit{rab-10(dx2)} null mutant. We also compared the subcellular localization of the two mutant forms of RAB-10 with that of the wild-type form. The endosomes labeled by Ce-GFP-RAB-10 or hRab10 proteins appeared as strong puncta throughout the cytoplasm and more weakly as a line just below the apical membrane (Figure 7, A and B, arrowheads). Interestingly we found that the predicted GTP-locked (predicted GTPase defective) form, GFP-RAB-10 (Q68L), also displayed strong punctate labeling, indicating an association with membranes as expected, and displayed partial rescuing activity (Figure 7, C and E). In addition, the GDP-bound form, GFP-RAB-10 (T23N), lacked rescuing activity completely and appeared diffuse in
the cytoplasm (Figure 7, D and E). These results indicate that GTP-binding, and a normal GDP/GTP cycle, are important for RAB-10 function and its localization to membranes in vivo. In a wild-type background neither of these predicted dominant forms of GFP-RAB-10 had sufficient interfering activity to produce obvious vacuoles similar to those found in \textit{rab-10} loss of function mutants (personal observation).

\textbf{RAB-10 is associated with endosomes and Golgi in the intestine}

To determine where RAB-10 is normally localized and to help test the hypothesis that RAB-10 functions directly in endocytic transport, we performed a series of colocalization studies using rescuing GFP-tagged-RAB-10 and a set of RFP-tagged endosomal markers very similar to the GFP markers described above. GFP-RAB-10 did not colocalize with RFP-RME-1 on basolateral recycling endosomes (Figure 8, M-R). However, we did find that a specific subset of intestinal GFP-RAB-10-labeled puncta colocalize very well with a subset of the early endosomes marked with RFP-RAB-5 (Figure 8, A-C). Most of the puncta positive for GFP-RAB-10 and RFP-RAB-5 are located very close to the basal PM and are best observed in the “Top” focal plane. Very few RAB-10 and RAB-5 double-positive structures were found in the “Middle” focal planes that offer better views of the medial and apical membranes (Figure 8, D-F). We also observed colocalization of GFP-RAB-10 with the early/late endosomal marker RFP-RAB-7, but the RAB-7 labeling of these structures was weaker (Figure 8, G-I).
Unlike RFP-RAB-5, which primarily colocalized with GFP-RAB-10 near the basal and lateral PM, RFP-RAB-11 extensively colocalized with GFP-RAB-10 in puncta of the medial cytoplasm (Figure 8, J-L, arrowheads). These results indicate that the medially localized RAB-10-positive structures are likely to be Golgi and/or apical recycling endosomes. We also observed localization of both GFP-RAB-10 and RFP-RAB-11 very near the apical PM (Figure 8, J-L, arrows). Given previously published reports that mammalian Rab10 is Golgi localized (Chen et al. 1993) and given my own observations showing extensive colocalization of GFP-RAB-10 with RFP-RAB-11, we next tested RAB-10 for Golgi localization using a specific C. elegans Golgi marker alpha-mannosidase II-GFP (MANS-GFP; (Rolls et al. 2002); Figure 9, A-F). Unlike vertebrate cells that contain one large juxtanuclear Golgi stack, most invertebrate cells such as those in C. elegans instead contain many small Golgi stacks per cell, with the individual “mini-stacks” dispersed throughout the cytoplasm (Figure 9, A and D). Although the size and shape of the Golgi “mini-stacks” marked by MANS-GFP were different from the puncta labeled by RFP-RAB-10 (Figure 9, B and E), most medially located RFP-RAB-10-labeled structures overlapped with or were found directly adjacent to MANS-GFP labeled Golgi (Figure 9, C and F). These results indicate that many of the medially located RAB-10-labeled structures are likely to be Golgi associated structures, probably TGN. Taken together these results are consistent with RAB-10 residing on a subset of basolateral early endosomes, where it could participate directly in basolateral cargo recycling. These results also indicate a significant residence of RAB-10 on TGN and/or apical recycling endosomes, where it could perform additional functions in secretion and/or apical recycling.
DISCUSSION

**RAB-10 is required for endocytic recycling in the worm intestine**

Here we present the first in vivo studies of *rab-10* function in any organism and demonstrate a requirement for *rab-10* in endocytic recycling. In *C. elegans* we found that expression of human Rab10 completely rescues the intestinal phenotype caused by loss of endogenous RAB-10 and that the distribution of human Rab10 within worm cells is quite similar to that of *C. elegans* RAB-10, establishing that the two proteins are functionally interchangeable and are true orthologues. Thus our studies of worm RAB-10 are very likely to be highly applicable to understanding Rab10 function in mammalian systems.

The intestinal phenotype caused by loss of *rab-10* is very similar to that caused by loss of *rme-1*, a known endocytic recycling factor (Grant *et al.* 2001; Lin *et al.* 2001). However, unlike *rme-1* mutants that also display obvious endocytic defects in oocytes and coelomocytes, we did not detect endocytic defects in oocytes or coelomocytes in *rab-10* mutant worms. Because our expression studies indicate that RAB-10 is broadly expressed, this lack of phenotype in other tissues likely indicates a redundancy in function for RAB-10, perhaps with another Rab protein, in at least some nonintestinal tissues. *C. elegans* intestinal cells are polarized epithelial cells with distinct apical and basolateral membrane compartments and thus display an increased complexity of membrane trafficking processes compared with nonpolarized cells. In this more complex context there may be less redundancy in the trafficking pathways such that loss of
individual components such as RAB-10 lead to more severe trafficking defects than occurs in nonpolarized cells.

In *rab-10* mutants, as in *rme-1* mutants, fluid-phase markers taken up through basolateral endocytosis accumulate in grossly enlarged endosomes. Endocytic tracers taken up by apical endocytosis never label these enlarged endosomes (unpublished data), suggesting that they are specifically of basolateral origin. The accumulation of endocytic tracers in these enlarged endosomes indicates that the internalization step of endocytosis is not significantly impaired in *rab-10* mutants, but rather the recycling of the fluid back to the body cavity is defective. We also found that basolaterally localized transmembrane cargo proteins thought to be endocytosed by both clathrin-mediated and clathrin-independent mechanism label the enlarged endosomes, consistent with the proposal that *rab-10*, like *rme-1*, regulates endocytic recycling but not endocytosis per se and that clathrin-dependent and clathrin-independent cargo are likely to meet in the endosomal system, as has been suggested in mammalian cell systems (Naslavsky *et al.* 2004; Weigert *et al.* 2004). Because all exogenous tracers that we have identified for studying endocytosis in *C. elegans* are sent to lysosomes when internalized apically but not recycled back to the intestinal lumen, we have not been able to determine if *rab-10* or *rme-1* mutations also affect apical recycling in the worm intestine. The only currently available transmembrane marker for the apical intestinal membrane is OPT-2, but it is not known if OPT-2 cycles through the apical endosomal system. In preliminary studies we find OPT-2-GFP to be normally localized in the intestine of *rab-10* mutant worms (S. Vashist and B. D. Grant, unpublished results).
RAB-10 may regulate transport between basolateral early and recycling endosomes

We took two additional approaches to determine the likely step in endocytic transport controlled by RAB-10. First we analyzed the number and morphology of all major endosome classes in rab-10 mutant intestinal cells in vivo, using a set of GFP-tagged endosome markers. It expected that a kinetic block in a particular transport step caused by the lack of RAB-10 would cause specific changes in endosome morphology. In general it expected that the RAB-10 donor compartment, or vesicles derived from that compartment, would become more numerous and/or enlarged in cells lacking RAB-10. It also expected that the target compartment, which normally receives cargo in a RAB-10-dependent manner, might be lost altogether and/or become smaller in size in cells lacking RAB-10. In fact we found that endosomes positive for RAB-5 and RAB-7 became more numerous and occasionally formed enormous endosomes (Figure 4), consistent with a block in export from the early endosomes. Conversely, the only compartment to become lost or greatly diminished was the RME-1-positive basolateral recycling endosomes. Taken together these results imply that transport from early endosomes to recycling endosomes is defective in rab-10 null mutants. An alternative view of these findings could be that RAB-10 functions with RME-1 in basolateral recycling endosome to PM transport and that RAB-10 functions to recruit RME-1 to endosomal membranes. We favor the first model where RAB-10 functions one step earlier than RME-1, because loss of RAB-10 leads to an abnormal accumulation of early endosomes, but loss of RME-1 does not lead to an abnormal accumulation of early endosomes, and because we could identify RAB-10 on early endosomes but not basolateral recycling endosomes (see
below). Consistent with previous results indicating that RME-1 mediates a distinct later recycling step, from basolateral recycling endosomes to the plasma membrane, we found that \textit{rme-1} null mutants accumulated the same recycling cargo as \textit{rab-10} mutants, but unlike \textit{rab-10} mutants, the number and size of the RAB-5-, RAB-7-, or RAB-10-labeled early endosomes was unaffected. Likewise the grossly enlarged endosomes evident in \textit{rme-1} null mutants did not label with these early endosome markers.

\textbf{RAB-10 colocalizes with endosome and Golgi markers in worm intestinal cells}

As a second approach to determine a likely step in endocytic transport controlled by RAB-10, we compared the subcellular localization of a rescuing GFP-RAB-10 fusion protein with a series of endosomal markers fused to RFP in the intestine of live animals. We found that a subset of the GFP-RAB-10-labeled puncta colocalize very well with a subset of the puncta labeled with early endosome markers. Medially localized GFP-RAB-10 showed extensive colocalization with medially localized RFP-RAB-11, a marker of Golgi and ARE, and MANS-GFP, a marker of Golgi ministacks, consistent with previously published evidence that Rab10 associates with Golgi in CHO cells and sea urchin embryos (Chen et al. 1993; Leaf and Blum 1998). Finally GFP-RAB-10 weakly labeled structures very near the apical PM, similarly to RFP-RAB-11 and RFP-RME-1, further suggesting possible association of RAB-10 with the ARE. Such apical GFP-RAB-10 was often only visible when expressed in a \textit{rab-10} null mutant background, suggesting that apical RAB-10-binding sites are easily saturated. These results suggest
that *C. elegans* RAB-10 is associated with multiple compartments. In particular the localization of RAB-10 to the Golgi suggests a role for RAB-10 in secretion.

Although we did not find evidence for basolateral (YP170-GFP) or apical (OPT-2-GFP) secretion defects in the *rab-10* null mutant, we sometimes observed some retention of intestinal YP170-GFP after *rab-10* RNAi (C. Chen, S. Vashist, B. D. Grant, unpublished observations). Such retention of the secretory reporter after *rab-10* RNAi could indicate that *rab-10* acts redundantly in secretion, such that removal of *rab-10* alone has no effect, but additional depletion of one or more closely related RAB proteins by RNAi crossover reveals a redundant requirement. Further analysis will be required to address this issue.

The lack of colocalization of basolateral RFP-RME-1 with any of the other compartment markers further supports our previous evidence that RME-1 localizes to a distinct basolateral recycling compartment. We did note however that in addition to the strong localization of GFP- or RFP-tagged RME-1 to basolateral endosomes, in live animals we could also detect weaker apical labeling with GFP- or RFP-tagged RME-1. Apical RME-1 partially overlaps with apical RAB-11, suggesting that some RME-1 is also present on the ARE (Z. Balklava and B. D. Grant, unpublished observations). This apical GFP-RME-1 is most clearly evident in live larvae, is less prominent in live adults, and is lost when animals are fixed and permeabilized for immunofluorescence, which is the most likely reason we did not detect such labeling previously.

*Comparisons with the mammalian system*
The worm intestine displays many of the defining characteristics of a polarized epithelium including well-defined apical and basolateral membrane domains separated by apical tight junctions that are thought to act as a molecular “fence”. The apical domain is actin-rich with prominent microvilli (Segbert et al. 2004). A well-defined subapical terminal web-rich in intermediate filaments and actin-binding proteins similar to that of mammalian intestinal epithelia is also present (Bossinger et al. 2004). In addition to the obvious role intestinal cells must play in the uptake and processing of nutrients and the subsequent distribution of those nutrients throughout the body, the worm intestine is also responsible for the synthesis and secretion of abundant lipoprotein complexes (yolk), a liver-like function (Kimble and Sharrock 1983). Finally the worm intestine is the major site of fat storage in the form of lipid droplets and thus also serves an adipose-like function for the animal (Ashrafi et al. 2003). It is likely that all of these functions as well as additional unknown functions for these cells contributed to the evolution of the complex trafficking pathways present in this tissue.

At present it is not clear how closely the membrane trafficking pathways of the worm intestine and those of mammalian polarized epithelial cells parallel one another, although our analysis of endomembrane markers presented here indicates significant similarity, including an apical compartment in the worm intestine that is highly enriched in RAB-11, a marker for the mammalian ARE (Casanova et al. 1999). One unique aspect of endomembrane organization in polarized MDCK cells is the presence of a common endosome that receives recycling or transcytotic cargo from both PM domains, which it then resorts for delivery to basolateral or apical membranes (Brown et al. 2000; Wang et
In polarized MDCK cells, GFP-Rab10 is enriched on the common endosomes (Babbey et al. 2006). In this study we show that RAB-10 in *C. elegans* localizes to several compartments including a basolaterally localized compartment enriched in RAB-5, a standard marker for early endosomes. In addition, we observed strong RAB-10 labeling of a medial compartment positive for RAB-11 and mannosidase, likely the worm TGN. Finally RAB-10 is often visible very close to the apical PM in close proximity to RAB-11, possibly labeling the ARE. One possibility is that the medially localized and/or apical compartments positive for RAB-10 and RAB-11 are involved in apical-basal sorting, although we cannot currently test this without identifying apically recycling cargo in the worm intestine. However in *rab-10* mutants we did not observe any steady miss-sorting of apical cargo (OPT-2-GFP) to the basolateral membrane or miss-sorting of basolateral cargo (YP170-GFP, hTAC-GFP, hTfR-GFP, or LMP-1-GFP) to the apical membrane.

Given our finding that human Rab10 can function in the context of the worm intestine, presumably recruiting and regulating endogenous effectors that mediate endocytic recycling, we anticipate that the molecular interactions that mediate RAB-10-dependent transport pathways in worms and humans are likely to be the same, and *C. elegans* genetics is likely to yield more important players in endocytic transport that are conserved among metazoans.
CHAPTER 3

Searching for RAB-10 Interacting Partners by

Yeast Two-hybrid Screens
INTRODUCTION

In Chapter 2, it was revealed that RAB-10 functions in basolateral endocytic trafficking, from early endosomes to recycling endosomes in the *C. elegans* intestine. In order to understand the mechanism by which RAB-10 promotes basolateral endocytic recycling, I decided to search for RAB-10-interacting proteins by screening a *C. elegans* cDNA library using the yeast two-hybrid system.

*Choice of yeast two-hybrid screens system*

In my study, I chose to use a *LexA*-based, galactose-inducible, yeast two-hybrid system to screen the *C. elegans* cDNA library. Different from the traditional *Gal4p*-based yeast two-hybrid system (Chien *et al.* 1991), the *LexA*-based system utilizes the same basic principle of the *Gal4p*-based system except that the DNA binding protein encoded by the bait plasmid pEG202 (HIS\(^+\)) is from the *E. coli* LexA protein and the transcription activation domain encoded by the prey plasmid pJG4-5 (TRP\(^+\)) is from the acid blob domain of the B42 protein (Fields and Song 1989; Gyuris *et al.* 1993). Because the DNA-binding and transcription activation domains are prokaryotic (from *E. coli*) rather than eukaryotic (from yeast *Gal4p*), the possibilities of false-positive results obtained from screens are thought to be reduced. In addition, a higher number of *LexA* DNA binding sites in the upstream promoter areas of reporter genes were included to increase the sensitivity of the assays. Identifying prey proteins having weak interaction with bait proteins maybe especially important in the case of RAB proteins which bind their
effectors weakly and transiently (Ren et al. 1996). So utilizing a yeast two-hybrid system with high sensitivity and less false background becomes an important issue in the case of searching for RAB-interacting proteins.

*Choice of the GTP-bound, constitutively active form of the RAB-10 protein as the bait in the yeast two-hybrid screens*

In addition, another way to improve detection of RAB-interacting partners is to use the GTP-bound, constitutively active form of RAB protein as the bait. Because the intrinsic GTPase activity of the RAB protein is disrupted in certain mutant forms, the rapid turnover cycles between GTP-bound and GDP-bound states of the RAB protein are disrupted, leaving the RAB protein mostly in the GTP-bound, active state. Thus, the GTP-bound RABs may have stronger interactions with their effectors and hopefully will be identified more easily in yeast two-hybrid screens. In searching for RAB-10-interacting proteins, I used the GTP-bound mutant form RAB-10(Q68L) as the bait protein.

*Choice of the mutant form of RAB-10 lacking C-terminal prenylation as the bait in yeast two-hybrid screens*

The last important issue we considered in bait design was to increase the entry of the bait protein into the nucleus where it could bind to the *LexA* operator. It has been reported that prenylation, a type of lipid modification, occurs in the C-terminal tails of RAB proteins. Most Rabs are covalently modified with 20-carbon geranylgeranyl isoprenoids
on the terminal cysteine residues. The prenylation of RAB tails is catalyzed by RAB geranylgeranyltransferase, which recognizes the “CAAX” (Cysteine-Aliphatic-Aliphatic-any amino acid) box in the C-terminus tails of RAB proteins (Seabra et al. 1992; Farnsworth et al. 1994; Casey and Seabra 1996; Desnoyers et al. 1996; Seabra and James 1998; Pereira-Leal et al. 2001; Ali and Seabra 2005; Leung et al. 2007). Processing of lipid modification in the C-terminus tails is important for the membrane association of RAB proteins. In order to increase the probability of nuclear entry of the RAB bait protein, the prenylation sites in the C-terminus of RAB-10(Q68L) bait were deleted; thus, avoiding the association of RAB-10 bait protein with membranes, likely improving entry into the yeast nucleus.
RESULTS

Eight candidates with RAB-10-interacting ability are identified from yeast two-hybrid screens

About 80 yeast colonies identified from the YNB (gal)-ura-his-trp-leu plates (positive colonies in the Leucine assay) were further analyzed using β-Galactosidase (β-Gal) assays. After a series of procedures including extraction, retransformation, and purification (please see Material and Methods, Chapter 5), about 70 plasmids isolated from individual yeast colonies and positive in both Leucine and β-Gal assays were identified. In order to determine which plasmids were likely to identify the same gene, plasmids were classified according to their restriction enzyme digestion patterns. Here, I chose XhoI and EcoRI enzymes for double digestion because all cDNA clones of library were inserted into the prey vector pJG4-5 between the XhoI and EcoRI sites. According to the restriction enzyme digestion patterns, these plasmids were classified into 16 subgroups and some of them from each subgroup were chosen for further sequence analysis (Figure 10, asterisk “*”). Sequence analysis of plasmids in these 16 subgroups corresponded to the following 8 proteins and the regions of amino acid sequences covered in each subgroup were listed in Table 1 and diagramed in Figure 11: they were EHBP-1 (subgroup 1-3, 16), HUM-2 (subgroup 4), GCK-2 (subgroup 5-6, 14, 15), F52E1.13 (subgroup 7), Y82E9BR.21 (subgroup 8), CNT-1 (subgroup 9), F20D1.2 (subgroup 10, 11), and ZK1248.10 (subgroup 12-13).
Four of the RAB-10 interacting proteins are known to associate with the actin-cytoskeleton system

Four candidates such as EHBP-1, HUM-2, GCK-2, and CNT-1 and their mammalian homologues may be associated with the actin-cytoskeleton system.

**EHBP-1**

In yeast two-hybrid screens, 27 positive clones were sequenced and were subdivided into three subgroups (subgroup 1-3) according to their restriction enzyme digestion patterns (Table 1). All of these 27 clones encoded regions of EHBP-1 C-terminus to the CH (Calponin Homology) domain (Figure 11A). This suggests that the CH domain is not required for the interaction between EHBP-1 and RAB-10.

The *ehbp-1* gene encodes a homolog of human Ehbp-1 (*EH-domain binding protein-1*, e value 7.1e-43, 90.3% in peptide length with human Ehbp-1) with 901 amino acids including a CH-domain (*Calponin Homology* domain) (Figure 11A). The CH domain belongs to a superfamily of actin-binding domains found in cytoskeletal bundling proteins such as actinin, spectrin, and plectin (Gimona *et al.* 2002; Borrego-Diaz *et al.* 2006). This implies that Ce-EHBP-1 and its mammalian homologue Ehbp1 may function in the processes of actin bundling formation. CeEHBP-1 and its human/mouse homologues Ehbp1 share about 25% identity (or 45% similarity) in amino acid sequences (Figure 12). It has been reported that human Ehbp1 can bind to mouse Ehd1 and Ehd2
(mammalian homologues of *C. elegans* RME-1) through its NPF (Asn-Pro-Phe) motifs to regulate the endocytotic internalization and recycling of Glut4 (Glucose transporter 4) (Guilherme et al. 2004; Guilherme et al. 2004). There are five NPF (Asn-Pro-Phe) motifs found in human Ehbp1 (four in mouse Ehbp-1) but no NPFs are found in CeEHBP-1 (Figure 12, green color). The lack of NPFs suggests that CeEHBP-1 may not be able to bind to RME-1 in worms. If EHBP-1 and RME-1 physically associate in worms, the mechanism would have to be different than in mammals.

**HUM-2**

The *hum-2* gene encodes an ortholog of human myosin Va/Vb/Vc heavy chain (e value 7.8e-285, 91.3% in peptide length with human myosin Vb). It contains an N-terminal motor domain with an ATPase motif, followed by a calmodulin-binding domain (IQ domain), and a C-terminal coiled-coil domain (DIL domain) (Figure 11B). There are three isoforms of mammalian myosin V heavy chains: Va, Vb, and Vc (Rodriguez and Cheney 2002). Human/mouse myosin Va and Vb have a closer evolutionary relationship with CeHUM-2 than does human/mouse myosin Vc (Figure 13A). In yeast two-hybrid screens, four positive clones were identified encoding HUM-2, and all of them encoding the same region with 208 amino acid stretch between the calmodulin binding domain and the C-terminal DIL domain (Table 1, Figure 11B). In this 208 amino acid-long region, CeHUM-2 and human myosin Va/Vb/Vc share about 20% identity (or 30% similarity) in amino acid sequence (Figure 13B). In mammals, myosin Vb was shown to be involved in endocytic recycling (Lapierre et al. 2001). Myosin Vb has also been reported to be
involved in trafficking of glutamate receptor subunit GluR-I, and in regulating synaptic function in the rat hippocampus (Lise et al. 2006). In addition, it has been found that mammalian Rab11a, involved in recycling and transcytosis, is associated with myosin Vb through the myosin Vb C-terminal tail (Hales et al. 2002; Lindsay and McCaffrey 2002; Lapierre and Goldenring 2005). It was shown that two regions in the C-terminal tail were required for association between rabbit myosin Vb and Rab11a (Lapierre et al. 2001). The more N-terminal region is highly conserved between rabbit myosin Vb and human myosin Va/Vb but not in CeHUM-2 (Figure 13B, bold in red); whereas the second region closer to the C-terminus is highly conserved in all myosin Vs (Figure 13B, underlined bold in red). In this region, CeHUM-2 and mammalian myosin Va/Vb share more than 50% similarity in amino acid sequences.

**GCK-2**

The gck-2 gene encodes an 829 aa-long protein belonged to the serine/threonine kinase MAP4K GCK (germin al center kinase) family. Based on particular structural variations, the GCK family members can be divided into eight subgroups (GCK I-VIII). GCK-2 belongs to the GCK I group (Dan et al. 2001). Members of the GCK I group such as GCK-2 contain an N-terminal serine/threonine-like kinase domain followed by a proline-rich domain and a C-terminal citron homology domain (Figure 11C). The citron homology domain was originally identified in citron kinase, which mainly interacts with the GTP-bound forms of the ras-like small GTPases such as Rho and Rac (Yamashiro et al. 2003; Loomis et al. 2006). The citron homology domain is only found in the CTDs
(C-terminal regulatory domain) of members in GCK group I and IV (Su et al. 1997). In addition, group I GCK members are in the MAPK (Mitogen activation protein kinase) cascades, functioning as MAP4Ks that activate MAP3K c-JNK (c-Jun Kinase) (Hu et al. 1996; Kiefer et al. 1996). In C. elegans, UNC-16, a c-JNK scaffolding protein, was shown to regulate the localization of glutamate receptors on the postsynaptic membrane. It has been suggested that c-JNK play a role in endocytosis of neuronal cells (Byrd et al. 2001). Mammalian Glk, also a member of GCK I group, was shown to associate with endophilin (a dynamin binding protein). Endophilin is involved in invagination of the plasma membrane for vesicle formation, probably as a sensor and/or regulator of membrane curvature (Ramjaun et al. 2001).

In yeast two-hybrid screens, nine positive clones were identified encoding regions of GCK-2. They were subdivided into two subgroups according to their digestion patterns after restriction digestion (Table 1). All of the regions covered by these clones were located into two overlapping areas, one was full-length and the other one was C-terminal to the kinase domain (Figure 11C). These results suggest that the kinase domain is not required for the interaction between GCK-2 and RAB-10.

CeGCK-2 and its mouse/human homologues share very high percentage of identity and similarity in amino acid sequence. They show about 66 % identity (or 84 % similarity) in the N-terminal kinase domain (Figure 14, bold in green). It was shown that the mouse Gck protein (called Rab8ip, Rab8-interacting protein) can bind to the GTP-bound, and wild-type, but not GDP-bound Rab8 (Ren et al. 1996). The Rab-8 interacting domain of
mouse Gck (Figure 14, residues 430-821, bold in red) located in its CTD (C-terminal regulatory domain) includes its C-terminal citron homology domain (Figure 14, bold in black). In this 391 amino acid-long region, CeGCK-2 and its mouse/human homologues share less identity (30 % identity or 50 % similarity) than that in the N-terminal kinase domains.

CNT-1

The cnt-1 gene encodes an ortholog of mammalian centaurin beta (also called Acap (Arf GAP, with Coil, ANK repeat, PH domain), e value 2.3e-102, 93.5% in polypeptide length with Acap2). It contains a N-terminal Bar (Bin-Amphiphsyn-Rvs) domain involving in membrane remodeling, a PH (Pleckstrin Homology) domain associated with phosphoinositides such as PtdIns(4,5)P2 (PIP2), a ARF (ADP-ribosylation factor) GAP domain, and a C-terminal ankyrin repeat domain (Figure 2H) (Lemmon 2003; Lemmon 2004; Randazzo and Hirsch 2004; Dawson et al. 2006; Nie and Randazzo 2006). Proteins with Bar domains such as amphiphysin, endophilins, sorting nexins, and centaurin have been reported to detect membrane curvatures and are involved in membrane remodeling (Casal et al. 2006). Human Acap1 and Acap2 are two Arf GAPs (GTPase activating protein) that stimulate the intrinsic GTPase activity of Arf6 (Jackson et al. 2000; Dai et al. 2004). It has been reported that Acap1, together with Arf6, is involved in regulating endocytic recycling (Jackson et al. 2000; Dai et al. 2004). In addition, it has been reported that PI4-directed PI5-kinase, that produces PIP2, and is
positively regulated by Arf6, could function together with Acap1 to enhance endosomal tubulation (Shinozaki-Narikawa et al. 2006).

Alignments of amino acid sequences of CeCNT-1, human Acap1 and Acap2 showed they were identical to each other with respect to domain structure, each bearing a Bar, PH, ARF GAP, and C-terminal ankyrin repeat domain (Figure 15B). Acap1 and Acap2 share 70% identity (or 84% similarity) in amino acid sequences. CeCNT-1 shares a little higher amino acid identity with Acap2 than does with Acap1 (28% vs. 25% identity or 47% vs. 43% similarity) (Figure 15A and 15B). Taken together these results suggest that CeCNT-1 may be an ARF-6 GAP, and could be involved in endocytic tubule formation.

In yeast two-hybrid screens, two positive clones were identified encoding CNT-1. Their prey plasmids may encode the same region because their enzyme digestion patterns were the same. The N-terminal ends of these two prey plasmids were partially sequenced and results are listed and diagramed (Table 1, Figure 11H).

**ZK1248.10 and F20D1.2 may be two RAB-10 GAPS (GTPase Activation Protein)**

The zk1248.10 gene encodes a homolog of human/mouse Rab Gap/Tbc1-2b proteins (e value 4e-68, Figure 16A) and yeast Mdr1p (e value 3.7e-29, 35% in polypeptide length, Figure 16B) which is a cytoplasmic GAP (GTPase-activating protein) for Ypt/Rab transport GTPases such as Ypt6p, Ypt31p and Sec4p. Mdr1p was reported to involve in recycling of internalized proteins and in the regulation of the Golgi secretory pathway.
(Albert and Gallwitz 1999; Lafourcade et al. 2003). ZK1248.10 protein contains an N-terminal PH (Pleckstrin Homology) domain and a RAB GAP/TBC binding domain found in the C-terminus (Figure 11F). PH domains are associated with lipid or protein binding and are found in a wide range of proteins involved in intracellular signaling or as constituents of the cytoskeleton (Lemmon et al. 2002; Lemmon 2003). The RAB GAP/TBC binding domain was originally identified in yeast Gyp6 and Gyp7, which are two GAPs of yeast Ypt6 and Ypt7 (Rak et al. 2000).

In yeast two-hybrid screens, three positive prey plasmids were identified for ZK1248.10. They were subdivided into two subgroups according to their restriction enzymes digestion patterns (Table 1). These plasmids were partially sequenced; one group of plasmids encoded a region extending from the N-terminus including the PH domain, and the other group of plasmids did not include the PH domain (Figure 11F). This suggests that the PH domain is not required for the interaction between ZK1248.10 and RAB-10.

The f20d1.2 gene encodes a homolog of human/mouse Rab Gap/Tbc1 number 23 proteins (e vaule7-89, Figure 17A) and yeast Gyl1p (amino acid alignment in Figure 17B) which is a putative GTPase activating protein and may have a role in Ypt1p/Sec4p-related polarized exocytosis (Chesneau et al. 2004). Gyl1p was found to stimulate Gyp5p GAP activity in Ypt1p-related trafficking from the ER to Golgi (De Antoni et al. 2002). Gyl1p colocalizes with Gyp5p at sites of polarized growth, and interacts with Gyp5p, Rvs161p, and Rvs167p (Elisabeth Bon 2000; Friesen et al. 2005). In structure, the F20D1.2 protein contains an N-terminal RAB GAP/TBC domain followed by a
Rhodanese-like domain. The Rhodanese-like domain is also found in other proteins such as rhodaneses (thiosulfate: cyanide sulfur transferases), MAP kinase phosphatase, and cdc25 phosphatase (Bordo et al. 2000; Bordo and Bork 2002).

In yeast two-hybrid screens, fifteen positive clones were identified for F20D1.2. Their prey plasmids were subdivided into two subgroups according to their restriction enzyme digestion patterns (Table 1). All of them encoded a region extending from the N-terminus including the RAB GAB/TBC domain. Since all clones were only partially sequenced (only the first 260 amino acids), it is still unknown if the Rhadanese-like domain is required for the interaction between F20D1.2 and RAB-10.

*F52E1.13 and Y82E9BR.21 are two proteins with unknown function*

*f52e1.13* encodes a homolog of human/yeast oxidation resistant protein Oxr1 which functions in mitochondria to protect cells from heat shock and oxidative damage (Volkert et al. 2000; Elliott and Volkert 2004). In structure, the F52E1.13 protein contains an N-terminal LysM (a peptidoglycan-binding repeat) followed by a GRAM domain, and a C-terminal TLD domain (Figure 11D and Figure 18). The LysM repeat with peptidoglycan binding ability is also found in various proteins such as glycoside hydrolase and chitinase (Bateman and Bycroft 2000). The GRAM domain (Glycosyltransferase, Rab-like GTPase activator, and Myotubularins) is also found in glucosyltransferases, myotubularins (lipid tyrosine phosphatases that specifically dephosphorylate phosphoinositides such as PI3P and PIP2) (Taylor et al. 2000; Walker et
al. 2001; Begley et al. 2003; Laporte et al. 2003; Schaletzky et al. 2003) and other putative membrane-associated proteins (Doerks et al. 2000). The GRAM domain may act similarly to PH (Pleckstrin Homology) domains, having the ability to bind phosphoinositides (Schaletzky et al. 2003). In C. elegans, it was reported that downregulating the functions of MTMs (myotubularin) family members such as MTM-1 and MTM-2 could rescue the larval lethality and endocytic defects caused by losing the function of let-512 (a homolog of yeast phosphatidylinositol 3-kinase, Vps34) (Xue et al. 2003). In addition, MTM-6 and MTM-9 were also shown to function in the RME-1/ARF-6-related endocytosis pathway (Dang et al. 2004). The TLD domain of unknown function is always found in proteins with LsyM and RAB GAP/TBC domain (Doerks et al. 2002).

The y82e9BR.21 gene encodes a 356 amino acid-long, hypothetical protein (Table 1 and Figure 11E). It seems to be a C. elegans-specific gene and there is no homolog found in other species. Its function remains unknown and it does not contain any currently known protein domains. The expression level of y82e9br.21 gene is very low, only 8.9% of average gene expression, and there is only one EST clone, yk1098g6, found in Kohara’s cDNA library (Thierry-Mieg and Thierry-Mieg 2006).

**RAB-10 interacting proteins identified in yeast two-hybrid screens specifically interact with wild-type, GTP-bound, but not GDP-bound RAB-10**
In yeast two-hybrid screens for RAB-10 interacting proteins, a GTP-bound, intrinsic GTPase-deficient mutant (Q68L), with presumed constitutive active function, was applied as the bait protein. It was reported that the GTP-bound RABs may have stronger binding affinity than do wild-type ones with their binding partners. So it might be more easy to identify interacting proteins especially with weak binding affinity using the GTP-bound form of the Rabs as baits in yeast two-hybrid screens (Ren et al. 1996). It is important to investigate if those candidates identified using the GTP-bound RAB-10(Q68L) could also interact with the wild-type, but not with the GDP-bound mutant RAB-10(T23N) with GTP-binding deficiency (Chen et al. 2006; Schuck et al. 2007). It was observed that all candidates (except Y82E9BR.21) could only interact with the wild-type and GTP-bound, but not the GDP-bound RAB-10 (Figure 19A, EHBP-1 (aa443-901) and GCK-2; Figure 19B, HUM-2 (aa1179-1387), F52E1.13, and Y82E9BR.21). Results were summarized in Table 2A. One of the two yeast colonies transformed with Y82E9BR.21 and RAB-10(T23N) showed positive results in both Leu and β-Gal assays, and indicated that the Y82E9BR.21 protein may be able to associate with all three forms of RAB-10. Taken together all these results suggest that all candidates identified from yeast two-hybrid screens by the GTP-bound, RAB-10(Q68L) bait protein are also able to have physical interactions with the wild-type but not the GDP-bound RAB-10.

*As in RAB-10, the wild-type and GTP-bound, but not GDP-bound RAB-8 could also interact with RAB-10 interacting proteins*
There are 29 RABs found in *C. elegans* and the one most close to RAB-10 is RAB-8 (Figure 20A and Figure 20B). RAB-8 and RAB-10 share more than 65% identity (or 75% similarity) in amino acid sequences in the N-terminal and middle conserved areas (Figure 20C). It has been reported that mouse Rab8 can bind to mouse Gck (Germinal center kinase) which is a homologue of CeGCK-2 (Ren et al. 1996). Since RAB-8 and RAB-10 share very high identity in amino acid sequences, it is interesting to investigate if those candidates interacting with RAB-10 could also be able to interact with RAB-8 (especially CeGCK-2). As in RAB-10, three forms of wild-type, GTP-bound, and GDP-bound of RAB-8 were tested for the ability to interact with the two-hybrid derived clones. Interestingly, all RAB-10-interacting proteins identified from yeast two-hybrid screens can also interact with the wild-type and GTP-bound, but not GDP-bound RAB-8 (Figure 21A, EHBP-1 (aa443-901) and GCK-2; Figure 21B, HUM-2 (aa1179-1387), F52E1.13, and Y82E9BR.21, and results are summarized in Table 2A). Like mouse Gck that interacts with the GTP-bound mammalian Rab8, CeGCK-2 showed a very strong interaction with wild-type, and the GTP-bound RAB-8 (Figure 21A). In addition, as we observed in the studies of RAB-10, the Y82E9BR.21 protein also showed weak interaction with the GDP-bound RAB-8. Taken together, all of these results suggest that all these RAB-10-interacting proteins may also interact and function with RAB-8.

*RAB-10 interacting proteins identified from yeast two-hybrid screens may be specific for RAB-8 and RAB-10*
All candidates identified in the yeast two-hybrid screens could interact with RAB-8 and RAB-10. It is important to investigate if interactions with these candidates are unique only for RAB-8 and RAB-10 or are universal for all other RABs. Here I tested GTP-bound RAB-5, RAB-7, RAB-11, and RAB-35 available in our laboratory as baits in combination with various RAB-10-interacting partners to check if there was any interaction between them. Interestingly, none of RAB-10-interacting proteins (except Y82E9BR.21) showed interaction with RAB-5, RAB-7, RAB-11, or RAB-35 (Figure 22A, EHBP-1 (aa443-901) and GCK-2; Figure 22B, HUM-2 (aa1179-1387), F52E1.13, and Y82E9BR.13), and results were summarized in Table 2A). One of the two colonies with Y82E9BR.21 and RAB-5(Q78L) showed positive results in both Leucine and β-Gal assays indicating that Y82E9BR.21 and RAB-5 may be able to interact. Taken together these results suggest that these RAB-10-interacting proteins (except Y82E9BR.21) identified from yeast two-hybrid are highly specific for RAB-8 and RAB-10.

**Three GAPs are not only specific for RAB-8 and RAB-10**

Three potential GAPs for small GTPase, ZK1248.10, F20D1.2, and CNT-1, were identified in the yeast two-hybrid screens. To investigate if these GAPs are RAB-10-specific or also interact with other RABs tested before, full-length proteins were used as preys in two-hybrid assay with various RABs as baits. It was found that all RAB-10-interacting GAPs could also interact with RAB-8. In addition, ZK1248.10 could also interact with RAB-5 and RAB-35; whereas CNT-1 could also interact with RAB-35 (Figure 23, Table 2B). Taken together these results suggest that these GAPs are not
specific for RAB-8 or RAB-10, but may also function with other RABs such as RAB-5 and RAB-35.

**RME-1 and LIN-10 do not interact with RAB-10-interacting proteins except Y82E9BR.21**

It has been shown that mammalian Ehbp1 could bind with Ehd1 and Ehd2 (mammalian homologues of CeRME-1) and regulate internalization and recycling of Glut4 (Guilherme et al. 2004; Guilherme et al. 2004). Interaction between mammalian Ehbp1 and Ehd1/Ehd2 is through five NPF motifs in Ehbp1 and the EH domain found in Ehd1 and Ehd2. It was of interest to check if CeEHBP-1 could bind to RME-1 even though there is no NPF motif found in the CeEHBP-1 protein. LIN-10, another possible endocytic regulator, was found to be involved in regulating trafficking of the EGF receptor in vulva formation and the AMPA-type glutamate receptor (GluR-I) in the postsynaptic membranes of the nerve system (Chang and Rongo 2005; Glodowski et al. 2005; Stetak et al. 2006). Recently, it was found that rab-10 mutants also caused a lin-10-like accumulation of GluR-1-GFP in the ventral nerve cord of *C. elegans* (Doreen R. Glodowski and Christopher Rongo, *Mol. Biol. Cell, submitted*). So I was interested to investigate if LIN-10 and RAB-10 utilize the same interacting proteins to regulate the endocytic trafficking of GluR-I. RME-1 (full length), RME-1 (EH domain), and LIN-10 were used as baits in combination with preys of various RAB-10-interacting proteins. It was found that none of RAB-10-interacting proteins (except Y82E9BR.21) were able to interact with RME-1 (either full length or EH domain only) or LIN-10 (Figure 24A,
EHBP-1 (aa443-901) and GCK-2; Figure 24B, HUM-2 (aa1179-1387), F52E1.13, and Y82E9BR.21, and results were summarized in Table 2A). Interestingly, prey Y82E9BR.21 with all three baits showed positive results in both Leu and β-Gal assays. It indicated that Y82E9BR.21 may be able to interact with RME-1 or LIN-10. Taken together these results suggest that none of RAB-10-interacting proteins (except Y82E9BR.21) could function together with RME-1 or LIN-10.

**Full-length EHBP-1 does not interact with RAB-8 or RAB-10 in yeast two-hybrid assay**

It was somewhat surprising to find that CeEHBP-1 did not interact with RME-1 even though their mammalian homologues Ehbp1 and Ehd1/Ehd2 interact. The clone with the longest cDNA identified in the yeast two-hybrid screens only encodes a half of full length EHBP-1 protein (aa443-901). It was possible that CeEHBP-1 and RME-1 would interact with each other but that the binding motif was not included in the area encoded by that prey plasmid. To address this question, full-length CeEHBP-1 (aa1-901) was used as prey to test with RME-1 and all other baits studied before. Interestingly, full-length CeEHBP-1 did not show any interaction with RME-1 (both EH domain only or full length), RAB-5, RAB-7, RAB-11, RAB-35, or even RAB-8 or RAB-10, which showed positive interactions with EHBP-1(aa443-901) before (Figure 25). Taken together these results showed that full-length CeEHBP-1 could not bind with any of baits tested in yeast two-hybrid assay. It remains to be determined if full length EHBP-1 was actually
expressed in the yeast cells and whether it entered the nucleus under the condition assayed.

**HUM-2 interacts with RAB-11 via a region distinct from that interacting with RAB-10**

It has been reported that extreme C-terminus of mammalian myosin Vb associates with Rab11a. The region of HUM-2 recovered in the yeast two-hybrid screens (aa1179-1387) only interacted with RAB-8 and RAB-10, but not RAB-11 in two-hybrid assay. In order to determine if HUM-2 may interact with RAB-11 in *C. elegans*, a new prey plasmid encoding the region of HUM-2 (aa899-1839) including the original RAB-8/RAB-10 interacting region (aa1179-1387) and the C-terminus region likely to include RAB-11 binding sequences was constructed. I found that HUM-2 (aa899-1839) could interact with RAB-8 and RAB-10, and in addition, it also interacted with RAB-11. HUM-2 (aa899-1839) did not interact with RME-1 (either full-length or EH domain only) RAB-5, RAB-7, or RAB-35 (Figure 25). Taken together these results show that HUM-2 and RAB-11 are able to associate to each in the context of the yeast assay, and suggest conservation of the myosin V/Rab11 interaction from worms to mammals. This would also suggest that HUM-2 interact with RAB-8/RAB-10 through a different motif than RAB-11.

*Functional analysis of RAB-10-interacting proteins by phenotype: deletion mutants or RNAi (RNA interference)*
To test for possible roles for individual RAB-10-interacting proteins in vivo, I analyzed their phenotypes using knockout mutants or RNAi. I assayed the subcellular localization of RAB-10, or looked for intestinal phenotypes such as vacuole accumulation, or abnormal diffusion of RME-1 as I had previously observed in rab-10 mutants. A deletion mutant, \textit{RB801 (ok596)} with a 2145 bp-long deletion (aa248-964) covering two thirds of the N-terminal motor domain and the whole calmodulin binding domain, was available for testing loss function of \textit{hum-2} (Figure 11B). For other RAB-10-interacting proteins such as EHBP-1, GCK-2, F52E1.13, and F20D1.2, I decided to use RNAi to knock down the function of each individual gene because there were no deletion mutants available at that time. RNAi clones corresponding to each gene were obtained from the AJ library (Kamath and Ahringer 2003; Kamath \textit{et al.} 2003) and the target areas in each gene were diagramed (Figure 26). No RNAi clone corresponding to \textit{y82e9br.21} gene was included in the AJ library. So I constructed a \textit{L4440-y82e9br.21} RNAi feeding construct covering the whole cDNA sequence of \textit{y82e9br.21} gene.

\textit{Subcellular localization of RME-1 and RAB-10 are affected by \textit{hum-2} mutants}

\textit{hum-2(ok596)} is presumably a null mutant of \textit{hum-2} because two important domains, motor and calmodulin binding, are deleted. To investigate if there is any effect on the subcellular localization of RAB-10 or RME-1 in the \textit{C. elegans} intestine caused by \textit{hum-2} mutation, transgenes expressing GFP-RME-1 or GFP-RAB-10 driven by the intestine-specific promoter \textit{vha-6} were crossed into the \textit{hum-2(ok596)} mutant background. In the wild-type intestinal cells, abundant GFP-RME-1-positive subcellular compartments
appeared as small punctate structures near the basolateral and apical membranes, with few punctate structures in the medial cytoplasm (Figure 27, A and C). Unlike \textit{rab}-10 mutants, \textit{hum}-2(ok596) did not accumulate intestinal vacuoles. In addition, GFP-RME-1-labeled structures in the area close to basal membrane appeared to be normal, abnormally large number of GFP-RME-1-labeled puncta were found to accumulate in the medial cytoplasm of intestinal cells in the \textit{hum}-2(ok596) mutant (Figure 27, B and D). The number and size of GFP-RME-1-labeled puncta near basal membrane were not obviously changed; whereas those in the medial cytoplasm were increased by about four fold or three fold, respectively, compared to those observed in wild-type animals (Figure 27, E and F, p<0.001). Taken together these results suggest that \textit{hum}-2 does affect RME-1 localization but in a different way than \textit{rab}-10.

Next, I assayed for effects on the subcellular localization of GFP-RAB-10 caused by the \textit{hum}-2(ok596) mutant. In wild-type intestinal cells, abundant GFP-RAB-10-labeled puncta were observed throughout the cell (Figure 28, A and B, E and F, arrowheads). \textit{hum}-2(ok596) mutants accumulated “foggy” patches of GFP-RAB-10 label. One possibility is that the patches represent groups of small vesicles (Figure 28, C and D, G and H, arrows). The number of large GFP-RAB-10-labeled puncta appeared to be normal but the size was slightly increased (about two-fold) (Figure 28, I and J, p<0.001). All these results suggest that endosomal membrane attachment of GFP-RAB-10 was not affected by \textit{hum}-2 mutation. Rather my results suggest that \textit{hum}-2 mutants have a defect in RAB-10 positive or RME-1 positive compartments.
**HUM-2 is broadly expressed in C. elegans**

To determine when and where HUM-2 is normally expressed in *C. elegans*, a transgene was created expressing a *hum-2*-GFP fusion, driven by a 3.7 kb of *hum-2* upstream sequences (the predicted promoter region). The HUM-2-GFP fusion protein was observed to express in the hypodermis, body wall muscle, intestine, pharynx, and nerve ring (Figure 29, A-D). The HUM-2-GFP fusion protein appeared punctate in most tissues. In the intestine, HUM-2-GFP localized to distinct puncta along the apical membranes (Figure 29C, arrows). Further experiments need to be done to test if the *hum-2* (*ok596*) mutant can be rescued by this HUM-2-GFP transgene.

**Depletion of EHBP-1, but not GCK-2, F52E1.13, Y82E9BR.21, or F20D1.2, affects the subcellular localization of RME-1 and RAB-10**

Here I continued to examine if RAB-10-interacting proteins could have any effect on the subcellular localization of either RME-1 or RAB-10. Since there were no deletion mutants available for each gene at the time, I decided to use RNAi by feeding method to knock down each individual gene. In order to have the maximum effect on gene knock down, the intestines of RNAi-treated animals in the F1, rather than P0 generation, were examined. The subcellular localization of GFP-RME-1 appeared to be normal in the areas close to basal membranes, apical membranes, or in the medial cytoplasm after RNAi of *gck-2*, *f52e1.13*, or *f20d1.2*. The number and size of GFP-RME-1-labeled puncta were not changed in animals treated with RNAi targeting *gck-2*, *f52e1.13*, or
In the intestines of animals treated with *ehbp-1* RNAi, the GFP-RME-1-labeled tubular and punctuate structures in the area near basolateral membranes became diffusive, similar in phenotype to *rab-10* mutants (Figure 30C). The number and size of GFP-RME-1-labeled puncta were reduced dramatically, about four-fold and three-fold respectively, compared to control animals (Figure 30, G and H, p<0.001). Taken together these results indicate that loss of EHB-1 function phenocopies the intestinal mutant phenotypes observed in *rab-10* mutants.

Next, the subcellular localization of GFP-RAB-10 in animals treated with RNAi of interacting proteins was examined. I found that there were no obvious changes in the number or size of GFP-RAB-10-labeled puncta in animals treated with RNAi targetting *gck-2, f52e1.13*, or *y82e9br.21* (Figure 31). However, animals treated with *ehbp-1* RNAi appeared to be abnormal in the subcellular localization of GFP-RAB-10. GFP-RAB-10 appeared to be more diffusive than that observed in control (*L4440*) animals (Figure 31C). The number and size of GFP-RAB-10-labeled puncta in *ehbp-1* RNAi-treated animals were reduced about two-fold and three-fold respectively, compared to control animals (Figure 31, G and H). Taken together these results suggest that EHB-1 functions with RAB-10 in vivo, and may be required for endosomal membrane association of RAB-10. More analysis of EHB-1 is presented in Chapter 4.

*Overexpression of GCK-2 causes a rab-10-like vacuole accumulation in the intestine*
Two types of transgenes expressing GFP-tagged gck-2 were made. One type, driven by the gck-2 promoter, was designed to investigate the cellular expression pattern of GCK-2. The other type driven by an intestine-specific promoter, was designed to determine the subcellular localization of GCK-2 in the intestinal cells. I found that GCK-2-GFP fusion driven by the gck-2 promoter was highly expressed in the pharynx and nerve ring, ventral nerve cord, and the intestine (Figure 32, A-C). In the intestinal cells, GFP-GCK-2 was found to label the apical/basolateral membranes and punctate structures in the cytoplasm (Figure 32, D-E). In addition, rab-10-like vacuole accumulation was also found in animals highly overexpressing GFP-GCK-2 (Figure 32F, arrow). These results suggest that GCK-2 is involved in endocytic recycling, perhaps serving as a negative regulator of RAB-10-driven transport.
DISCUSSION

**RAB-10 may play an important role in the association of basolateral endocytic trafficking and the actin-cytoskeleton system**

Through yeast two-hybrid screens of *C. elegans* cDNA library, eight candidates with RAB-10-interacting abilities were identified and four of them, EHBP-1, Myosin V, GCK-2, and CNT-1, based upon work with their orthologs in other systems, are expected to be involved with the actin-cytoskeleton. RAB-10 may play an important role in coupling the basic endocytic machinery and actin-cytoskeleton system through these four predicted actin-cytoskeleton-associated factors. RAB-10 and its human ortholog Rab10 have already been shown to be required in trafficking departing from basolateral early endosomes to recycling endosomes in the *C. elegans* intestine and mammalian MDCK cells (Babbey et al. 2006; Chen et al. 2006; Schuck et al. 2007). Since human Rab10 can substitute for loss of CeRAB-10 function in the *C. elegans* intestine, it is very likely that mammalian Rab10 functions in a very similar way to CeRAB-10. It will be important to further investigate if mammalian Rab10 can interact with mammalian homologues of the RAB-10-interacting proteins identified in the yeast two-hybrid screens in *C. elegans*.

*The key amino acids in RAB-10 responsible for the associations with RAB-10-interacting proteins may be identified by comparison of various RAB protein amino acid sequences*
It has been shown that Rabs are highly conserved in the PM (Phosphate-Magnesium binding)/G (guanine base binding) domains, and four RabF domains (RabF1-F4) (Valencia et al. 1991; Pereira-Leal and Seabra 2000). On the contrary, some less conserved regions such as the RAB CDR (Complementary-Determining Region) and the switch I/II regions are more variable and are thought to be potential binding domains for various Rab effectors (Olkkonen and Stenmark 1997; Ostermeier and Brunger 1999; Pereira-Leal and Seabra 2000). It is important to identify the key amino acids in individual RABs responsible for interacting with their effectors. In my yeast two-hybrid interacting assays, most of RAB-10-interacting proteins such as EHBP-1, HUM-2, GCK-2, CNT-1, and F52E1.13 only interact with RAB-8 and RAB-10 but not with other RABs such as RAB-5, -7, -11, or RAB-35. So the amino acids located in the RAB CDB or the switch I/II regions only identical or similar in RAB-8 and RAB-10 but not in other RABs may be responsible for the interaction with individual RAB-10-interacting proteins. Further experiments such as site-directed mutagenesis could be done to mutate these amino acids and to investigate if it will affect interactions between RAB-8/RAB-10 and individual interacting proteins.

**RAB-8 and RAB-10 may function redundantly in some membrane trafficking processes**

RAB-8 and RAB-10 are the closest relatives of yeast Sec4p in metazoans and are very similar to each other. So far, all RAB-10-interacting proteins identified in the yeast two-hybrid screens could also interact with RAB-8. It is possible that RAB-8 and RAB-10
function redundantly in some of membrane trafficking processes, utilizing the same effectors. In basolateral endocytic recycling, intestinal vacuole accumulation is only observed in rab-10 mutants but not in rab-8 RNAi animals (personal observation). In addition, overexpressed GFP-RAB-8 cannot substitute for loss of RAB-10 function to the intestinal vacuole phenotype (Figure 47). These results suggest that RAB-8 and RAB-10 function in two distinct processes in the intestine.

On the other hand, RAB-8 and RAB-10 may still function redundantly in some other membrane trafficking processes in other tissues. For instances, developmental defects in gonad and embryo, was observed in rab-10 mutant animals treated with rab-8 RNAi but not observed in rab-10 mutants alone or in wild-type animals treated with rab-8 RNAi. In addition, polarized MDCK cells treated with Rab8 and Rab10 siRNA had more severe exocytosis defects than single knockout cells (Schuck et al. 2007). These results imply that, for some membrane trafficking processes (like exocytosis), RAB-8 and RAB-10 may function together in a redundant manner.

**Is F20D1.2 or ZK1248.10 a GAP for RAB-8 or RAB-10?**

Recently, it has been reported that Rab8 and Rab10 were found to associate with the Glut4 (Glucose Transporter 4)-positive microsomal vesicles insolated from 3T3-L1 adipocyte cells (Larance et al. 2005; Miinea et al. 2005; Ishikura et al. 2007; Sano et al. 2007). In addition, a 160 kDa protein AS160 (Akt substrate of 160 kDa) with potential GAP activity for various Rabs, was found to serve as a negative regulator in Glut4
translocation from intracellular compartments to the plasma membrane. In addition, AS160 specifically binds to Rab8 and Rab10 proteins in vitro and in vivo (Ishikura et al. 2007; Sano et al. 2007). Through the yeast two-hybrid screens, I identified two other potential GAPs, F20D1.2 and ZK1248.10, the RAB-8 and RAB-10 interacting proteins. Further experiments could be done such as GAP activity assays with ZK1248.10 or F20D1.2 for RAB-8 and RAB-10 to investigate if they are true GAPs for RAB-8 or RAB-10.

Loss of HUM-2, F52E1.13, or Y82E9BR.21 function alone may not be enough to contribute the rab-10 intestinal phenotypes

So far, only RNAi of EHBP-1, but not other candidates such as HUM-2, F52E1.13, or Y82E9BR.21, could cause rab-10-like intestinal phenotypes. It is possible that loss of RAB-10 function may disrupt function of several downstream effectors at once; therefore, loss of individual candidates alone may contribute only a part of the effects observed in rab-10 mutants. Further experiments such as combinatorial RNAi or isolation of effectors deletion mutants could be done to disrupt the functions of more than one gene at once, and to investigate if it will produce rab-10-like phenotypes in the intestine. In addition, only loss of EHBP-1 function but not function of other candidates affected the apparent association of RAB-10 with endosomes. It is important to investigate if loss of RAB-10 function will affect the morphology or subcellular localization of these candidates in the intestine. For HUM-2, F52E1.13, or Y82E9BR.21, it is possible that RAB-10 may recruit these downstream effectors to endosomes. Loss of RAB-10
function would be expected to disrupt their membrane association. On the other hand, it is also possible that loss of individual RAB-10-interacting protein function will cause a \textit{rab-8}-like, but not \textit{rab-10}-like, intestinal phenotypes. In the case of the \textit{hum-2(ok596)} mutants, GFP-RME-1-labeled tubular structures accumulate excessively in cross-sections of the intestine. However, GFP-RME-1 becomes almost totally cytosolic diffusive in \textit{rab-10} mutant animals. Since HUM-2 can interact with RAB-8 in the yeast two-hybrid assay, it is possible that the mutant phenotype observed in \textit{hum-2(ok596)} mutant is actually a \textit{rab-8}-like but not \textit{rab-10}-like phenotype. Recently, a \textit{rab-8} deletion mutant became available. Further experiments should be done using the \textit{rab-8} deletion mutant to investigate whether the intestinal mutant phenotypes caused by \textit{rab-8(-)} or \textit{hum-2(-)} are similar.

\textit{GCK-2 may serve as a negative regulator in the endocytic trafficking from early to recycling endosomes}

Results from yeast two-hybrid binding assays have suggested physical interaction between RAB-10 and GCK-2. Overexpression of GFP- or RFP-tagged GCK-2 in the intestine caused a \textit{rab-10}-like intestinal vacuole phenotype. In addition, GCK-2 RNAi had no obvious effects on the subcellular localization of RAB-10 or RME-1. Several possibilities could explain these results. First, GCK-2 may serve as one of several downstream effectors recruited by RAB-10 and function together with RAB-10 to enhance trafficking from basolateral early to recycling endosomes. Lose of GCK-2 function alone, as in the \textit{hum-2} mutant, may not be enough to cause a \textit{rab-10}-like
intestinal phenotype. In addition, overexpression of GCK-2 in the intestines may cause “dominant negative” effect by titrating out most of the cellular factors needed for RAB-10’s normal function.

On the other hand, it is also possible that GCK-2 serves as a negative regulator in endocytic trafficking from early to recycling endosomes. Since GCK-2 is a serine/threonine protein kinase, it is possible that GCK-2 may phosphorylate RAB-10 (upstream of RAB-10) and negatively regulate the function of RAB-10, or that GCK-2 may cause phosphorylation of other downstream cellular factors and result in reducing trafficking from early to recycling endosomes. In this case, RAB-10 may serve as a negative regulator of GCK-2 function (downstream of RAB-10). Further experiments could be done such as epistasis analysis to help understanding the upstream or downstream relationship between RAB-10 and GCK-2.

**CNT-1 may serve as an intermediary between RAB-10 and ARF-6**

The ADP-ribosylation factor (Arf) family of proteins belongs to the Ras superfamily of small GTPases shown to regulate membrane trafficking (Kahn et al. 2006). Six mammalian Arfs (Arf1-6) and many more Arf-like proteins such as ARL (Arf-like, Arl1-5) have been found (Kahn et al. 1992; Kahn et al. 1992; Breiner et al. 1996). Like other GTPases such as Rab proteins, Arf proteins cycle between an inactive GDP-bound and active GTP-bound states. In the active GTP-bound state, Arfs interact with effectors to carry out their functions. Among six mammalian Arf proteins, Arf1 and Arf6 are the
most well studied members of this family. Arf1 physically resides at the Golgi complex and is involved in traffic departing the Golgi (Menetrey et al. 2007; Price et al. 2007); whereas Arf6 is found at the plasma membrane and on endosomes, and is involved in membrane trafficking and actin cytoskeleton regulation (Klein et al. 2006; Venkateswarlu et al. 2007).

So far, more than 15 candidates for Arf GAPs have been found in the human genome (Klein et al. 2006; Tanabe et al. 2006; Yoon et al. 2006). These predicated Arf GAPs contain multiple functional motifs such as pleckstrin homology, Src homology 2/3, and proline rich motifs and may be able to interact with a variety of signaling molecules that have great impact on the rearrangement of the actin cytoskeleton. In addition, two Arf GAPs were shown to have effects on the GTPase activity of Arf6 in vitro and in vivo. They are mammalian Acap1 and Acap2 (Jackson et al. 2000). It has been reported that mammalian Acap1, collaborating with PIP5K (Ptdln-4-phosphate-5 kinase) which is a downstream effector of Arf6 and produces PI(4,5)P2 (Ptdln-(4,5)-bisphosphate), enhances microtubule formation for endosomal recycling (Shinozaki-Narikawa et al. 2006). Mammalian Arf6 and its C. elegans homolog ARF-6 share 88% identity in amino acid sequences, and CNT-1 may be a C. elegans ortholog of mammalian Acap1 and Acap2 (Jackson et al. 2000). It will be interesting to investigate if CNT-1 physically associates with ARF-6 in worms, and has GAP activity toward ARF-6. Thus, CNT-1 may serve as an intermediary between two major endocytic recycling players, ARF-6 and RAB-10.
CHAPTER 4

Functional Analysis of RAB-10 Interacting Partner EHBP-1 During Endocytosis in the C. elegans Intestine
INTRODUCTION

The role of actin-cytoskeleton in endocytosis

The role of the actin-cytoskeleton in endocytosis is still not well understood. In yeast, it has been shown that loss of function in actin, fimbrin or several other genes disrupts both endocytosis and actin polymerization (Kubler and Riezman 1993; Benedetti et al. 1994; Munn et al. 1995; Tang et al. 1997; Wesp et al. 1997). On the contrary, it was also reported that some mutant genes affecting actin polymerization have no effects on endocytosis (Riezman et al. 1997). In mammalian cells, many reports have revealed that intact function of actin-cytoskeleton is required for maintaining normal endocytic processing (Durrbach et al. 1996; Lamaze et al. 1997; Fujimoto et al. 2000; Qualmann et al. 2000; Taunton et al. 2000; Lanzetti et al. 2001; Zaslaver et al. 2001). Most of these reports focused on the early event of endocytosis such as vesicle formation, budding/invagination, and departure from the plasma membrane, but did not focus on the later events such as movement of cargo between different endosomal compartments. It has been reported that actin comet tails are associated with endosomes in a cell-free system (Moreau and Way 1998). This suggests association between the actin-cytoskeleton system and movement of intracellular endosomes, although a detailed mechanism still remains unclear (Nakagawa and Miyamoto 1998; Qualmann et al. 2000; Gruenberg 2001).
In the internalization step of endocytosis, several factors such as syndapin may serve as connectors between basic endocytic machinery and the actin-cytoskeleton. Syndapin, through its SH3 (Src homology 3) domain, binds to dynamin and N-WASP (Neural Wiskott-Aldrich-Syndrome Protein), an activator of the Arp2/3 complex in regulating actin polymerization (Qualmann and Kelly 2000; Kessels and Qualmann 2004; Anggono et al. 2006; Kessels and Qualmann 2006; Anggono and Robinson 2007). Thus syndapin couples dynamin-mediated vesicle fission and actin polymerization, and promotes the departure of newly formed endocytic vesicles from the plasma membrane. In addition, syndapin was also shown to regulate endocytic recycling back to the plasma membrane through interactions with mammalian Ehd1/mRme1 (Xu et al. 2004; Braun et al. 2005). Other cellular factors such as the CART (Cytoskeleton-Associated Recycling or Transport) complex, an Hrs/Actinin-4/BERP/Myosin-V protein complex, has also been reported to be involved in early endosome sorting and trafficking departing from early endosomes to recycling endosomes (Yan et al. 2005).

**EHBP-1 may serve as an intermediary between endocytic trafficking and actin-cytoskeletal system**

The CeEHBP-1 protein is a homolog of human Ehbp1 (EH-domain binding protein 1) with 901 amino acids including a CH-domain (Calponin Homology domain). The CH domain belongs to a superfamily of actin-binding domains found in cytoskeletal bundling proteins such as actinin, spectrin, and plectin (Gimona et al. 2002; Borrego-Diaz et al. 2006). The presence of this domain suggests that mammalian Ehbp1 may associate with
actin and function in the processes of bundling actin fibers. It has been reported that human Ehbp1 binds to Ehd1 and Ehd2 (mammalian homologues of *C. elegans* RME-1) through its five NPF (Asn-Pro-Phe) motifs, regulating the endocytotic internalization and recycling of Glut4 (Glucose transporter 4) (Guilherme et al. 2004; Guilherme et al. 2004). Because no NPF motifs are found in the CeEHBP-1, CeEHBP-1 may not be able to bind directly with CeRME-1.

In Chapter 3, I discussed my findings that CeEHBP-1 could have physical interactions with RAB-8 and RAB-10. The preliminary results from chapter 3 have shown that *rab-10*-like intestinal mutant phenotypes were also observed in the *ehbp-1* RNAi treated animals. In this chapter, I pursue a more detailed analysis to investigate if the actin-cytoskeleton system is involved in the basolateral endocytic recycling trafficking in *C. elegans* intestines, and also investigate the function of EHBP-1 protein in endocytic trafficking and the interaction between EHBP-1 and RAB-10 proteins.
RESULTS

Efficient knockdown of ehbp-1 by RNAi feeding

In Chapter 3, it was demonstrated that knockdown of \textit{ehbp-1} by RNAi feeding method generates a \textit{rab-10}-like intestinal phenotype in the F1 generation. It has been reported that the expression level of \textit{ehbp-1} mRNA is about 1.3 times higher than that of average gene (Thierry-Mieg and Thierry-Mieg 2006). It is important to know if the intestinal phenotype found in the \textit{ehbp-1} RNAi animals truly results from loss of EHBP-1 expression. To determine whether the intestinal expression of EHBP-1 is truly knocked down by \textit{ehbp-1} RNAi leading to intestinal vacuoles, \textit{ehbp-1} RNAi was applied to transgenic strain expressing GFP-EHBP-1 driven by an intestine-specific promoter from the \textit{vha-6} gene. Very strong GFP-EHBP-1 expression was observed in the intestine of control animals (Figure 33, A-C). However, the expression level of GFP-EHBP-1 was reduced dramatically and only autofluorescent background could be observed in the \textit{ehbp-1} RNAi animals (Figure 33, D-F). In control animals, about 94\% of animals were GFP-EHBP-1-positive; whereas only 2.5\% of animals treated with \textit{ehbp-1} RNAi were GFP-EHBP-1-positive (Figure 33I). These results suggest that knockdown of \textit{ehbp-1} by RNAi feeding is likely efficient to disrupt the endogenous expression of EHBP-1.

\textit{ehbp-1 (RNAi) animals display rab-10-like endocytosis defects in the C. elegans intestine}
rab-10 mutants display an endocytic trafficking defect in C. elegans intestine. In rab-10 mutant animals, gigantic vacuoles were observed in the intestine. These intestinal vacuoles can be filled specifically by the fluid-phase marker ssGFP taken up from the basolateral membrane of intestine. This phenotype indicates that RAB-10 protein is involved in basolateral recycling in the intestine. Loss of RAB-10 function also specifically causes accumulation of early endosomes labeled by various endosomal markers such as RAB-5, -7, -8 (Appendix-1), and causes diminution of recycling endosomes labeled by basolateral recycling marker GFP-RME-1. These results formed the basis of our one previous work showing that RAB-10 protein is involved in the trafficking between early endosomes and recycling endosomes, and the gigantic intestinal vacuoles may be formed through fusion of accumulated early endosomes in the intestine.

On the other hand, there is no effect of loss RAB-10 on the subcellular localization of apical recycling marker GFP-RAB-11 suggesting that RAB-10 functions in basolateral but not apical recycling pathways of the intestine. Finally, several GFP-fused, endocytic transmembrane cargo proteins such as hTAC-GFP, hTfR-GFP, and LMP-1-GFP are also observed to label the limiting membranes of intestinal vacuoles found in rab-10 mutants.

The preliminary results from Chapter 3 showed that loss of function in RAB-10 interacting protein EHBP-1 by RNAi causes a rab-10-like, cytosolic disruption of basolateral recycling endosomes labeled by GFP-RME-1. I sought to further investigate if loss of ehbp-1 function has further similarity to phenotypes observed in rab-10 mutants. In the ehbp-1 RNAi animals, transparent vacuoles accumulate in the worm intestine similar to those observed in rab-10 mutant animals (Figure 34, A-B). To determine if the
abnormal vacuoles found in the *ehbp-1* RNAi intestine are enlarged basolateral endosomes like those observed in *rab-10* mutants, *ehbp-1* RNAi was applied to the *cup-4; arIs37* animals where GFP is secreted from body-wall muscle cells into the body cavity and accumulates there. Most of secreted GFP is not endocytosed by the coelomocytes because of the *cup-4* mutation. The GFP from the body cavity is taken up by the intestine through the basolateral membrane and is then recycled back to the body cavity through early endosomes and recycling endosomes. I found that the gigantic vacuoles in the intestinal cells of *ehbp-1* RNAi animals accumulated GFP from the body cavity, indicating that they enlarged endosomes involved in basolateral recycling similar to those previously found in *rab-10* mutants (Figure 34, C-D). These results indicate that *ehbp-1* RNAi animals, like *rab-10* mutants, have severe defects in basolateral endocytic recycling of the intestine.

Next I wanted to determine if transmembrane cargo proteins such as hTAC and hTfR also accumulated in the abnormal endocytic compartments of the intestine in the *ehbp-1* RNAi animals. These GFP-fused, transmembrane cargo proteins were previously found to accumulate on the limiting membranes of intestinal vacuoles in *rab-10* and *rme-1* mutants (Chen *et al.* 2006). In *ehbp-1* RNAi animals, the limiting membranes of intestinal vacuoles were labeled by both hTAC-GFP (Figure 34H) and hTfR-GFP (Figure 34J), similar to that observed in *rab-10* mutants. In control animals, hTAC-GFP was found to label basolateral membranes and cortical tubular structures (Figure 34, E and G). However, the hTAC-GFP labeling in basolateral membranes and tubular structures was dramatically disrupted in *ehbp-1* RNAi animals (Figure 34, F and H).
Our previous studies indicated that \textit{rab-10} mutants only display endocytic recycling defects in the intestine and in the postsynaptic membranes of inter neurons (Doreen Glodowski and Christopher Rongo). No other endocytic recycling defect was found in other tissues such as oocytes or coelomocytes in \textit{rab-10} mutants. To determine if \textit{EHBP-1} protein also functions in other tissues such as oocytes or coelomocytes, \textit{ehbp-1} RNAi was applied to animals bearing transgenes that direct expression of YP170-GFP (Grant and Hirsh 1999) or fluid-phase marker ssGFP (Fares and Greenwald 2001; Fares and Greenwald 2001). I found that there is no endocytic defect in yolk uptake by oocytes (Figure 34, K-L), or in fluid phase marker uptake by coelomocytes (Figure 34, M-N). Taken together these results indicate that loss of \textit{ehbp-1} function does not affect the endocytic recycling process of oocytes or coelomocytes. In addition, similar to \textit{rab-10} mutants, basolateral secretion of YP170-GFP by the intestine and secretion of fluid-phase marker GFP by muscle cells appeared normal in \textit{ehbp-1} RNAi animals.

\textit{ehbp-1} RNAi results in a dramatic increase in RAB-5-positive early endosomes and with a loss of RME-1-positive recycling endosomes

These results suggest that loss of \textit{ehbp-1} function, like loss of \textit{rab-10} function, results in defective basolateral recycling in the worm intestine. In chapter 2, a set of GFP-tagged transgenes, encoding endosomal marker proteins such as RAB-5, -7, -8, -11 and RME-1, were used to assay for effects on endosomal morphology, and to determine which step in trafficking is impaired in \textit{rab-10} mutants. Loss of \textit{rab-10} function causes accumulation of early endosomes labeled by GFP-RAB-5, -7, -8 and disappearance of recycling
endosomes labeled by GFP-RME-1. In addition, loss of RAB-10 function has no effect on GFP-RAB-11. To determine if EHBP-1 and RAB-10 function in the same step of endocytosis and loss of *ehbp-1* function may have similar effects on subcellular localization of various endosomal markers tested in *rab-10* mutants, *ehbp-1* RNAi was applied to animals bearing transgenes directing expression of various endosomal GFP-tagged markers. In the control animals (L4440 only), there were no changes in the subcellular localization, or in the number of the various GFP-tagged endosome markers. (Chen *et al.* 2006) (Figure 35, A, C, E, G, I, K, M, O, Q, respectively). Abnormal accumulation of early endosomes labeled by GFP-RAB-5, -7, -8 was found in *ehbp-1* RNAi animals (Figure 35, B, D, F, and H). The limiting membrane of intestinal vacuoles was also strongly labeled by GFP-RAB-5, GFP-RAB-7, and GFP-RAB-8, suggesting that many of these structures are grossly enlarged early endosomes (Figure 35, B, D, and F, respectively). The number and size of GFP-tagged RAB-5, -7, -8-labeled endosomes in *ehbp-1* RNAi animals were significantly different compared to those in wild type animals (Figure 35, S and T, *ehbp-1*(-) vs. L4440: 64.6±11.5 vs. 37.7±8.4, p<0.001 in puncta number and 0.58±0.15 vs. 0.3±0.08, p<0.001 in puncta size for GFP-RAB-5; 65.7±15.2 vs. 31.8±7.8, p<0.001 in puncta number and 0.55±0.21 vs. 0.4±0.21, p>0.05 (not statistically significant) in puncta size for GFP-RAB-7; 38.9±5.7 vs. 25.6±4.2, p<0.001 in puncta number and 1.29±0.21 vs. 0.41± 0.16, p<0.001 in puncta size for GFP-RAB-8). The effects caused by *ehbp-1* RNAi on the numbers of GFP-RAB-5/-7/-8-labeled endosomes are very similar (1.5 to 2.0 fold higher). On the contrary, subcellular morphology and localization of GFP-RAB-10-labeled endosomes were reduced dramatically and appeared to be more cytosolically diffuse in *ehbp-1* RNAi animals.
Figure 35, J (Top) and L (Middle), respectively). Quantification results showed reduction in puncta number (ehbp-1(-) vs. L4440: 24±7.1 vs. 50.1±8.6, p<0.001) and in size (0.2± 0.08 vs. 0.62± 0.31, p<0.001) (Figure 33, S and T). The reduction of GFP-RAB-10-labeled endosome number in ehbp-1 RNAi animals may indicate a defect in recruiting RAB-10 to basolateral early endosomes, or it could indicate a loss of basolateral early endosomes altogether (and thus a loss of endosomal binding sites for RAB-10). The first model, in which loss of EHBP-1 leads to a defect in recruiting RAB-10 to basolateral early endosomes, might better explain the observed reduction in GFP-RAB-10-labeled early endosome number because basolateral early endosomes labeled by other RAB markers (RAB-5, -7, -8) were increased and not disrupted under the same conditions. EHBP-1 also interacts physically with RAB-10, suggesting recruitment as a possible mechanism. In addition, disruption of basolateral recycling endosomes labeled by GFP-RME-1 was also observed in ehbp-1 RNAi but not in control animals (Figure 35, P (Top), and T (Middle), respectively). The number and size of GFP-RME-1-labeled endosomes were dramatically decreased (Figure 35, S and T, ehbp-1(-) vs. L4440; 16.2±6.1 vs. 63.3 ± 14.2, p<0.001 in puncta number; 0.15±0.06 vs. 0.46±0.23, p<0.001 in puncta size, respectively). There was no effect on subcellular localization or number of RAB-11-labeled apical recycling endosomes after ehbp-1 RNAi (Figure 35, N, S and T). Taken together these results suggest that EHBP-1, like RAB-10, functions in basolateral transport from early to recycling endosomes in the intestine. In addition, EHBP-1 protein may be important in recruiting or maintaining of RAB-10 protein on the basolateral early endosomes.
**ehbp-1 RNAi-induced intestinal vacuoles labeled by transmembrane cargo hTAC-GFP are enlarged RAB-5-positive early endosomes**

Individual labeling of intestinal vacuoles caused by *ehbp-1* RNAi by hTAC-GFP or GFP-RAB-5 suggested that such structures contain both markers. To test this directly, *ehbp-1* RNAi was applied to animals expressing both hTAC-GFP and RFP-RAB-5. I found that both hTAC-GFP and RFP-RAB-5 can colocalize to the intestinal vacuoles in *ehbp-1* RNAi animals (Figure 36, A-C, D-F, and G-I), confirming my hypothesis.

**Loss of EHBP-1 function affects the subcellular localization of ACT-5-GFP-labeled compartments in the intestine**

In one major respect, however, *ehbp-1* RNAi did not resemble *rab-10* mutants. In appendix-1, I described accumulation of abnormal ACT-5-GFP-labeled intracellular structures in the intestine of both *rab-10* and *rme-1* mutants. In addition, the ACT-5-GFP-labeled fibers were found on the limiting membrane of enlarged endosomes in *rab-10* mutants. These results suggested the actin-cytoskeleton is involved in basolateral early endosomal recycling. In addition, both CeEHBP-1 and mammalian Ehbp1 have an actin-bundling motif (Calponin Homology). Human Ehbp1 has been predicated to be involved in the bundling of actin fibers (Guilherme et al. 2004). To investigate if there is any actin cytoskeleton defect caused by loss of *ehbp-1* function, *ehbp-1* RNAi was applied to animals bearing an ACT-5-GFP transgene. In wild type animals (*L4440* only), ACT-5-GFP was found to label the apical and basolateral membranes and a few
cytoplasmic puncta in the intestinal cells (Figure 37, A and D). However, in \textit{ehbp-1} RNAi animals, ACT-5-GFP became very cytosolically diffuse. ACT-5-GFP was barely found to label on lateral membrane or limiting membrane of enlarged endosomes (Figure 37, B and E). I quantified a two-fold reduction in the number of ACT-5-GFP-labeled endosomes in \textit{ehbp-1} RNAi animals (Figure 37G, \textit{ehbp-1(-): L4440}, 6.66±2.84 vs. 14.63±3.1, p<0.001). There was also a significant difference in the size of ACT-5-GFP-labeled puncta after \textit{ehbp-1} RNAi (Figure 37H, \textit{ehbp-1(-):L4440}, 0.16±0.07 vs. 0.26±0.1, p>0.001). Finally, unlike in \textit{rab-10} mutants, no accumulation of ACT-5-GFP-labeled fibers was found on the limiting membrane of enlarged endosomes in \textit{ehbp-1} RNAi animals (Figure 37, C and F). Taken together these results suggest that EHBP-1 may be involved in formation of actin bundles, and possibly endosomal recruitment or polymerization of actin.

\textit{Loss of \textit{ehbp-1} function decreases the accumulation of ACT-5 on the enlarged endosomes of \textit{rab-10} mutants}

I previously showed that loss of RAB-10 results in accumulation of ACT-5-GFP-labeled puncta in the cytoplasm and accumulation of actin fibers on the limiting membrane of intestinal vacuoles. Conversely, \textit{ehbp-1} RNAi decreased the number of ACT-5-GFP-labeled puncta in the cytoplasm and reduced accumulation of F-actin in wild-type animals. I was therefore interested in investigating if loss of \textit{ehbp-1} function could also decrease the accumulation of ACT-5-labeling in \textit{rab-10} mutants. In control animals (\textit{L4440 only}), accumulation of ACT-5-GFP-labeled puncta was found in the cytoplasm of
intestinal cells (Figure 38, B-D). In addition, strong labeling of ACT-5-GFP fibers was observed on the limiting membranes of intestinal vacuoles (Figure 38, C). *ehbp-1* RNAi in *rab-10* mutant background resulted in a dramatic reduction of ACT-5-GFP-labeled endosomal compartments. The ACT-5-GFP-labeling became more cytosolically diffuse (Figure 38, F-H), and number and size of cytoplasmic puncta labeled by ACT-5-GFP were about 2.5-fold lower compared to those in control animals (Figure 38, I-J, *ehbp-1(-): L4440, 17.4±5.22 vs. 42.36±10.31, p<0.001 in puncta number; 0.34±0.1 vs. 0.77±0.27, p<0.001 in puncta size, respectively). In addition, accumulation of ACT-5-GFP-labeled fibers observed on the limiting membrane of intestinal vacuoles in *rab-10* mutants was reduced by *ehbp-1* RNAi (Figure 38G). There was no effect on the number or size of *rab-10*-induced intestinal vacuoles by *ehbp-1* RNAi. These results suggest that *ehbp-1* may be involved in actin polymerization or bundling on endosomes and perhaps elsewhere.

**EHBP-1 is broadly expressed in *C. elegans***

To determine *ehbp-1* expression and subcellular localization in *C. elegans*, I created transgenic animals expressing an *ehbp-1*-GFP fusion gene, driven by 3.2 kb of *ehbp-1* upstream sequences (the predicted promoter region). It was found that this transgene was expressed almost ubiquitously. Expression of EHBP-1-GFP was observed in the pharynx and nerve ring, body-wall muscles, seam cells, intestine, oviduct sheath cell and spermatheca (Figure 39, A-F). EHBP-1-GFP fusion protein appeared punctate or tubular in most tissues. I also made a GFP-EHBP-1 transgene driven by the intestinal promoter
vha-6. In the intestine N-terminally or C-terminally GFP-tagged EHBP-1 localized to the apical and basolateral plasma membranes (Figure 39, D-E, and G-H, arrows), and distinct cytoplasmic puncta close to basolateral membranes resembling endosomes ranging in size from 0.5 to 1.0 µm (Figure 39, D, H, and I, arrowheads). In addition, GFP-EHBP-1-labeled, tubular structures, close to basolateral plasma membrane were observed in animals expressing the vha-6-GFP-EHBP-1 transgene (Figure 39, H and I, arrows and arrowheads indicate tubular or puncta-like structures, respectively).

**EHBP-1 is associated with endosomes but not Golgi in the intestine**

To determine where EHBP-1 is normally localized and to help test the hypothesis that EHBP-1 functions together with RAB-10 in endocytic transport in the intestine, I performed a series of colocalization studies by using GFP-tagged EHBP-1 and a set of RFP-tagged endosomal markers described previously in our RAB-10 studies. First, I found that GFP-EHBP-1 colocalized very well with early endosome marker RFP-RAB-5 on apical and basolateral cortex (Figure 40, A1-A3 and B1-B3). Some of the GFP-EHBP-1-labeled structures that colocalized with RFP-RAB-5 appearing tubular or punctate (Figure 40, A1-B3). In addition, a high degree of colocalization of GFP-EHBP-1 with both endosomal marker RFP-RAB-8 and RFP-RAB-10 was observed in the cytoplasmic puncta and tubular structures (Figure 40, C1-D3 and E1-F3). On the other hand, GFP-EHBP-1 only slightly colocalized with basolateral recycling endosome marker RFP-RME-1 (Figure 40, I1-J3) and not at all with apical recycling/TGN marker RFP-RAB-11 (Figure 40, G1-H3). Finally, GFP-EHBP-1 was found not to colocalize
with MANS-RFP at all on the Golgi (Figure 40, K1-K3). Taken together these results indicate that EHBP-1 is enriched on endosomes but not Golgi, where it could interact with RAB-10 to promote endocytic recycling.

*Overexpression of GFP-EHBP-1 induces tubulation of endosomes*

In single label experiments, RFP-RAB-5, RFP-RAB-8, and RFP-RAB-10 are only observed in the puncta (Figure 41, A and E, arrowheads). However, in the colocalization studies of GFP-EHBP-1 with RFP-RAB5, RFP-RAB-8, or RFP-RAB-10, the double-labeled endosomes often appeared as long tubules (Figure 40, A1-A3; Figure 41, B and F, arrowheads representing puncta and tubules). Cells expressing the highest levels of GFP-EHBP-1 produced the most tubular morphology.

*EHBP-1 labels the enlarged endosomes of rab-10 mutants*

Earlier in this chapter I showed that GFP-RAB-10 became more cytosolic diffusive in appearance, and somewhat surprisingly the number of GFP-RAB-10-labeled endosomes was reduced, by *ehbp-1* RNAi. This suggested that EHBP-1 may be required for association of RAB-10 with endosomal membranes. Rather, we might have expected that loss of RAB-10 would result in loss of membrane associated EHBP-1, since Rab proteins are thought to recruit their effectors to membranes. Two kinds of GFP-tagged EHBP-1 transgenes were made for this study. The first transgene encodes an EHBP-1 fusion protein with a C-terminal GFP-tag, driven by the *ehbp-1* promoter. The second reporter
encodes an EHBP-1 with an N-terminal GFP-tag, driven by the intestine-specific vha-6 promoter. The former (pwIs451/RT1125) is an integrated line with lower level, but more even, expression throughout all intestinal cells. The latter (pwEx104/RT1449) is an exchromosomal array, and shows stronger, but more mosaic, expression in the intestine. Visible expression could only be observed in animals at the L4 larval, or young adult stages. The expression level drops dramatically after that.

I introduced both transgene pwIs451 and transgene pwEx104 into the rab-10(q373) mutant background individually, and assayed the subcellular localization of GFP-tagged EHBP-1. Loss of rab-10 function resulted in accumulation of GFP-tagged EHBP-1-labeled puncta and vesicles of variable sizes (Figure 42, A-D and E-L). Abnormally numerous puncta, and tubule/fiber-like labeling by GFP-tagged EHBP-1 was observed on the limiting membrane of enlarged rab-10 mutant endosomes in both tagged forms of EHBP-1 (Figure 42, C-D and G-L, respectively). In adult animals, I found enlarged endosomes with much longer and more complicated tubular/fiber-like labeling by GFP-tagged EHBP-1 on their surfaces (Figure 42, C-D). In younger animals (L4 larva/young adult), accumulation of GFP-EHBP-1-labeled puncta and small vesicles was found in the cortical area close to basal membrane (Figure 42, G-J), lateral membranes or medial cytoplasm (Figure 42, K-L). Many of them clustered as if preparing to fuse together to form even larger vacuoles (Figure 42, I-L). Many tubular structures extended from these local gathering sites (Figure 42, G-H and I-J). Taken together these results suggest that, unlike the effect on the subcellular localization of RAB-10 caused by loss of ehp-1 function, rab-10 mutation does not interfere with the ability of EHBP-1 to associate with
endosomal membranes. Rather, EHBP-1 remains associated with the abnormal endosomes that are produced in such a rab-10 mutant background.

**Loss of RME-1 function disrupts formation of GFP-EHBP-1-labeled tubules and changes the subcellular localization of GFP-EHBP-1**

RAB-10 and RME-1 are involved in two consecutive steps in the same basolateral endocytic recycling process, and loss of either one results in similar appearing vacuoles accumulating in the worm intestine. rab-10 mutations interfere with transport from early to recycling endosomes, and the intestinal vacuoles are enlarged basolateral early endosomes. On the contrary, rme-1 mutations disrupt the trafficking from the downstream recycling endosomes back to basolateral plasma membrane, so the rme-1-specific intestinal vacuoles are enlarged basolateral recycling endosomes. In the following experiment I wanted to determine the phenotype of rme-1 mutations on the subcellular localization of GFP-tagged EHBP-1. The same two transgenes, driven by the ehbp-1 or vha-6 promoter, were introduced individually into the background of rme-1(b1045). It was observed that EHBP-1-GFP labeled the limiting membrane of the intestinal vacuoles in rme-1(b1045) mutant animals (Figure 43C), but unlike in the rab-10 mutants, there was no accumulation of GFP-tagged EHBP-1-labeled tubule/fiber-like structures on the intestinal vacuoles (Figure 43, A-B (young adult), and D-E (L4 larva), respectively). In addition, abnormally numerous GFP-tagged EHBP-1-labeled tubular puncta were observed to accumulate in the cortical areas close to the basal membrane (Figure 43, A-B (young adult), and D-E (L4 larva)), or in the apical or lateral membranes
Taken together these results indicate that RME-1 is not required for endosomal membrane association of GFP-EHBP-1. However, the accumulation of GFP-tagged EHBP-1 in \textit{rme-1} mutants suggests that EHBP-1 may have an additional function in the late RME-1-driven step in addition to its role in the earlier RAB-10 driven step.
DISCUSSION

The actin-cytokeleton system may be involved in basolateral endocytic recycling

The role of the actin-cytoskeleton in mammalian cell endocytosis is still quite controversial (Qualmann et al. 2000; Qualmann and Kessels 2002; Engqvist-Goldstein and Drubin 2003). In my study, accumulation of actin-5-labeled endosomes and endosomal tubules were found in the intestine in rab-10 mutants. In addition, another actin-cytoskeletal regulator, syndapin involved in the internalization step from plasma membranes and the recycling step back to the plasma membrane, was also found on the limiting membrane of rab-10-specific intestinal vacuoles (Appendix-2). This indicates that actin-cytoskeleton may be involved in endocytic recycling. Recently, the mammalian early endosomal marker Hrs was found to associate with actinin-4 interacts with BERP and myosin V to form the mammalian CART (Cytoskeleton-Associated Recycling or Transport) complex. CART was proposed to regulate endocytic recycling, suggesting that actin regulation may be a phylogenetically conserved feature of endocytic recycling trafficking.

EHBP-1 may function together with RAB-10 in basolateral recycling process

General speaking, knockdown of EHBP-1 function causes rab-10-like intestinal phenotypes such as early endosomal vacuole accumulation and loss of basolateral
recycling endosomes. These phenotypes indicate that EHBP-1 functions together with RAB-10 protein in basolateral recycling process.

Mammalian Ehbp1 has been shown to interact with Ehd1 or Ehd2 (mammalian homologue of *C. elegans* RME-1) through its NPF (Asparagine-Proline-Phenylalanine) repeats and function together in regulating endocytic internalization or recycling steps of Glut4 in the 3T3-L1 adipocyte cells (Guilherme et al. 2004; Guilherme et al. 2004). On the contrary, there is no NPF motif found in CeEHBP-1 and no interaction detected by yeast two-hybrid assay for CeEHBP-1 and RME-1 proteins. In colocalization studies, GFP-EHBP-1 and RFP-RME-1 only colocalized slightly in the *C. elegans* intestine. My results indicate that EHBP-1 may not be involved in other endocytic steps such as internalization, but rather only the recycling process with RAB-10 in the *C. elegans* intestine.

**EHBP-1 may be required to stabilize membrane association of RAB-10 to basolateral early endosomes**

In this chapter I showed that RNAi of EHBP-1 affected the subcellular localization of GFP-RAB-10. The number of GFP-RAB-10-labeled puncta was reduced and GFP-RAB-10 became more diffusive in appearance. Two possibilities may explain the observed effects on the subcellular localization of GFP-RAB-10. First, the number of basolateral early endosomes could be reduced such that RAB-10 has fewer structures to bind to. The second possibility is that the membrane association of RAB-10 to early endosomes is
disrupted by loss of EHBP-1. The former situation seems unlikely because RNAi of EHBP-1 actually increased number of GFP-RAB-5-labeled early endosomes, suggesting that early endosomes are still present after EHBP-1 RNAi. Rather, I favor the hypothesis that EHBP-1 may regulate the membrane attachment of RAB-10 to basolateral early endosomes. If EHBP-1 associates with a RAB-10 GEF (Guanine nucleotide exchange factor), as is the case for some Rab effector/Rab GEF pairs in mammalian cell, then loss of EHBP-1 might reduce GEF activity on the endosome and reduce the level of active GTP-bound RAB-10 associating to the endosomal membrane.

**rme-1 mutation affects the morphology of EHBP-1-labeled structures**

As mentioned above, *rab-10* mutation does not obviously affect the morphology of GFP-EHBP-1-labeled tubular endosomes, although the intestinal vacuoles produced in *rab-10* mutants are GFP-EHBP-1 positive. However, I found that *rme-1* mutation does affect the morphology but not endosomal membrane association of GFP-EHBP-1. It has been previously reported that mammalian Ehd2 (the *C. elegans* homologue of RME-1) interacts with Arp2/3 complex, an activator of actin nuclear polymerization, through several acidic amino acids in the N-terminal upstream of EH domain. Overexpression of full-length Ehd2 in 3T3-L1 adipocyte cells induced cortical actin-cytoskeleton rearrangement, while expression of the EH domain of Ehd2 alone disrupted the cortical actin-cytoskeleton (Guilherme et al. 2004; Guilherme et al. 2004). These results suggest that RME-1 may be involved in the polymerization of endosomal actin. Therefore, loss
of RME-1 function may disrupt the morphology of compartments labeled by EHBP-1, if actin polymerization regulates their formation or function.

**EHBP-1 may be a true actin-binding protein with the ability to enhance actin polymerization or bundling formation to endosomes**

The proposed function of EHBP-1 in actin binding, and bundling of actin fibers, remains unproven. *ehbp-1* RNAi disrupted ACT-5-GFP morphology in the intestine, consistent with a possible role for EHBP-1 in promoting actin polymerization or bundling. In addition, overexpression of GFP-EHBP-1 induces tubular endosomal structures in the intestine. Are these GFP-EHBP-1-labeled tubular endosomal structures truly associated with F-actin? Several experiments could be done to address this question. First, dissected worm intestine could be labeled in situ for actin fibers with rhodamine-conjugated phalloidin. Alternatively, this experiment could also be done by introducing transgenes expressing GFP-EHBP-1 and ACT-5-RFP into the same animals using two-color imaging to determine if the GFP-EHBP-1-labeled structures are also labeled by ACT-5-RFP. If this turns out to be the case, it would suggest that overexpression of GFP-EHBP-1 induces actin polymerization or bundling on endosomes.

Several CH domain containing actin-binding proteins have been found including fimbrin, α-actinin, spectrin, calponin, and Scp1 (Bramham et al. 2002; Goodman et al. 2003; Klein et al. 2004; Liu et al. 2004). The CH domains in fibrin, α-actinin, and spectrin are presented as tandem pairs (CH1 and CH2) but only one CH domain is found in calponin
and Scp1. It was found that actin binding in single CH domain proteins such as calponin is cooperative (Galkin et al. 2006). This indicates that proteins with single CH domain may need to be dimerized to associate with actin, offering at least two CH domains to bind actin and crosslink actin fibers. Since only one CH domain is found in the mammalian Ehbp1 and CeEHBP-1, it is possible that EHBP-1 may act like calponin, forming homo-dimers to bind and enhance bundling of F-actin. In addition to direct bundling activity, EHBP-1 may also serve as a locator to recruit other factors to actin fibers and regulate actin rearrangement like other single CH domain-containing actin binding proteins (Gimona et al. 2002; Galkin et al. 2006).

**EHBP-1 may be a true RAB-8 or RAB-10 effector**

Through yeast two-hybrid binding assays in vitro, EHBP-1 protein was shown to interact with RAB-8 and RAB-10. In addition, results from colocalization studies of GFP-EHBP-1 with RFP-RAB-8 or RFP-RAB-10 show very strong evidences that EHBP-1 are present on the same endosomes with RAB-8 or RAB-10 in vivo. Overexpression of GFP-EHBP-1 induces accumulation of tubular endosomes also labeled by RFP-RAB-8 or RFP-RAB-10. The RFP-RAB-8, -10-labeled endosomes are only long and tubular in animals coexpressing GFP-EHBP-1. They are much more punctate in animals expressing RFP-RAB-8 or RFP-RAB-10 alone.
CHAPTER 5

Materials and Methods
**General methods and strains**

All *C. elegans* strains were derived originally from the wild-type Bristol strain N2. Worm cultures, genetic crosses, and other *C. elegans* husbandry were performed according to standard protocols (Brenner 1974). A complete list of strains used in this study was listed in Table 4.

**Tissue-specific endocytosis assays**

Three assays were performed to monitor steady-state endocytosis in oocytes, coelomocytes, and the intestine of worms. Oocyte endocytosis was assayed by using a transgenic strain *bIs1[vit-2::GFP]* expressing a YP170-GFP fusion protein (Grant and Hirsh 1999). Transgenic strain *arls37[myo-3::ssGFP, dpy-20(+); dpy-20(e1282)]* expressing a secreted form of GFP expressed by muscle cells and released into the body cavity was used for coelomocyte assays (Fares and Greenwald 2001). Basolateral endocytosis by the intestine was also be visualized using muscle-secreted GFP as a fluid-phase marker. In some cases a *cup-4* mutation was also included in the assay to reduce uptake by coelomocytes and increase availability of the marker in the body cavity (Patton *et al.* 2005).

**Mutant isolation, genetic mapping, and molecular cloning**
The q373 allele was originally isolated as a spontaneous mutant arising from a Bristol N2 stock. *gum-1(q373)* was mapped to the interval between *bli-4* and *unc-13* on chromosome I by standard three-factor mapping. The *dx2* allele was isolated in a non-complementation screen performed by crossing EMS-treated N2 males with *gum-1(q373) unc-13(e51)* hermaphrodites screening F1 progeny for the Gum-nonUnc phenotype (Eric Lombie performed mapping). To identify *gum-1*-containing DNA fragments, cosmids (10-20 µg/ml) containing *C. elegans* genomic DNA together with the dominant transgenic marker *rol-6(su1006)* (plasmid pRF4, 100 µg/ml) were microinjected into *gum-1(q373)* worms to establish transgenic strains and assayed for rescue of the intestinal phenotype. Only the cosmid clone T23H2 displayed rescuing activity. The rescuing activity was further narrowed to the T23H2.5 gene by microinjection of PCR products of individual predicted genes within T23H2. Subsequently it was found that RNAi of T23H2.5 was able to phenocopy the *gum-1* intestinal phenotype in wild-type animals. To identify mutations in *gum-1* mutants, the complete T23H2.5 genomic region was amplified by PCR and sequenced directly using nested primers. To confirm the coding region of *gum-1/rab-10*, the apparently full-length cDNA clone yk586g1 was sequenced (Barth Grant and Peter Schweinsberg performed the experiments).

**Plasmids and integrated transgenic strains**

To construct the GFP-RAB-10 transgene driven by its own promoter, 2.9 kb of RAB-10 promoter sequence was PCR amplified from *C. elegans* genomic DNA with primers `ggatctctcttgtctatatggtgc` (Bam-t23h2.5promF) and `ggtaccaagttcctctct` (Bam-t23h2.5promF)
(Asp-t23h2.5promR) containing BamHI and Asp718I restriction sites and cloned into the same sites in the *C. elegans* GFP vector pPD117.01 (gift of Andrew Fire) to generate the plasmid 2.9GFP. The entire genomic exon/intron and 3’UTR was then PCR amplified with primers gaattcatgetgccgacctgatgac (RI-t23h2.5genebodyF) and (Ngo-t23h2.5genebodyR) gccggctcgctggaaggtggaattgctc including EcoRI and NgoMIV restriction sites and cloned into the same sites downstream of GFP in the 2.9GFP vector to generate the GFP-rab-10 plasmid. Integrated transgenic lines *pwIs214* and *pwIs215* for GFP-rab-10 were generated and produced similar expression patterns. The *pwIs214* line was crossed into a *rab-10(dx2)* background and was found to rescue the intestinal vacuole phenotype.

Most analysis presented here used the *pwIs214* line. Five integrated lines (*pwIs262–266*) were isolated for the 2.9GFP promoter construct. To construct GFP or RFP fusion transgenes for expression in the worm intestine, two Gateway destination vectors were prepared using the promoter region of the intestine-specific gene vha-6 cloned into the *C. elegans* pPD117.01 vector, followed by GFP or RFP coding sequences, a Gateway cassette (Invitrogen, Carlsbad, CA), and *let-858* 3’UTR sequences, followed by the *unc-119* gene of *C. briggsae*. The genomic or cDNA sequences of *C. elegans* rab-5, rab-7, rab-8, rab-10, rab-11, rme-1, or human Rab10 genes were cloned individually into entry vector pDONR221 and then transferred into vha-6-GFP (or RFP)-vectors by Gateway recombination cloning to generate N-terminal fusions (experiments performed by Barth Grant, Peter Schweinsberg). The human *TfR* (transferrin receptor), human *TAC* (alpha-chain of the IL-2 receptor), *C. elegans* act-5, syndapin, and a fragment of *C. elegans* alpha-mannosidase II (F58H1.1, first 82 aa including signal sequence/TM-anchor domain (Rolls et al. 2002). were cloned into a similar vector upstream of GFP to generate
C-terminal fusions (by Barth Grant, Peter Schweinsberg, and Shilpa Vashist). The GFP- or RFP-tagged plasmids (8 µg) were cobombarded with plasmid MM016B encoding the wild-type unc-119 gene (8 µg) for plasmids lacking Cb-unc-119 into unc-119(ed3) mutant worms to establish low copy integrated transgenic lines by the microparticle bombardment method (Praitis et al. 2001). Integrated transgenic lines used in this study were listed in Table 4.

To construct the GFP-tagged hum-2, gck-2, ehbp-1, or y82e9br.21, or lin-10 transgenes driven by their own promoters, DNA fragments of hum-2, gck-2, ehbp-1, y82e9br.21, or lin-10 including their whole genomic sequences, and presumed promoter sequences, were PCR amplified from C. elegans genomic DNA using primers listed below and cloned into the entry vector pDONR221. They were transferred into the C. elegans pPD117.01-GtwyB (Asp 718)GFP-cbunc-119 vector, including a Gateway cassette (Invitrogen, Carlsbad, CA) followed by GFP coding sequences, let-858 3’ UTR sequences, and the unc-119 gene of C. briggsae, to generate C-terminal fusions. The GFP-tagged plasmids (10 µg each) were bombarded into unc-119(ed3) mutant animals to establish low copy integrated transgenic lines by micro-particle bombardment method (Praitis et al. 2001). Integrated transgenic lines used in this study were listed in Table 4.

**Primer lists (for C-terminal fusions driven by their promoters)**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F25B3.1ehbp-1promF</td>
<td>GGGGACAACTTTGTACAAAAAAGTTGctgtcacttactgtgagcac</td>
<td>ZC404.9gck-2promF</td>
</tr>
<tr>
<td>F25B3.1ehbp-1nostopR</td>
<td>GGGGACAACTTTGTACAAGAAAGTTGaacggcgccaccattgaataaagtc</td>
<td></td>
</tr>
<tr>
<td>ZC404.9gck-2promF</td>
<td>GGGGACAACTTTGTACAAAAAAGTTGacatttcacggctttccatga</td>
<td></td>
</tr>
</tbody>
</table>
To construct the GFP-tagged gck-2, ehbp-1, or y82e9br.21 transgenes driven by the intestine-specific vha-6 promoter, the genomic sequences of gck-2, ehbp-1, or y82e9br.21 genes were PCR amplified from C. elegans genomic DNA with primers listed below. They were cloned into the entry vector pDONR221 and then were transferred into vha-6-GFPtwyB(EcoRI)-cbunc-119 vector by Gateway recombination cloning to generate N-terminal fusions. The GFP-tagged plasmids (10 µg each) were bombarded into unc-119(ed3) mutant animals to establish low copy integrated transgenic lines as mentioned above.

**Primer lists (for N-terminal fusions driven by the intestine-specific vha-6 promoter)**

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGGGACAACTTTTGTCACAGAAAAAGTTGatccagcgatgctgatg</td>
<td>ZC404.9gck-2nostopR</td>
</tr>
<tr>
<td>GGGGACAACTTTTGTCACAGAAAAAGTTGtgcagctacagaaagtgc</td>
<td>F36D4.3hum-2promF</td>
</tr>
<tr>
<td>GGGGACAACTTTTGTCACAGAAAAAGTTGatacaagtctacaaacactgtgc</td>
<td>F36D4.3hum-2nostopR</td>
</tr>
<tr>
<td>GGGGACAACTTTTGTCACAGAAAAAGTTGcgcattctctataaggaggctgtgc</td>
<td>Y82E9BR.21promGtwyF</td>
</tr>
<tr>
<td>GGGGACAACTTTTGTCACAGAAAAAGTTGatgtcctagctaaagcttcgc</td>
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</tr>
<tr>
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<td>Lin-10promF</td>
</tr>
<tr>
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<td>Lin-10nostopR</td>
</tr>
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<td>Y82E9BR.21-RRBPnostopGtwyR</td>
</tr>
</tbody>
</table>
To construct the LIN-10-GFP transgene driven by the intestine-specific vha-6 promoter, the genomic sequences of lin-10 gene were PCR amplified from C. elegans genomic DNA with primers GGGGACAACTTTGTACAAAAAAATTTTGttcaggtatccttgaage (Lin-10startF) and GGGGACAACTTTGTACAAGAAATTTGcttatttggtttcttgacccg (Lin-1 0nostopR) and cloned into an entry vector pDONR221 and then transferred into vha-6-Gtwy B(Asp718)GFP-chunc-119 vector by Gateway recombination cloning to generate C-terminal fusions. Transgenic lines for lin-10-GFP were generated by the microparticle bombardment method and listed in Table 4.

**Microscopy and image analysis**

Live worms were mounted on 2% agarose pads with 10 mM levamisol as described previously (Sato et al. 2005). Epi-fluorescence images were obtained using an Axiovert 200M (Carl Zeiss MicroImaging, Oberkochen, Germany) microscope equipped with a digital CCD camera (C4742–12ER, Hamamatsu Photonics, Hamamatsu, Japan), captured using Metamorph software (Universal Imaging, West Chester, PA), and then deconvolved using AutoDeblur software (AutoQuant Imaging, Watervliet, NY). Images taken in the DAPI channel were used to identify broad-spectrum intestinal autofluorescence caused by lipofuscin-positive lysosomes (Clokey and Jacobson 1986). To obtain images of GFP fluorescence without interference from autofluorescence, I used a Zeiss LSM510 Meta confocal microscope system (Carl Zeiss MicroImaging). It was determined that the GFP fluorescence peak at 510 nm lacked significant contributions from autofluorescent lysosomes. Thus confocal images shown depict only this wave-
length peak and depict GFP only. Most of confocal images were taken from worms at the young adult stage (16-24 hr after the L4 larval stage). Quantification of images was performed with Metamorph software (Universal Imaging). Data was collected from 8 animals of each genotype. Puncta number and area measurement were sampled in three different regions of each intestine defined by a 100 x 100 (pixl²) box positioned at random. Analysis of standard deviations was performed by the student’s T-test. Most GFP/RFP colocalization analysis was performed on L3 larva generated as F1 cross-progeny of GFP marker males crossed to RFP marker hermaphrodites.

*Identification and cloning of RAB-10-interacting proteins*

*Bait, prey plasmids, and C. elegans cDNA library*

A yeast two-hybrid screen to identify candidates of RAB-10-interacting proteins was performed according to the procedure of the DupLEX-A yeast two-hybrid system (OriGene Technologies, Rockville MD). To construct the “bait” plasmids, the cDNA sequences of *C. elegans rab-8*(wt-, Q67L-, and T22N-), and *rab-10*(wt-, Q68L-, T23N-) in the entry vector pDONR221 were transferred into the pEG202-Gtwy bait vector (provided by Miyuki Sato) by Gateway recombination cloning (Invitrogen, Carlsbad, CA) to generate N-terminal fusions with the *lex A* DNA binding domain. The *pEG202-rab-5*(Q78L), *-rab-7*(Q68L), *-rab-11*(Q70L), *-rme-1*(full-length, EH only), or *-lin-10* were made in the same ways. The prepylation motifs for membrane attachment at the C-
terminal ends of RAB were also deleted to improve entry of bait fusion proteins into the yeast nucleus.

To construct the prey plasmid with the full-length coding sequence of the \textit{ehbp-1} gene, the coding region was PCR amplified from \textit{C. elegans} EST clone \textit{yk1543d03} provided by Dr. Yuji Kohara (National Institute of Genetic, Japan) with primers GGGGACAACTTTGTACAAAAAAAGTTGtggeggttatctctcg (F25B3.1EHBP-1nostartGtwyF) and GGGGACAACTTTGTACAAAAAAAGTTGttatacaagtctacaaacactctgc (F25B3.1EHBP-1stopGtwyR), followed by cloning into an entry vector pDONR221 and then transferred into the pJG4-5-Gtwy prey vectors (provided by Miyuki Sato) by Gateway recombination cloning (Invitrogen, Carlsbad, CA) to generate a N-terminal fusion with the B42 transcription-activating domain.

To construct the prey plasmid encoding the C-terminal half of the HUM-2 protein (aa889-1839), coding sequences were PCR amplified from \textit{C. elegans} EST clone \textit{yk20f7} provided by Dr. Yuji Kohara (National Institute of Genetic, Japan) with primers GGGGACAACTTTGTACAAAAAAAGTTGaaaagcttcgctatgagaaatc (yk20f7-hum-2-nostartGtwyF) and GGGGACAACTTTGTACAAAAAAAGTTGttatacaagtctacaaacactctgc (yk20f7-hum-2-stopGtwyR), followed by cloning into an entry vector pDONR221 and transfer into the pJG4-5-Gtwy prey vector as above.

The \textit{C. elegans} cDNA library was purchased from the \textit{DupLEX-A} yeast two-hybrid system (OriGene Technologies, Rockville MD). This cDNA library was made by oligo
d(T)-priming followed by cloning into the B42 transcription activating domain between the XhoI and EcoRI sites of the pJG4-5 prey vector. Expression from this vector is regulated by a galactose-indusible promoter GAL1.

To verify the sequence in the LexA-bait junction, all pEG202-bait plasmids were sequenced by using primer ttcgtcagcagagcttcacc (pEG202/Seq, designed by Miyuki Sato).

**Yeast C. elegans library transformation**

A 20 ml overnight culture of *Saccharomyces cerevisiae* strain EGY48 bearing bait plasmid pEG202-rab-10(Q68L) and lacZ reporter plasmid pSH18-34 was prepared in YNB (glu)-his-ura medium. The overnight culture was diluted in 300 ml of YPD medium to a final OD$_{600}$=0.1, and then grown at 30°C to an OD$_{600}$ =0.5-0.7 (about 5-6 hours). To prepare yeast for transformation, cell pellets were harvested by centrifugation at 1500 x g for 5 minutes, resuspended in 30 ml of sterile ddH$_2$O, spun-down, and then resuspended in 30 ml of LiOAc buffer (0.1 M LiOAc, 10 mM Tris-HCl (pH=8.0), 1 mM EDTA), spun-down, and were resuspended again in 3 ml of LiOAc buffer (100 µl/per transformation).

In each transformation, 1 µg bait plasmid pEG202-rab-10(Q68L) and 1 µg prey plasmid pJG4-5-cDNA library were added in 100 µl of EGY48 cell suspension solution with LiOAc buffer (total DNA volume should not exceed 10 µl (10%). 100 µg single stranded carrier DNA (salmon sperm testis genomic DNA (Sigma) boiled at 100 °C for 5 minutes,
and adjusted a final concentration of 10 µg/µl in TE buffer and kept it cool on ice) was added to each transformation and left it at room temperature for 5 minutes. 280 µl of PEG solution (50% w/v PEG3350 (M.W.=4000), 0.1 M LiOAc, 10 mM Tris-HCl (pH=8.0), 1 mM EDTA, warmed slowly to dissolve, and then sterilized it by 0.22 µm filter filtration) added in yeast cell suspension, mixed completely by gentle inversion, and then incubated it at 30 ºC incubator for 45 minutes (or at room temperature and mixed once every 15 minutes). At the end of incubation, the cells were heat shocked at 42 ºC for 5 minutes, and then transformation tubes were immediately chilled on ice for 2 minutes. Cell pellets were spun down at 2500 x g for 2 minutes and resuspended in 500 µl of sterile ddH2O. 10 µl of transformation solution was taken from one of tube, diluted in 990 µl of ddH2O, and 100 µl of diluted cell solution was spread onto a 100 mm YNB(glu)-his-ura-trp plate, and then incubated at 30 ºC for 3 days to count colony number.

The rest of tubes were spun down, and resuspended in 250 µl of sterile ddH2O (or culture medium), and spread evenly onto 24 cm x 24 cm YNB (glu)-his-ura-trp plates (one plate for one tube, 29 plates in total) and incubated at 30 ºC for 3 days until colonies appeared.

Three days later, 10 ml of sterile ddH2O was added to each plate and colonies were recovered from each plate by scraping into 50 ml falcon tubes. The cells were pelleted at 1500 x g. The volume of cell pellets was estimated and an equal volume of sterile ddH2O and sterile 50% glycerol was added. Cells were then frozen in 1 ml aliquots/ per tube at -70 ºC.
Calculation of transformation efficiency

To calculate the transformation efficiency, the number of transformants was obtained by counting total colony number on the 100 mm plate with $10^3$ fold dilution. The transformation efficiency was calculated by total colony number /per 1 µg library plasmids/per transformation. So the total number of transformants for screens is the transformation efficiency x number of transformation. To perform a saturating screen with the *C. elegans* library, at least $2 \times 10^6$ transformants in total should be screened. In my study, $4.9 \times 10^6$ transformants were screened.

Titering the number of viable cells in each frozen tube

An aliquot (1 ml) of frozen EGY48 transformants was thawed out and added to 9 ml of YNB (gal)-his-ura-trp medium (10 x dilution) followed by incubating at 30 ºC for 4 hours. 100 µl of a 1000 x dilution incubated solution was spread onto a 100 mm YNB (gal)-his-ura-trp plate and was incubated at 30 ºC for 2-3 days until colonies appeared. Total number of colony-forming units (cfu)/per ml (per frozen aliquot tube) equals $10^5$ times the total colony number counted on the 100 mm YNB (gal)-his-ura-trp plate.

Screens for Leu-positive colonies

Frozen aliquots with a total viable cell number (cfu) equal to 5-7 fold the original total transformant number were thawed. YNB (gal)-his-ura-trp medium was added to adjust
cell concentration to $OD_{600} = 0.5$ (1 $OD_{600} = 2 \times 10^7$ yeast cells (or cfu)/ml), and then was incubated at 30 ºC for 4 hours. 100 µl of cell solution was spread onto each 100 mm YNB (gal)-his-ura-trp-leu plate (maximum 1 x 10⁶ cfu/per plate) and was incubated at 30 ºC for 2-3 days (or until colonies appeared). Colonies were then picked onto new YNB (gal)-his-ura-trp-leu plates and incubated at 30 ºC for 3 days, and then were kept at 4 ºC to create a master plate for β-Galactosidase assays (see below).

**Screens for β-Galactosidase-positive colonies**

Four copies of each colony strip were made from the original YNB(gal)-his-ura-trp-leu master plates and were transferred onto the following four types of plates: YNB(glu)-his-ura-trp-leu, YNB(gal)-his-ura-trp-leu, YNB(glu)-his-ura-trp+X-gal, and YNB(gal)-his-ura-trp+X-gal, and then were incubated at 30 ºC for 2 days. The truly positive transformants should grow on the (gal)-leu and (gal) +X-gal plates but not on the (glu)-leu or (glu) +X-gal plates.

**Recovery of positive prey plasmids encoding RAB-10-interacting candidates from yeast**

Each positive transformant was grown in 2 ml of YNB (glu)-trp medium at 30 ºC overnight. The cells were then pelleted. 200 µl of plasmid rescue solution (2% Triton-X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH=8.0), 1 mM EDTA), 0.3 g of glass beads, and 100 µl of phenol (pH=8.0) and 100 µl of 24:1 chloroform: isoamyl alcohol was added, and then vortexed for 2 minutes. The aqueous phase was collected, 1/10
volume of 3 M NaOAc and at least 2 volume of 95% ethanol was added to precipitate the plasmid. Plasmid pellets were resuspended in 70% ethanol, air dry, and were resuspended in 5 µl of sterile ddH₂O ready for KC8 cells transformation.

**Purification of positive prey plasmids encoding RAB-10-interacting candidates from bacteria KC8 cells**

1 µl of plasmid solution recovered from yeast was used to transform *E. coli* strain KC8 cells by electroporation followed by spreading onto a minium-trp plate at 37 ºC for 2 days because of its slow growing rate on the minium-trp plate. Two colonies were picked from each plate and set up in mini-cultures in the minium-trp medium at 37 ºC for 18-24 hours. Prey plasmids were purified from the overnight culture of KC8 cells. Purified DNA was resuspended in 30 µl of 1 x TE buffer (10 mM Tris-HCl pH=7.5, and 1 mM EDTA). All potential positive pJG4-5 prey plasmids purified from KC8 cells were retransformed back to EGY48 yeast cells with bait plasmid pEG202-rab-10(Q68L) and reporter plasmid pSH18-34 to confirm their interactions with RAB-10(Q68L).

**Subgrouping of pJG4-5 prey plasmids by restriction enzyme digestion**

Before sending for sequence analysis, all pJG4-5 prey plasmids encoding potential RAB-10-interacting candidates identified from yeast two-hybrid screens were first double-digested with XhoI/EcoRI (because individual cDNA fragment of library was inserted into the pJG4-5 prey plasmid between XhoI and EcoRI sites). The cutting patterns of
double digestion were monitoring on 1% ethidium bromide agarose gel and were subdivided into several subgroups based on the size of bands. Only one or two samples representing each subgroup were sent for further DNA sequencing analysis. Two sequencing primers were used for further sequencing analysis: tgcctgatggaatctcctcc (pJG4-5/Seq) for 5’ forward sequencing, and ccttgattggagaacctgacc (pJG4-5 3'Seq) for 3’ reverse sequencing.

List of primers used for further sequencing analysis of various pJG4-5 prey plasmid identified in yeast two-hybrid screens

<table>
<thead>
<tr>
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RNA interference method

RNAi was performed by the feeding method (Timmons and Fire 1998). E. coli strain HT115 with RNAi plasmids were cultured in 6 ml of LB medium (100 µg/ml Ampicillin) at 37 ºC overnight. Resulting cell pellets were spun down and were resuspended in 400 µl of LB medium (100 µg/ml Ampicillin), added onto four RNAi plates (100 µl/per plate), and were left at room temperature for 1-2 days to air dry. Eggs harvested from bleached animals were placed onto each RNAi plate. Once these animals reached adulthood (about 4 days), 12 adult animals (P0) were picked and moved to a new RNAi plate, allowed to lay eggs for 6 hours, and then were removed. Phenotypes were scored when F1 animals reached the adult stage (about 20-24 hours after L4 larval stage).

All HT115 RNAi bacteria strains of RAB-10 interacting proteins except y82e9br.21 were from AJ library (Kamath et al. 2003) and their positions in AJ library collection boxes were listed below: ehbp-1(f25b3.1), V6C-H02; gck-2(zc404.9), V4D-H04, hum-2(f36d4.3), V6C-E08; f52e1.13, V6A-B11; cnt-1(y17g7b.15), II8A-F02; zk1248.10, II4C-G05; and f20d1.2, X6D-G12. All RNAi plasmids were confirmed by sequencing and only the RNAi clone of zk1248.10 was found to be wrong.

The L4440-y82e9br.21 RNAi feeding construct was prepared by PCR amplification from EST clone yk1098g06 provided by Dr. Yuji Kohara (National Institute of Genetic, Japan) with primers GGGGACAACTTTTGATACAAAAAGTTGgagctcatggccgctaggaatgatgg (Y82E 9BR.21-SacI startGtwyF) and GGGGACAACTTTTGATACAAGAAAAGTTGggtacc
ctagctatcgattacgtc (Y82E9 BR.21-KpnI stopGtwyR) containing SacI and KpnI restriction sites and was cloned into the same sites in the RNAi vector L4440 (Timmons 2006). The feeding construct was then transformed into E. Coli strain HT115.
CHAPTER 6

Conclusion and Future Directions
CONCLUSION

Establishment of groundwork for endocytic trafficking studies in the *C. elegans* intestine

In my studies, one of the main objectives was to establish a system of molecular markers for analysis of subcellular organelles mainly using GFP or RFP-tagged endosomal markers such as RAB-5, -7, -8, -10, -11, LAMP-1, and RME-1 proteins and endocytic transmembrane cargo such as hTfR, hTAC in the *C. elegans* intestine. In wild-type animals, most of endosomal markers and transmembrane cargo act as predicted from previous studies of their mammalian homologues. Because the cells are large and complex (polarized epithelial cells with apical/basolateral cellular compartments), the *C. elegans* intestine is an ideal tissue for studies of polarized membrane trafficking.

*RAB-10 protein regulates basolateral endocytic recycling in the *C. elegans* intestine*

In Chapter 2, my results showed that *gum-1* is Ce-RAB-10 as confirmed by genetic mapping, sequencing, and rescue experiments. In addition, I showed that the human Rab10 is a true ortholog of Ce-RAB-10, able to rescue the defects associated with loss of endogenous RAB-10. *rab-10* mutations result in a dramatic increase in early endosome number with a concomitant loss in basolateral but not apical recycling endosomes. These results suggested that the gigantic vacuoles may be formed by homotypic fusion of accumulated early endosomes. Finally, RAB-10 is present on basolateral early
endosomes where it could directly regulate such endosomal transport. My additional observation that RAB-10 also labels the Golgi implies that RAB-10 may be also involved in some aspect of exocytosis.

**Several RAB-10-interacting proteins identified using yeast two-hybrid screens may be helpful to reveal how RAB-10 controls basolateral endocytic recycling trafficking**

In Chapter 3, eight candidates of RAB-10 interacting proteins were identified. Four of these are likely to be associated with the actin-cytoskeletal system. In term of binding specificity, most of them appear to be specific for RAB-8 and RAB-10. In addition, all of them showed specificity for the active GTP-bound RABs the assays.

Among these eight RAB-10-interacting candidates, I found that *hum-2(ok596)* mutant has defects in the subcellular localization of RME-1 and RAB-10. Only loss of *ehbp-1* but not other candidates by RNAi results in a *rab-10*-like intestinal vacuole phenotype.

**EHBP-1 functions together with RAB-10 in basolateral endocytic recycling trafficking**

Through analysis of various endosomal markers and transmembrane cargo, it was revealed that EHBP-1 displays an RNAi-phenotype mainly identical to that of *rab-10* loss of function mutants. In addition, EHBP-1 may facilitate the endosomal membrane association of RAB-10. EHBP-1 colocalized extensively with RAB-8 and RAB-10 on early endosomes consistent with the inferred physical interaction between EHBP-1 and
RAB-8 and/or RAB-10 in vivo. Finally, I found evidence that the actin-cytoskeleton system may be involved in basolateral recycling. EHBP-1 may function in bundling actin fibers on endosomes.

**Possible working model for the function of an EHBP-1/RAB-10/Myosin V complex in the endocytic recycling pathway**

Figure 44 shows a working model for how RAB-10 and its putative effectors could function together in the endocytic recycling pathway of the worm intestine. First, the EHBP-1 protein may associate with the membranes of early endosomes directly, perhaps through its C-terminus which contains the CAAX motif, and indicating potential prenylation upon activation by active RAB-10. EHBP-1 may help to bundle actin associate with the endosomes, or it might bring the endosomes into contact with preexisting actin fibers. On the other hand, membrane-bound EHBP-1 could also help to stabilize the endosomal membrane association of active GTP-bound RAB-10. This might happen if for instance, EHBP-1 associates with a RAB-10 GEF. GTP-bound active RAB-10 could then recruit more downstream effectors such as the actin motor HUM-2 (Myosin V) to associate to local endosomal vesicles. Taken together, this model predicts that endosomal vesicles bearing EHBP-1/RAB-10 could associate with actin motor HUM-2 and move from early endosomes to recycling endosomes along the “Track” formed by actin-cytoskeletal fibers.
FUTURE DIRECTIONS

**RAB-8 and RAB-10 may be partially redundant in other biological processes**

My studies indicated that RAB-8 and RAB-10 do not function redundantly in basolateral endocytic recycling trafficking of in the *C. elegans* intestine. However, I also found that *rab-10* mutant animals treated with *rab-8* RNAi but not *rab-10* mutants or wild-type animals treated with *rab-8* RNAi, produced dead embryos or became sterile adults. In addition, my yeast two-hybrid RAB-10-interacting proteins could all also interact with RAB-8 in this assay system. This implies the possibility that RAB-8 and RAB-10 may function redundantly, perhaps in the secretory pathway. Consistent with this idea, it has been suggested that mammalian Rab8 and Rab10 display a more severe secretory defect in secretion of vsvg-gfp in non-polarized MDCK cells when depleted in combination rather than single (Schuck *et al.* 2007). It will be important to further investigate if RAB-8 and RAB-10 function redundantly to mediate exocytosis.

**Searching for RAB-10-specific binding domains in various RAB-10-interacting proteins and other evidence for binding specificity**

So far, I have not narrowed down the interacting sequences in individual RAB-10-interacting proteins responsible for binding to RAB-10. Further investigation could be done using yeast two-hybrid assays. In addition, more biochemical assays in vitro such
as immuno pull-down assays using tissue culture cells or worm lysates should be performed to test the results from yeast two-hybrid.

Functional analysis of RAB-10-interacting proteins using available deletion mutants

Of the candidate RAB-10 effectors identified in my screen, only *ehbp-1* recapitulated the *rab-10*-like intestinal mutant phenotype. Without extensive controls to determine the efficiency of RNAi knockdown, I cannot determine the validity of these negative results. Recently, deletion mutants for all of eight candidates became available from the Japanese *C. elegans* knockout group. It will be important to investigate the functions of these candidate RAB-10-interacting proteins again using these available deletion mutants. In case of the *ehbp-1*, it will be important to see if all the *ehbp-1* (RNAi) phenotypes are also observed in the deletion mutant animals.
APPENDIX-1

Results related to Chapter 2 but not included in the paper published in

*Molecular Biology of the Cell* (Chen et al. 2006).
RESULTS

*rab-10 mutants accumulate enlarged intestinal vacuoles labeled by trans-membrane cargo proteins and RAB-5*

Since many of the intestinal vacuoles found in *gum-1* mutants are labeled by the trans-membrane cargo proteins hTAC-GFP, hTfR-GFP, LMP-1-GFP and early endosome marker GFP-RAB-5 in single label experiments, I sought to investigate if these abnormal structures labeled with cargo markers and RAB-5 simultaneously. *rab-10 (q373)* mutants were crossed into the background of both *vha-6*-driven transgenes of RFP-RAB-5 and hTAC-GFP, hTfR-GFP, or LMP-1-GFP individually. I observed that all three cargo proteins colocalized with RFP-RAB-5 on the limiting membrane of the abnormal intestinal vacuoles in *rab-10(q373)* mutants (Figure 4, A-F (hTAC-GFP/RFP-RAB-5); Figure 46, A-F (hTfR-GFP/RFP-RAB-5), and G-L (LMP-1-GFP/RFP-RAB-5), arrows). These results suggest that the hTAC-GFP/hTfR-GFP/LMP-1-GFP-labeled intestinal vacuoles are gigantic early endosomes.

*rab-10 mutants accumulate abundant endosomes labeled by GFP-RAB-8*

It has been reported that there are 29 different RAB proteins found in *C. elegans* involved in the membrane trafficking in various cell types (Perea-Leal and Seabra 2000). Among these 29 RAB proteins, RAB-8 is the most similar to RAB-10. RAB-8 and RAB-10 are homologues of yeast Sec-4 which is associated with Golgi to the plasma membrane
transport in yeast (Salminen and Novick 1989; Walworth et al. 1989). It was predicated that RAB-8 is involved in the secretary pathway in mammalian cells (Linder et al. 2007). In addition, it has been reported that RAB-8 physically resides in the Golgi, which is consistent with the idea that RAB-8 functions in trafficking from the Golgi (Schuck et al. 2007). I was interested in investigating if there is any effect on the subcellular localization of RAB-8 caused by rab-10 mutants in the C. elegans intestine. Because of high identity in amino acid sequence between RAB-8 and RAB-10, I also wanted to investigate if overexpression of RAB-8 can substitute for loss of RAB-10 and rescue the intestinal vacuoles phenotype in rab-10 mutants. In addition, I performed a parallel analysis in an rme-1 null mutant background for comparison. In the wild-type intestinal cells, abundant GFP-RAB-8-positive subcellular compartments appeared as small punctate structures near the basolateral and apical membranes, with some larger structures in the medial cytoplasm (Figure 47, A and C, arrowheads). I found abnormally numerous GFP-RAB-8-labeled puncta and small vesicles in gum-1(q373) mutant (Figure 47, B and D). The number and size of GFP-RAB-8-labeled subcellular compartments were twofold to threefold higher than those observed in the wild-type animals (Figure 47, F and G, p<0.001). In addition, like GFP-RAB-5 and GFP-RAB-7, GFP-RAB-8 also appeared on the limiting membranes of the abnormal intestinal vacuoles of rab-10(q373) mutants (Figure 47, D and E, arrows). The number and size of intestinal vacuoles was not affected by overexpressing GFP-RAB-8. Taken together these results suggest that RAB-8 is also involved in endocytic recycling in the C. elegans intestine but with a distinct function from RAB-10.
rme-1 mutations also cause accumulation of GFP-RAB-8-labeled subcellular compartments

I also sought to investigate if the subcellular localization of GFP-RAB-8 was also changed in the rme-1 mutants. Again, a transgene with GFP-RAB-8 was introduced into the rme-1(b1045) null mutant background and the subcellular localization of GFP-RAB-8 was characterized. Unlike in the cases of GFP-RAB-5 or GFP-RAB-7, which were not affected by rme-1 mutations, abnormal accumulation of GFP-RAB-8 was found in rme-1(b1045) mutants. This phenotype differed in appearance from that observed in rab-10 mutants. Large patches of GFP-RAB-8 were observed in areas close to basolateral membranes and in the medial cytoplasm (Figure 48, B and D, arrows). The number and size of GFP-RAB-8-labeled compartments in the rme-1 mutants were increased 1.7 fold and 2.5 fold respectively compared to those in wild-type control animals (Figure 48, E and F). Taken together these results suggest that RAB-8 could be associated with early endosomes and recycling endosomes.

RAB-5, RAB-7, and RAB-11 but not MANS can colocalize to a compartment near the basal plasma membranes of intestinal cells

McBride and Zerial have shown that a single endosome can harbor multiple Rab proteins, with each Rab defining a different subdomain within the endosome (Zerial and McBride 2001). My previous results showed that GFP-RAB-10 can colocalize with RFP-RAB-5, RFP-RAB-7, and RFP-RAB-11 in areas near basolateral intestinal membranes; whereas
GFP-RAB-10 only colocalizes with RFP-RAB-11 in the medial cytoplasm. In addition, RFP-RAB-10 also partially colocalizes with the TGN marker MANS-GFP in areas close to basolateral membranes and in the medial cytoplasm. In mammalian cells, it has been shown that Rab4 and Rab5 are on early endosomes, Rab7 is in the early/late endosomes, MANS is in the medial Golgi, and Rab11 is in both TGN and recycling endosomes. Some fraction of early endosome contains Rab4, Rab5, and Rab7. In polarized cells, early endosomes could be found in the cortical areas near apical/basolateral plasma membranes, whereas apical recycling endosomes and TGN could be found in cortical apical plasma membranes and/or medial perinucleus cytoplasm. Since GFP-RAB-10 was highly colocalized with all three endosomal markers RFP-RAB-5, RFP-RAB-7, RAB-11 in endosomes locating in the area near basal plasma membranes, it suggested some intestinal endosomes might exist that label for all four markers. To test this idea, colocalization between RAB-5, RAB-7, RAB-11, and MANS in the cortical areas of apical/basal plasma membranes or medial cytoplasm were investigated. Male worms with the GFP-RAB-5 were crossed with hermaphrodite worms bearing RFP-RAB-7, RFP-RAB-11, or MANS-RFP transgenes. GFP-RAB-5 and RFP-RAB-7 were highly colocalized in endosomes found in the areas near basal plasma membranes and medial cytoplasm (Figure 49, A-C and D-F, arrowheads). In the colocalization of GFP-RAB-5 and RFP-RAB-11, GFP-RAB-5 was highly colocalized with RFP-RAB-11 in areas close to basal plasma membranes (Figure 49, G-I, arrowheads), whereas it was only partially colocalized with RFP-RAB-11 in the apical plasma membranes and medial cytoplasm (Figure 49, J-L). Finally, GFP-RAB-5 and MANS-RFP were only found to be colocalized very occasionally probably by coincidence. Very few GFP-RAB-5 and
MANS-RFP double positive structures were found in either area close to apical/basal plasma membranes or area in medial cytoplasm (Figure 49, M-O and P-R). Taken together these results indicate that RAB-5, RAB-7, and RAB-11 but not MANS can colocalize on endosomes located in the area near basal plasma membrane of intestine cells, suggesting that endosomes bearing multiple subdomains exist in this area. One reasonable hypothesis would be that these endosomes are the worm equivalent of the so-called “common recycling endosomes” defined in MDCK cells (Babbey et al. 2006). Such common endosomes receive recycling cargo from basolateral and apical domains and act as a sorting station for polarized transport.

**RAB-8 is associated with endosomes and Golgi in a similar manner of RAB-10**

Like GFP-RAB-5 and GFP-RAB-7, GFP-RAB-8 was also found to accumulate on the limiting membranes of enlarged endosomes in *rab-10* mutants. I was interested to determine if RAB-8 is also physically associated with the basolateral early endosomes. I compared the subcellular localization of GFP- or RFP-RAB-8 with RFP-RAB-5, GFP-RAB-10, or MANS-RFP in animals heterozygous for each transgene. GFP-RAB-8 was found to colocalize with RFP-RAB-5 in areas throughout the cell (Figure 50, A-C and D-F). It appeared that there were more RAB-8 and RAB-5 double positive endosomes than those labeled by both RAB-10 and RAB-5, especially near apical plasma membranes and in the medial cytoplasm (Figure 50, D-F, and Figure 10, D-F). GFP-RAB-10 and RFP-RAB-8 colocalized in puncta close to the basal plasma membranes (Figure 50, G-I) and were less frequently colocalized in the area near apical plasma membranes or medial
cytoplasm (Figure 50, J-L). In addition, GFP-RAB-8 was also found to partially colocalize with the medial Golgi marker MANS-RFP in the areas near the basal plasma membranes and medial cytoplasm, suggesting that like RAB-10, RAB-8 is resident on Golgi in addition to endosomes (Figure 50, M-O and P-R). The main difference in the subcellular localization of RAB-8 and RAB-10 is that RAB-8 seems to have a greater presence than RAB-10 near apical plasma membranes and in the medial cytoplasm.

**GFP or RFP-tagged RAB endosomal markers have similar subcellular localizations**

There is always a concern in studying the subcellular localization of a protein using the GFP or RFP-tagged fusions, rather than antibody staining for endogenous protein, as to whether the GFP/RFP-tagged fusion proteins accurately reflect the endogenous proteins in terms of localization and function. As I have shown previously, expression of N-terminally-tagged GFP-CeRAB-10 or GFP-hRab10 rescues the intestinal mutant phenotypes caused by loss of CeRAB-10. This indicates that the GFP-tagged RAB-10 behaves similarly to the endogenous RAB-10, likely reflecting the function and subcellular localization of the endogeneous protein. On the other hand, members of RAB family share a lot of common features such as C-terminal lipid modification. In addition, Rab family proteins in other systems have been used extensively with fluorescent protein tags and are believed to localize normally. In my studies, I presumed that various GFP-tagged RABs such as RAB-5, RAB-7, RAB-8, and RAB-11 could also act like their endogenous version of proteins.
Several RFP-tagged RAB proteins were also created and used in the studies of intestinal subcellular localization to serve as endosomal markers. Since the GFP-tagged forms were better characterized, I decided to compare our newly made collection of RFP-tagged RABs with the GFP-tagged set. Here I used RAB-5 and RAB-10 as examples to test if the GFP- and RFP-tagged versions of RAB-5 or RAB-10 colocalize with each other as would be expected. I found that GFP- and RFP-tagged RAB-5 colocalized very well with each other (Figure 51, A-C, arrowheads). Very nice colocalization was also found between GFP- and RFP-tagged RAB-10 (Figure 51, D-F, arrowheads). Taken together these results indicate that the subcellular localization of various endosomal RAB markers shown by GFP or RFP-tagged versions are similar to each other and could represent the true subcellular locations of their endogenous counterparts.

*Intestinal subcellular localization of Golgi marker MANS-GFP is not affected by rab-10 mutants*

Mammalian Rab10 was reported to colocalize with a Golgi marker in mammalian CHO and BHK cells, or oocytes/embryos of sea urchins (Chen et al. 1993; Leaf and Blum 1998). In my studies, in addition to colocalization with early endosomes in the areas close to the basal plasma membranes, Ce-RAB-10 also was found to partially colocalize with Golgi marker MANS. This result implies that RAB-10 may function in secretion in addition to basolateral recycling. In order to address if RAB-10 might also be involved in the secretary pathway of intestinal cells, the MANS-GFP transgene was crossed into the *rab-10(q373)* mutant background and the subcellular localization of MANS-GFP was
examined. If trafficking from Golgi to the plasma membrane is disrupted in *rab-10* mutants, then the size or number of MANS-GFP-labeled Golgi puncta may change. I found that the subcellular localization of MANS-GFP was not affected in *rab-10(q373)* mutants (Figure 52, A-B). In addition, there was no accumulation of MANS-GFP observed on the intestinal vacuoles in *rab-10(q373)* mutants (Figure 52, B-C, arrows). Quantification of images in terms of number or size of MANS-GFP-labeled puncta showed there was no difference in the wild-type and *rab-10(q373)* mutant animals (Figure 52, D-E).

*Intestinal subcellular localization of apical transmembrane cargo OPT-2-GFP is affected by rab-10 mutants*

RAB-10 has been shown to be involved in endocytic recycling of basolateral intestine membranes. It was of interest to investigate if RAB-10 also functions in the apical recycling. Currently, the only available transmembrane marker for the apical intestinal membranes is OPT-2, but it remains unclear if OPT-2 cycles through the apical endosomal system (Nehrke 2003). Preliminary studies by epi-fluorescence microscopy indicated that OPT-2-GFP was normally localized to the apical membranes of intestinal cells in *rab-10* mutants (S. Vashist and B. D. Grant, unpublished results). Due to very strong fluorescence expression of OPT-2-GFP near the intestinal lumen, it was very difficult to observe OPT-2-GFP localized in other areas of the intestinal cells such as basolateral membranes or medial cytoplasm. In order to solve this problem, worms bearing an OPT-2-GFP transgene were examined again by confocal microscopy. In
confocal microscopy, signals from GFP had better spatial resolution and non-apical signals could be separated and identified. In wild-type worms, OPT-2-GFP was found to strongly label the apical lumen. In addition, some extra OPT-2-GFP-labeled structures including puncta and ring-like vesicles of variable size were also found in the medial and basal cytoplasm (Figure 53, A and D, arrowheads). In *rab-10(q373)* mutants, labeling on the apical lumen appeared to be normal. However, abnormally high accumulation of OPT-2-GFP-labeled puncta and ring-like structures were observed in the medial and basal cytoplasm (Figure 53, B and E, arrowheads). The number and size of OPT-2-GFP-labeled structures in *rab-10(q373)* mutants were increased about twofold and fourfold respectively when compared with that in the wild-type animals (Figure 53, G and H). In addition, a low level of OPT-2-GFP was also found to label the limiting membrane of intestinal vacuoles in the *rab-10(q373)* mutant, suggesting possible redirection of some OPT-2-GFP to the basolateral endocytosis system. (Figure 53, C and F, arrows).

*Negative control: intestinal expression of GFP shows cytosolic diffusion and no labeling on the limiting membrane of intestinal vacuoles in *rab-10* or *rme-1* mutants*

Many GFP-tagged endosomal markers and transmembrane cargo proteins have been used in the studies of *rab-10* and *rme-1* mutants to address the endocytic processes involved. In the subcellular localization studies of various GFP-tagged markers, it is important to know that the GFP tag alone does not also label the limiting membrane of intestinal vacuoles in *rab-10* or *rme-1* mutants. To test this, I crossed an intestine-specific GFP-only transgene into the background of *rab-10* or *rme-1* mutants and observed the
subcellular localization of GFP in the worm intestine. The GFP tag expressed in the intestinal cells was totally diffuse in appearance in all areas of the cell, including the nucleus (Figure 54, A and B, arrows). In *rab-10* or *rme-1* mutants, GFP tags remained diffuse and there was no labeling of the limiting membrane of intestinal vacuoles (Figure 54, C and D, arrows). Taken together these results indicate that the GFP tag itself does not become enriched on any subcellular structures on its own, and is a suitable tag to use in our experiments.
APPENDIX-2

The Actin-cytoskeleton and Basolateral Endocytic Recycling in
the *C. elegans* Intestine
RESULTS

The actin-cytoskeleton may be involved in the basolateral endocytic recycling in the C. elegans intestine

The actin-cytoskeleton system has been shown to be involved in endocytosis in mammalian cells and yeast cells (Samaj et al. 2004; Gachet and Hyams 2005; Yarar et al. 2005; Hyman et al. 2006; Hernandez et al. 2007). Actin polymerization on endosomes may be important for producing transport carriers, since several actin regulators associated with endosomes such as Arf6 and syndapin are required for recycling (Caplan et al. 2002; Prigent et al. 2003; Kessels and Qualmann 2004; Braun et al. 2005; Kessels and Qualmann 2006; Gong et al. 2007). In order to investigate if the actin-cytoskeleton system is involved in the basolateral recycling of C. elegans intestine, worms bearing the ACT-5-GFP transgene were crossed into the rab-10(q373) or rme-1(b1045) mutant backgrounds. There are five actin proteins found in C. elegans. ACT-1, -2, and -3 are mainly found in the body-wall muscles or germline/early embryos; whereas ACT-4 and ACT-5 are mainly found in the intestinal and excretory (kidney-like) cells (Files et al. 1983; Gobel et al. 2004; MacQueen et al. 2005; Willis et al. 2006). If the actin-cytoskeleton is involved in the basolateral endocytic recycling in the C. elegans intestine, we might expect to observe accumulation or loss of ACT-5-GFP-labeled structures in the intestinal cells of rab-10 or rme-1 mutants. In wild-type animals, ACT-5-GFP was highly enriched on or near the apical intestinal lumen (Figure 55, A and E, arrows). Very weak labeling in the lateral plasma membranes and a small number of labeled puncta
were observed in cross-sections of intestinal cells (Figure 55E). I observed abnormally high level accumulation of ACT-5-GFP-labeled structures close to the basolateral membranes and in the medial cytoplasm of \textit{rab-10(q373)} or \textit{rme-1(b1045)} mutants (Figure 55, B and F, D and H, arrowheads). The number and size of ACT-5-GFP-labeled structures were increased about threefold and fourfold respectively in \textit{rab-10(q373)} or \textit{rme-1(b1045)} mutants (Figure 55, I and J). In addition, ACT-5-GFP-labeled cytoskeletal fibers were observed to accumulate on the periphery of intestinal vacuoles in the \textit{rab-10(q373)} mutants (Figure 55, C and G, arrows). I did not observe ACT-5 cable-like structures in the \textit{rme-1(b1045)} mutants even though ACT-5-GFP still labeled the limiting membrane of abnormal vacuoles (Figure 55, D and H, arrows). Taken together these results suggest that the actin-cytoskeleton system may be involved in the basolateral endocytic recycling in the \textit{C. elegans} intestine.

\textbf{Different effects on the subcellular localization of Syndapin-GFP are observed in the intestine of \textit{rab-10} or \textit{rme-1} mutants}

Syndapin is an N-WASP and Ehd1/mRme-1 binding protein that couples endocytosis with the actin-cytoskeleton system. It was reported that mammalian syndapin is involved in the internalization and recycling steps of endocytosis (Braun \textit{et al.} 2005; Anggono \textit{et al.} 2006; Anggono and Robinson 2007). I was interested in determining if the subcellular localization of worm SDNP-1/Syndapin-1 was affected in the \textit{rab-10} or \textit{rme-1} mutants. Worms expressing a SDNP-1-GFP transgene were crossed into the \textit{rab-10(q373)} or \textit{rme-1(b1045)} mutant background and the subcellular localization of SDNP-1-GFP was
examined. In wild-type animals, SDNP-1-GFP was found to label the apical plasma membranes and punctate, tubular structures in the areas close to basal plasma membranes (Figure 56, A and D, arrowheads). It has been shown that the SDNP-1-labeled puncta and tubule-like structures were also labeled by RME-1 (Saumya Pant and Barth Grant, unpublished data), indicating that the SDNP-1-GFP-labeled punctate and tubular structures are basolateral recycling endosomes. In \textit{rab-10(q373)} mutants, the subcellular localization of SDNP-1-GFP was abnormal (Figure 56, B and E). SDNP-1-GFP appeared cytosolically diffuse in the cortical areas close to basal plasma membranes, with slightly enhanced labeling on the basolateral plasma membranes (Figure 56B). In addition, SDNP-1-GFP was observed to weakly label the limiting membranes of intestinal vacuoles (Figure 56E, arrows). Labeling of apical plasma membranes was normal and enhanced labeling in the basal plasma membranes was also observed in the cross-sections of the intestine (Figure 56E, arrowheads). The number and size of SDNP-1-GFP-labeled puncta and tubules were reduced dramatically which were about twelvefold and threefold as compared with that in wild-type control animals (Figure 56, G and H, p<0.001).

In \textit{rme-1(b1045)} mutants, the subcellular localization of SDNP-1-GFP was also affected, but in a different manner from that in \textit{rab-10(q373)} mutants. SDNP-1-GFP-labeled punctate and tubular structures are disrupted and became diffuse in appearance, and large patches labeled by SDNP-1-GFP were found in area close to the basal plasma membranes (Figure 56C, arrows). In addition, weak labeling of SDNP-1-GFP was found on the limiting membrane of small vacuoles, and vacuoles were often surrounded by SDNP-1-
GFP-labeled puncta (Figure 56C, arrowheads). In a cross-sectional view of the intestine, accumulation of SDNP-1-GFP-labeled patches were also observed near the basolateral plasma membranes and in medial cytoplasm. Apical labeling by SDNP-1-GFP was reduced (Figure 56F, arrowheads). The number of SDNP-1-GFP-labeled puncta in the cortical areas of basal plasma membranes was reduced about fourfold, but the size of SDNP-1-GFP-labeled structures was increased about threefold compared to wild-type (Figure 56, E and F, p<0.001). Taken together these results showed that *rab-10* and *rme-1* mutants affect the subcellular localization of SDNP-1 albeit in different manners, and suggest that syndapin may function in this pathway *in vivo*.

It has been reported that the mammalian Syndapin is involved in both internalization from the plasma membrane and recycling back to plasma membrane from endosomes (Kessels and Qualmann 2004; Braun *et al.* 2005). In *rab-10* mutants, accumulation of SDNP-1-labeled small vesicles was observed near basal membrane and on the limiting membrane of intestinal vacuoles, with concomitant loss of downstream tubular recycling endosomes. In *rme-1* mutants, large SDNP-1-labeled patches accumulated, and the puncta and tubules in between were disrupted. These results suggest that worm SDNP-1 is associated with the basolateral recycling endosomes in the intestine. Further analysis will be required to determine its function there.
APPENDIX-3

Functional Analysis of ARF-6 in the *C. elegans* Intestine
RESULTS

I performed some pilot studies for the functional analysis of ARF-6 during endocytosis in the *C. elegans* intestine and other tissues such as oocytes or coelomocytes. I crossed worms with various transgenes of endocytic GFP markers into the *arf-6(tm1447)* mutant background (Table 4). Some of the GFP-tagged markers such as RME-1, RAB-10, ACT-5, and Syndapin/SDNP-1 in the *arf-6(tm1447)* mutant background were examined by confocal microscopy. The *arf-6(tm1447)* mutation contains a 2263 bp deletion in genomic sequences covering the first five exons and introns (Figure 57). *arf-6(tm1447)* is therefore predicted to be an null mutation.

*Basolateral recycling endosomes labeled by GFP-RME-1 and Syndapin-GFP are affected by arf-6 mutants*

It was found that GFP-RME-1 and Syndapin-RFP colocalize on the tubulo-vesicular endosomes near basal plasma membranes (Saumya Pant and Barth Grant, unpublished data). This indicates that Syndapin could function in basolateral recycling. Animals expressing GFP-RME-1 or Syndapin-GFP were crossed into the *arf-6 (tm1447)* mutant background, and effects on the subcellular localization of GFP-RME-1 or Syndapin-GFP were examined. In wild-type animals, both of GFP-RME-1 and Syndapin-GFP labeled on the tubular, punctate structures (recycling endosomes) in the areas close to basal plasma membrane (Figure 58, A and E). They also label apical structures (Figure 58, C and G). However, in *arf-6(tm1447)* mutants GFP-RME-1- or Syndapin-GFP-labeled
basolateral structures largely disappeared, concomitant with increased cytosolic GFP signal (Figure 58, B and F, arrowheads). GFP-RME-1 or Syndapin-GFP on or near apical membranes appeared normal (Figure 58, D and H). Quantification revealed that the number and size of GFP-RME-1 or Syndapin-GFP-labeled puncta in arf-6(tm1447) mutants were reduced (threelfold and sevenfold lower in number and threelfold and threelfold lower in size, for GFP-RME-1 or Syndapin-GFP, respectively) compared to those observed in wild-type animals (Figure 58, I and J, p<0.001). Taken together these results indicate that loss of arf-6 function causes a rab-10-like effect on basolateral recycling endosomes labeled by GFP-RME-1 or Syndapin-GFP. These results suggest that ARF-6 may function upstream of RME-1 in endocytic recycling.

The subcellular localization of GFP-RAB-10 is not affected in arf-6 mutants

In chapter 2, I showed that RAB-10 functions in trafficking from basolateral early to recycling endosomes. In addition, it was shown previously in mammals mRme-1/Ehd1 functions in the Arf6 pathway (Caplan et al. 2002). To investigate if ARF-6 functions before or after RAB-10, animals expressing GFP-RAB-10 were crossed into the arf-6(tm1447) mutant background and the subcellular localization of GFP-RAB-10 was examined. In arf-6(tm1447) mutants with GFP-RAB-10-labeled structures appeared normal (Figure 59, A and B, C and D) with no obvious change in the number or size of GFP-RAB-10-labeled puncta (Figure 59, E and F). These results indicate that the subcellular localization of GFP-RAB-10 does not depend on arf-6 function. Future experiments should investigate if there is any effect on the subcellular localization of
ARF-6-GFP in *rab-10* mutants; in particular, it would be interesting to determine if the *rab-10*-specific intestinal vacuoles are labeled by ARF-6-GFP.

*The subcellular localization of ACT-5-GFP is affected in arf-6 mutants*

Mammalian Arf6 is involved in regulating actin cytoskeletal rearrangements (Donaldson and Honda 2005; Venkateswarlu *et al.* 2007). To test for a similar function in *C. elegans*, I assayed for effects on the subcellular localization of ACT-5-GFP in *arf-6* mutants. Animals expressing ACT-5-GFP were crossed into the *arf-6(tm1447)* mutant background and subcellular localization of ACT-5-GFP in the intestine was examined. In wild-type animals, ACT-5-GFP was observed to strongly label apical membranes and to weakly label basolateral membranes, whereas occasional cytoplasmic puncta were also observed (Figure 60, A and D). In *arf-6(tm1447)* mutant animals, ACT-5-GFP was found to accumulate on or near lateral membranes, and in excess cytoplasmic puncta (Figure 60, B and E). In addition, ACT-5-GFP-labeled fibers were also found to accumulate on the limiting membranes of rare intracellular vacuoles that were somewhat similar to those observed in *rab-10* mutants but smaller in size (Figure 60, C and F, arrows). Quantification of images indicated that the number of ACT-5-GFP-labeled puncta in *arf-6(tm1447)* mutant animals increased by about twofold compared to wild-type animals (Figure 60G, *p*<0.001), but there was no obvious change in the size of labeled puncta (Figure 60H). Taken together these results suggest that ARF-6 does regulate actin assembly in the worm intestine.
APPENDIX-4

Functional Analysis of LIN-10 in the *C. elegans* Intestine
RESULTS

Recently, LIN-10 has been shown to be involved in the endocytic trafficking of the EGF receptor during vulval development, and in trafficking of the AMPA-type glutamate receptor in the postsynaptic membranes of neuronal cells (Chang and Rongo 2005; Glodowski et al. 2005; Stetak et al. 2006). Loss of lin-10 function caused intracellular accumulation of GLR-1-GFP. In addition, the rab-10 mutation was also found to cause accumulation of lin-10-like GLR-1-GFP-labeled intracellular patches in the ventral nerve cord of *C. elegans* (Doreen Glodowski and Christopher Rongo, *Mol. Biol. Cell*, submitted). These results suggest that LIN-10 and RAB-10 may both play roles in regulating endocytic trafficking of GLR-1 in postsynaptic membranes. Since RAB-10 regulates basolateral endocytic recycling in the intestinal cells, it was of interest to investigate LIN-10 function in the intestine.

I performed pilot studies to test lin-10 mutants for defects during endocytosis in the *C. elegans* intestine, oocytes, and coelomocytes.

*LIN-10 is broadly expressed in C. elegans*

LIN-10 expression has been reported using immunofluorescence techniques in larve, but GFP-tagged transgenic lines for LIN-10 have been restricted to vulval cells and cells of the nervous system. First, I sought to determine where LIN-10 is expressed, and in particular if LIN-10 is expressed in the adult intestine. In order to address this question, I
created transgenic lines expressing a lin-10-GFP fusion gene including all exons and introns and was driven by 7.0 kb upstream sequences from the lin-10 gene (the predicted promoter region). LIN-10-GFP fusion protein expressed from the lin-10 promoter was observed in the pharynx, nerve ring, ventral nerve cord, body-wall muscle, posterior touch neurons, spermatheca, hypodermis, coelomocytes, vulva, and intestine (Figure 61, A-J). The LIN-10-GFP appeared to label punctate structures in most tissues. Later, a second transgene of the same lin-10-GFP fusion gene driven by the intestine-specific promoter vha-6 was also created to facilitate examination of the subcellular localization of LIN-10-GFP in the intestinal cells. In the intestine, LIN-10-GFP localized to distinct puncta throughout the cell (Figure 61, K-L). Further experiments need to be done to test if lin-10 mutants are rescued by these two LIN-10-GFP transgenes.

**lin-10 mutation affects the subcellular localization of GFP-RME-1 in the C. elegans intestine**

First, I assayed lin-10 mutants for any coelomocyte uptake defect. It was found that ssGFP uptake by coelomocytes from body cavity appeared to be normal in lin-10 mutants (Figure 62, A-B). In addition, the subcellular localization of GFP-RAB-10 appeared to be normal in lin-10 mutants (Figure 62, C-D and E-F). The number of GFP-RAB-10-labeled puncta in lin-10 mutants was increased, a little less than twofold compared to wild-type animals (Figure 62K, p<0.001). There was no obvious change in the size of labeled puncta (Figure 62L).
Interestingly, lin-10 mutation affected the subcellular localization of GFP-RME-1 in a hum-2-like, but not in a rab-10-like manner. In lin-10 mutants, GFP-RME-1-labeled puncta in the areas close to basal membranes of the intestine appeared to be normal (Figure 62, G-H and I-J, arrowheads). However, large accumulations of GFP-RME-1-labeled puncta were observed in the medial cytoplasm, with diminished labeling along the apical plasma membranes (Figure 62, I-J, arrows). In lin-10 mutant animals the number of GFP-RME-1-labeled puncta in the medial cytoplasm was increased about fourfold higher compared to that in wild-type animals (Figure 62M, p<0.001). These results indicate that loss of lin-10 function affects the subcellular localization of GFP-RME-1, but not in a rab-10-like manner. These results suggest that both RAB-10 and LIN-10 function in endocytic recycling but perhaps in two distinct pathways.

**LIN-10 is associated with both early endosomes and Golgi in the C. elegans intestine**

As mentioned previously, loss of lin-10 function caused intracellular accumulation of GLR-1-GFP, likely trapping GLR-1 in endosomes. In addition, lin-10 mutations slightly increased the number of GFP-RAB-10-labeled, basolateral early endosomes in the intestine. These results suggest that LIN-10 may physically reside in basolateral early endosomes and function in trafficking departing early endosomes. To address this question, colocalization studies of LIN-10-GFP with various RFP-tagged endocytic markers such as RAB-5 and RAB-10 were performed. Animals expressing LIN-10-GFP were crossed with animals expressing RFP-RAB-5 or RFP-RAB-10, and the intestines of cross progeny animals in the F1 generation were examined. It was found that LIN-10-
GFP and RFP-RAB-5 partially colocalized in the basal cortex and the medial cytoplasm (Figure 63, A-C and D-F). I also found that LIN-10-GFP and RFP-RAB-10 colocalized extensively in basal cortex and medial cytoplasm (Figure 63, G-I and J-L). Taken together these results suggest that LIN-10 physically resides on early endosomes and Golgi.
FIGURES
Figure 1. Anatomy of the worm intestine. The worm intestine consists of 20 individual epithelial cells forming nine donut-like intestinal rings (Int I-IX). Int I consists of 4 epithelial cells while all other rings are formed by 2 epithelial cells. (A) From left to right, two intestinal rings (Int VIII-IX,) followed by an int-rec valve are shown. Image (B) represents a 90 degree x-axis rotation of (A).
Figure 2. *gum-1* mutants display an intestinal phenotype similar to that of *rme-1* mutants. (A–C) Nomarski images of the intestine in wild-type, *gum-1(dx2)*, and *rme-1(b1045)* worms. (A) No large transparent vacuoles were observed in the intestines of wild-type worms. (B) Very large transparent vacuoles are found in the intestines of *gum-1(dx2)* and (C) *rme-1(b1045)* mutant worms. Arrows indicate the positions of vacuoles. Intestinal endocytosis of the basolateral recycling marker ssGFP in (D) wild-type, (E) *gum-1(dx2)*, and (F) *rme-1(b1045)* mutants. In wild type worms very little secreted GFP accumulates in intestinal endosomes because of efficient recycling back to the body cavity (D). The *gum-1-* and *rme-1-*specific vacuoles accumulate basolaterally endocytosed GFP (E and F). A *cup-4(ar494)* mutation was also included in the strain shown in E to impair coelomocyte function, increasing steady-state levels of secreted GFP in the body cavity. Similar results are seen in *gum-1* single mutants but the endocytosed GFP is less abundant (personal observation). (G–O) Confocal images of the worm intestine expressing GFP-tagged endocytic transmembrane cargo markers in wild-type, *gum-1(dx2)*, and *rme-1(b1045)* mutant animals. In wild-type worms, the basolateral membranes and basolateral endocytic compartments are labeled by hTAC-GFP (G), hTfR-GFP (human transferrin receptor; J), and LMP-1-GFP (M). In *gum-1(dx2)* and *rme-1(b1045)* mutant worms, all three transmembrane cargo markers accumulate in the enlarged endosomes: hTAC-GFP (H and I), hTfR-GFP (K and L), and LMP-1-GFP (N and O), respectively. Arrows indicate the enlarged endosomes. (P–R) Endocytosis of YP170-GFP by the oocytes of adult hermaphrodites. In wild type, YP170-GFP is endocytosed efficiently by oocytes (P). In the *rme-1(b1045)* mutant, internalization of YP170-GFP by oocytes is dramatically reduced and YP170-GFP accumulates in the body cavity (R). However, in *gum-1(dx2)* mutant, the secretion of YP170-GFP by the intestine and the endocytosis of YP170-GFP by oocytes is normal (Q). Arrows indicate the YP170-GFP accumulation in the oocytes and embryos. OO, oocyte; SP, spermatheca; Emb, embryo. (S–U) Endocytosis of ssGFP by the coelomocytes of adult hermaphrodites. In the *rme-1(b1045)* mutant, coelomocyte endocytosis of GFP secreted by muscle cells is reduced and secreted GFP accumulates in the body cavity and in abnormally small peripheral vesicles of the coelomocytes (U). However, in *gum-1(dx2)* mutants, the secretion of GFP by muscle and the endocytosis of GFP by coelomocytes is relatively normal (T). Bar, 10 µm.
Figure 3. *gum-1* mutants but not *rme-1* mutants accumulate endosomes marked with GFP-RAB-5 and GFP-RAB-7 and lose most endosomes marked with GFP-RME-1. Confocal images in wild-type background are shown for GFP-RAB-5 (A), GFP-RAB-7 (C), GFP-RAB11 (E), or GFP-RME-1 (G and I). Confocal images in *gum-1*(*q373*) background are shown for GFP-RAB-5 (B), GFP-RAB-7 (D), GFP-RAB11 (F), or GFP-RME-1 (H and J). Similar defects were found in *gum-1(dx2)* mutants. Arrowheads indicate punctate, tubular, or ring-like endosomes labeled by GFP-RAB-5, GFP-RAB-7, GFP-RAB-11 and GFP-RME-1 in the apical, cytosolic, lateral, or basolateral compartments. Arrows indicate enlarged intestinal endosomes (vacuoles) labeled by GFP-RAB-5 (B) or GFP-RAB-7 (D). Quantification of endosome number as visualized by the markers is shown in K. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixel²) box positioned at random). Quantification of GFP-RAB-5-positive endosome size in *gum-1* mutants is graphed in L. Bar, 10 μm.
Figure 4. *gum-1* mutant-specific enlarged intestinal vacuoles labeled by transmembrane cargo protein hTAC-GFP are RFP-RAB-5-positive endosomes. (A-F) Epi-fluorescence images of colocalization of RFP-RAB-5 and hTAC-GFP are shown on the limiting membranes of intestinal vacuoles in *gum-1(q373)* mutants. Images in higher magnification are shown in D, E, and F. Arrows indicate enlarged intestinal vacuoles labeled by RFP-RAB-5 and hTAC-GFP. Blue color indicates autofluorescent intestinal granules. Bar, 10 µm.
Figure 5. Amino acid alignment of Ce-RAB-10 and human Rab10. The *C. elegans* and human proteins share more than 90% amino acid identity outside of the hyper-variable C-terminal domain. Mutations deduced from genomic sequencing of *rab-10* mutants alleles *dx2* and *q373* are shown mapped onto the predicted protein sequence. Identical amino acids are shown in black; whereas similar amino acids are shown in gray.
Figure 6. RAB-10 is broadly expressed in *C. elegans*. Expression of a rescuing GFP-RAB-10 transgene driven by the *rab-10* promoter is indicated in A, intestine, arrowheads indicate cytoplasmic puncta; in B, hypodermis, arrows indicate nucleus and arrowheads indicate cytoplasmic puncta; in C, seam cells (arrow); in D, nerve cord (arrow) and body-wall muscle (arrowhead); in E, spermatheca (arrow) and oviduct sheath cells (arrowhead); in F coelomocyte (arrow) and cytoplasmic puncta (arrowhead); and in G, pharynx (arrow) and nerve ring (arrowhead). Bar, 10 μm.
Figure 7. GFP-tagged Ce-RAB-10 or GFP-tagged human Rab10 can rescue the intestinal phenotype of rab-10 null mutants.  (A–D) Confocal images of intestines in the rab-10(dx2) strain expressing GFP-tagged Ce-RAB-10 or human Rab10.  (A) rab-10(dx2) is rescued by expression of GFP-Ce-RAB-10 driven by the intestine-specific vha-6 promoter.  (B) rab-10(dx2) is rescued by expression of GFP-human Rab10 driven by the vha-6 promoter.  (C) rab-10(dx2) is partially rescued by expression of GFP-Ce-RAB-10(Q68L) driven by the vha-6 promoter.  (D) rab-10(dx2) is not rescued by expression of GFP-Ce-RAB-10(T23N) or GFP alone driven by the vha-6 promoter.  Arrowheads indicate punctate endosomes and apical plasma membrane.  Arrows indicate vacuoles with reduced sizes (C) or enlarged sizes (D).  (E) Quantification of vacuoles in rescue experiments.  Rescue efficiency was determined by counting vacuoles in transgenic young adult animals (n =10 each).  Bar, 10 µm.
Figure 8. RAB-10 colocalizes with endosome markers in the intestine. (A-F) Colocalization of GFP-RAB-10 with early endosomal marker RFP-RAB-5. Epi-fluorescent images of the “TOP” focal plane (A-C) show basolateral membranes, whereas images of the “MIDDLE” focal plane (D-F) show the intestine in cross-section. Puncta labeled by both GFP-RAB-10 and RFP-RAB-5 are indicated by arrowheads (A-C) or arrows (D-F). Puncta labeled by GFP-RAB-10 or RFP-RAB-5 only but not both markers are indicated by arrowheads. (G-I) Colocalization of GFP-RAB-10 with early/late endosomal marker RFP-RAB-7. Arrowheads indicate puncta labeled by both GFP-RAB-10 and RFP-RAB-7. (J-L) Colocalization of GFP-RAB-10 with marker RFP-RAB-11. Arrowheads indicate medial puncta labeled by both GFP-RAB-10 and RFP-RAB-11. Arrows indicate apical puncta labeled by RFP-RAB-11. (M-R) Lack of colocalization of GFP-RAB-10 and RFP-RME-1. Arrowheads indicate puncta labeled only by GFP-RAB-10 or RFP-RME-1 and arrows indicate apical intestine labeled by RFP-RME-1. “L” indicates the position of the intestinal lumen. Bar, 10 µm. In each image autofluorescent lysosomes can be seen in all three channels with the strongest signal in blue, whereas GFP appears only in the green channel and RFP only in the red channel. Signals observed in the green or red channels that do not overlap with signals in the blue channel are considered bone fide GFP or RFP signals, respectively. Bar, 10 µm.
Figure 9. RFP-RAB-10 partially colocalizes with a Golgi marker (MANS-GFP) in the intestine. Images of the “TOP” focal plane (A-C) show basolateral membranes and cortex, whereas images of the “MIDDLE” focal plane (D-F) show the intestine in cross-section. Arrowheads indicate overlapping signals generated by MANS-GFP and RFP-RAB-10. Arrows indicate puncta positive for RFP-RAB-10 only. The asterisk (*) indicates the lateral plasma membrane and “L” indicates the position of apical lumen of the intestine. Blue color, intestinal autofluorescent granules. Bar, 10 µm.
Figure 10. *XhoI and EcoRI restriction digestion patterns of plasmids identified in yeast two-hybrid screens of *C. elegans* cDNA library.* About 70 plasmids were isolated from individual yeast colonies which were positive in both Leucine and β-Gal assays. They were classified into 16 subgroups according to their *XhoI/EcoRI* digestion patterns. M, 2-Log DNA ladder (New England BioLabs Inc., Beverly, MA). The asterisk “*” indicates plasmids were sequenced.
Figure 11. Diagrams of protein motifs in each RAB-10-interacting protein. (A) EHBP-1. (B) HUM-2. (C) GCK-2. (D) F52E1.13. (E) Y82E9BR.21. (F) ZK1248.10. (G) F20D1.2. (H) CNT-1. One plasmid representing each subgroup is listed and their coding sequences are aligned together with each full-length protein with detailed protein motifs. Number indicates the position of each protein motif.
Figure 12. Amino acid alignment of CeEHBP-1, human/mouse Ehbp1, and mouse Tangerin B. Sequences of CeEHBP-1 (Wormbase accession no. CE05711), human Ehbp1 (GenBank accession no. AAS48537), mouse Ehbp1 (GenBank accession no. NP_694718), and mouse Tangerin B (GenBank accession no. Q99J97) were compared. The C. elegans and human/mouse proteins share more than 50% amino acid similarity in the N-terminal/C-terminal domains (bold in purple), and central CH (Calponin Homology) domain (bold in red). The NPF motifs in human/mouse Ehbp1 are shown in green, whereas one corresponding NPA in CeEHBP-1 is shown in yellow, and the CAAX (AA, two aliphatic amino acids) box in the C-terminus is shown in blue (Guilherme et al. 2004). In yeast two-hybrid screens, the shortest fragment corresponding to CeEHBP-1 protein (H9 clone) is located in the C-terminal domain (underlined region). Sequence alignment was created and refined using Clustal W 1.83 (EMBL-EBI, UK) followed by Boxshade 3.21 (Swiss Node, EMBnet). Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray. Ce, C. elegans; Hu, Homo sapiens (human); and Mus, Mus musculus (mouse).
Figure 13A. Phylogenetic relations among CeHUM-2 and human/mouse myosin Va, Vb, and Vc.

Sequences of CeHUM-2a (Wormbase accession no. CE35927), human myosin Va (GenBank accession no. Q9Y4I1), myosin Vb (GenBank accession no. Q9ULV0), myosin Vc (GenBank accession no. Q9NQX4), mouse myosin Va (GenBank accession no. Q99104), mouse myosin Vb (GenBank accession no. Q91L70), and mouse myosin Vc (GenBank accession no. Q91L70) were analyzed and the amino acid alignment is listed in Figure 13B. The phylogenetic tree was created using Clustal W 1.83 (EMBL-EBI, UK) in phylogram style, where levels of inferred evolutionary change are proportional to the branch lengths of tree. According to the phylogenetic distances, CeHUM-2 has closer evolution relation to human/mouse myosin Vb and Va, and then to human/mouse myosin Vc. Ce, C. elegans; Hu, Homo sapiens (human); and Mus, Mus musculus (mouse).
Figure 13B. Amino acid alignment of CeHUM-2 and human/mouse myosin Va, Vb, Vc and rabbit Vc. The *C. elegans* and human/mouse proteins are highly conserved in the N-terminal motor domains (bold in yellow) including the ATPase motif (bold in red), calmodulin binding (IQ) domain (bold in green), and C-terminal DIL domain (bold in purple). In yeast two-hybrid screens, the RAB-10-interacting region encoded in the L1 clone is indicated in blue (1177-1372). The peptide sequences of rabbit Vc (GenBank accession no. AAG09237.1) contains only the last 589 amino acids to the C-terminus; whereas two regions required for association with Rab11a are also shown (140-161, bold in red, and 539-554, underlined bold in red, in OryMyoVb sequence) (Lapierre et al. 2001). The sequence alignment was created and refined using Clustal W 1.83 (EMBL-EBI, UK) followed by Boxshade3.21 (Swiss Node, EMBnet). Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray. Ce, *C. elegans*; Hu, *Homo sapiens* (human); Mus, *Mus musculus* (mouse), and Ory, *Oryctolagus cuniculus*, (rabbit).
MusGck-Rab8ip    1 MALLEDSVSQEDGREFRKKRERELQVGCAGTVGDYKADFVTSELAAKIVKLDPDDISSG
HuGck           1 MALLEDSVSQEDGREFRKKRERELQVGCAGTVGDYKADFVTSELAAKIVKLDPDDISSG
CeGCK=2        1 GSA----GKGSGG---GKKRERELQVGCAGTVGDYKADFVTSELAAKIVKLDPDDISSG

MusGck-Rab8ip    60 QITILRECFRRMNTPVAYGIVY-NLRPRLLCMFGSCGGQQGLQCEYTHATPGLEEBRQIAKYCR
HuGck           60 QITILRECFRRMNTPVAYGIVY-NLRPRLLCMFGSCGGQQGLQCEYTHATPGLEEBRQIAKYCR
CeGCK=2        59 QITILRECFRRMNTPVAYGIVY-NLRPRLLCMFGSCGGQQGLQCEYTHATPGLEEBRQIAKYCR

MusGck-Rab8ip    120 ALKGLRHLRQKIQRDGKLYQVIFLQGQGDYKADFVTSELAAKIVKLDPDDISSG
HuGck           120 ALKGLRHLRQKIQRDGKLYQVIFLQGQGDYKADFVTSELAAKIVKLDPDDISSG
CeGCK-2        119 ALKGLRHLRQKIQRDGKLYQVIFLQGQGDYKADFVTSELAAKIVKLDPDDISSG

MusGck-Rab8ip    180 PEVAAVERKGGYNELCDVWALGITAIELGELQPPLFHLHPMRALMLMSKSSFQPPKLDP
HuGck           180 PEVAAVERKGGYNELCDVWALGITAIELGELQPPLFHLHPMRALMLMSKSSFQPPKLDP
CeGCK-2        179 PEVAAVERKGGYNELCDVWALGITAIELGELQPPLFHLHPMRALMLMSKSSFQPPKLDP

MusGck-Rab8ip    240 TRWTQNFHHFLKLALTKNPKKRPTAERLLQ--HPPF--TTQHLPPALLTQLLDKASDPHLGTLS
HuGck           240 TRWTQNFHHFLKLALTKNPKKRPTAERLLQ--HPPF--TTQQLPRALLTQLLDKASDPHLGTPS
CeGCK-2        239 TRWTQNFHHFLKLALTKNPKKRPSPEKLLTSHPFVLGSLARMTRDLLDKVNG---APTD

MusGck-Rab8ip    299 PEDSELETHDMFPDTIHSRSHHGPAERTPSEIQFHQVKFGAPRRKETDPLNEPWEEEWT-
HuGck           299 PEDSELETHDMFPDTIHSRSHHGPAERTPSEIQFHQVKFGAPRRKETDPLNEPWEEEWT-
CeGCK-2        296 PEDSELETHDMFPDTIHSRSHHGPAERTPSEIQFHQVKFGAPRRKETDPLNEPWEEEWT-

MusGck-Rab8ip    358 LLGKEELSGSLLQSVQEALEERSLTIRPALELQELDSPDDAIGTIKRAPFLGLPHTESTS
HuGck           358 LLGKEELSGSLLQSVQEALEERSLTIRPALELQELDSPDDTMGTIKRAPFLGPLPTDPP-
CeGCK-2        352 LLGKEELSGSLLQSVQEALEERSLTIRPALELQELDSPDDTMGTIKRAPFLGPLPTDPP-

MusGck-Rab8ip    418 GDNAQSCSPGTLSAPP-AGPGSPALLPTAWATLKQQEDRERSSCHGLPPTPKVHMGACFS
HuGck           417 AEEPLSSPPGTLPPPP-SGPNSSPLLPTAWATMKQREDPERSSCHGLPPTPKVHMGACFS
CeGCK-2        412 TLRAPRAPPRTLRAAQ---EESDMDDAPSLLTP---DAAPIRIGLPAHLNGLRLDARCLEQLSS

MusGck-Rab8ip    477 KVMDRTELK
HuGck           476 KVMDRTELK
CeGCK-2        472 KVMDRTELK

MusGck-Rab8ip    537 VNNVLLSLSGKSTHIWAHDLPGLFEQRRLQHQAPLSIPTNRITQRIIPRRFALSTKIPDT
HuGck           536 VNNVLLSLSGKSTHIWAHDLPGLFEQRRLQHQAPLSIPTNRITQRIIPRRFALSTKIPDT
CeGCK-2        532 VNNVLLSLSGKSTHIWAHDLPGLFEQRRLQHQAPLSIPTNRITQRIIPRRFALSTKIPDT

MusGck-Rab8ip    597 KGCLQCRVVRN
HuGck           596 KGCLQCRVVRN
CeGCK-2        592 KGCLQCRVVRN

MusGck-Rab8ip    653 AEPLVLDGKELPQVCVGAEGPEGPG
HuGck           652 AEPLVLDGKELPQVCVGAEGPEGPG
CeGCK-2        649 AEPLVLDGKELPQVCVGAEGPEGPG

MusGck-Rab8ip    705 AQQVIQVDRDTILVSFERCVRIVNLQGEPTAALAPELTFDFTIETVVCLQDSVLAFWSHG
HuGck           704 AQQVIQVDRDTILVSFERCVRIVNLQGEPTAALAPELTFDFTIETVVCLQDSVLAFWSHG
CeGCK-2        709 AQQVIQVDRDTILVSFERCVRIVNLQGEPTAALAPELTFDFTIETVVCLQDSVLAFWSHG

MusGck-Rab8ip    765 MQGRSLDTNEVTQEITDETRIFRVLGAHRDIILESIP
HuGck           764 MQGRSLDTNEVTQEITDETRIFRVLGAHRDIILESIP
CeGCK-2        768 MQGRSLDTNEVTQEITDETRIFRVLGAHRDIILESIP

MusGck-Rab8ip    --
HuGck           --
CeGCK-2        828 AG
Figure 14. Amino acid alignment of CeGCK-2, mouse, and human Gck proteins. Sequences of CeGCK-2 (Wormbase accession no. CE07599), Human Gck (GenBank accession no. NP_004570), mouse Gck-Rab8ip (GenBank accession no. Q61161) were compared. The *C. elegans* and human/mouse proteins are highly conserved in the N-terminal kinase domains (bold in green), and C-terminal citron-like domain (bold in red). The region underlined (starting from A(431)PPAGP in MusGck-Rab8ip protein) includes amino acids required for association with mammalian Rab8 (Ren *et al.* 1996). The exact amino acids responsible for association with Rab8 still remain unknown. The sequence alignment was created and refined using *Clustal W 1.83 (EMBL-EBI, UK)* followed by *Boxe-shade3.21 (Swiss Node, EMBnet).* Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray. Ce, *C. elegans*; Hu, *Homo sapiens* (human); and Mus, *Mus musculus* (mouse).
Figure 15A. Phylogenetic relations among CeCNT-1 and human/mouse centaurin β1, β2, and β5. Sequences of CeCNT-1a (Wormbase accession no. CE28116), human centaurin β1 (or called Acap1, for AIP GAP with coiled-coil, ANK repeat, and PH domain), human centaurin β2 (Genbank accession no. NP_055531), centaurin β2 (or called Acap2, Genbank accession no. CAB41450), centaurin β5 (Genbank accession no. CAI23171), mouse centaurin β1 (Genbank accession no. NP_722483), mouse centaurin β2 (Genbank accession no. BAC 97851), and mouse centaurin β5 (Genbank accession no. AAH67016) were aligned, and the amino acid alignment is listed in figure 15B. The phylogenetic tree was created using Clustal W 1.83 (EMBL-EBI, UK) in phylogram style where levels of inferred evolutionary change are proportional to the branch lengths of tree. According to the phylogenetic distances among various subtypes of centaurin, CeHUM-2 has closer evolution relation to human/mouse centaurin β2, human/mouse centaurin β5, and then to human/mouse centaurin β1. Ce, C. elegans; Hu, Homo sapiens (human); and Mus, Mus musculus (mouse).
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Figure 15B. Amino acid alignment of CeCNT-1 and human/mouse centaurin β1, β2, and β5. The *C. elegans* and human/mouse proteins are highly conserved in the N-terminal Bar domain (bold in green), PH domain (bold in yellow), ARF GAP domain (bold in red), and C-terminal Ankyrin repeat domain (bold in purple). Two coil-coil domains (bold in pink) are shown in the N-terminus of Bar domain and PH domain, respectively. Within Bar domain, residues possible for GTPase-binding sites are underlined in red (compared with amino acid sequences of Arfapin2, GeneBank accession no. NP_036534), residues possible involved in tubule formation in the plasma membranes are underlined in blue (compared with the amino acid sequences of Amphiphysin1, GeneBank accession no.NP_001626), and the “PI-PLC X BOX” are underlined in purple (Jackson *et al.* 2000; Habermann 2004). The sequence alignment was created and refined using Clustal W 1.83 (EMBL-EBI, UK) followed by Boxshade3.21 (Swiss Node, EMBnet). Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray. Ce, *C. elegans*; Hu, Homo sapiens (human); Mus, Mus musculus (mouse).
Figure 16A. Amino acid alignment of ZK1248.10 and human/mouse Tbc1-2b proteins. Sequences of ZK1248.10 (Wormbase accession no. CE29155), human Tbc1-2b protein (GenBank accession no. NP_055894), and mouse Tbc1-2b protein (GenBank accession no. NP_919315) were compared. The *C. elegans* and mouse proteins are highly conserved in the N-terminal PH domain (bold in green), and C-terminal RABGAP/TBC domain (bold in red). The sequence alignment was created and refined using *Clustal W 1.83 (EMBL-EBI, UK)* followed by *Boxshade3.21 (Swiss Node, EMBnet)*. Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray. Ce, *C. elegans*; Hu, *Homo sapiens* (human); and Mus, *Mus musculus* (mouse).
Figure 16B. Amino acid alignment of ZK1248.10 and yeast Mdr1 proteins. Sequences of ZK1248.10 (Wormbase accession no. CE29155), yeast Mdr1 protein (GenBank accession no. NP_011614) were compared. The N-terminal PH domain of C. elegans protein is highlighted in bold green; whereas the C-terminal RABGAP/TBC domain is highlighted in bold red. The sequence alignment was created and refined using Clustal W 1.83 (EMBL-EBI, UK) followed by Boxshade3.21 (Swiss Node, EMBnet). Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray. Ce, C. elegans; Yeast, Saccharomyces cerevisiae.
Figure 17A. Amino acid alignment of F20D1.2 and human/mouse RabGap/Tbc1 number 23 proteins. Sequences of F20D1.2 (Wormbase accession no. CE31485), human RabGap/Tbc1- number 23 protein (GenBank accession no. NP_060779), and mouse RabGap/Tbc1- number 23 protein (GenBank accession no. NP_080530) were compared. The C. elegans and human/mouse proteins are highly conserved in the N-terminal RABGAP/TBC domain (bold in red), and central Rhodanese-like domain (bold in green). The sequence alignment was created and refined using Clustal W 1.83 (EMBL-EBI, UK) followed by Boxshade3.21 (Swiss Node, EMBnet). Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray. Ce, C. elegans; Hu, Homo sapiens (human); and Mus, Mus musculus (mouse).
**Figure 17B.**  Amino acid alignment of F20D1.2 and yeast Gyl1 proteins.  Sequences of F20D1.2 (Wormbase accession no. CE31485), yeast Gyl1 protein (GenBank accession no. NP_013917) were compared. The N-terminal RABGAP/TBC domain of *C. elegans* protein is highlighted in bold red; whereas the C-terminal Rhodanese-like domain is highlighted in bold green.  The sequence alignment was created and refined using *Clustal W 1.83* (*EMBL-EBI, UK*) followed by *Boxshade3.21* (*Swiss Node, EMBnet*).  Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray.  Ce, *C. elegans*; Yeast, *Saccharomyces cerevisiae*.
Figure 18. Amino acid alignment of F52E1.13 and human/mouse Oxr1 proteins. Sequences of F52E1.13 (Wormbase accession no. CE33101), human Oxr1 protein (GenBank accession no. NP_851999), and mouse Oxr1 protein (GenBank accession no. NP_570955) were compared. The *C. elegans* and human/mouse proteins are highly conserved in the N-terminal LysM domain (bold in yellow), central Gram domain (bold in green), and C-terminal TLD domain (bold in red). The sequence alignment was created and refined using *Clustal W 1.83 (EMBL-EBI, UK)* followed by *Boxshade3.21 (Swiss Node, EMBnet)*. Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray. Ce, *C. elegans*; Hu, Homo sapiens (human); and Mus, Mus musculus (mouse).
Figure 19A. RAB-10 interacting proteins identified in yeast two-hybrid screens specifically interact with wild-type, GTP-bound, but not GDP-bound RAB-10. (A-D) Interaction of EHBP-1(443-901) or GCK-2 with various forms of RAB-10 by yeast two-hybrid assay. (A-B) β-Galactosidase assay, and (C-D) Leucine assay. (A, C) without galactose-induction, and (B, D) with galactose-induction. Yeast cells EGY48 previously transfected with Lac Z reporter plasmid pSH18-34 were cotransfected with combinations of prey plasmids (ehbp-1(Q4, 443-901) or gck-2(Q1)) in pJG4-5 vector and bait plasmids (rab-10(+), wt; rab-10(Q68L), GTP-bound form; or rab-10(T23N), GDP-bound form) in pEG202 vector. Results of various combinations were summarized in Table 2A and strains with pairs of prey and bait plasmids were listed in Table 3. Prey plasmids Q4 (ehbp-l) and Q1 (gck-2) isolated from yeast colonies were originally identified from C.elegans cDNA library. Plasmid Q4 only encodes EHBP-1 protein partially (the region of amino acid 443 to 901); whereas plasmid Q1 encodes the full length of GCK-2 protein (Figure 11 and Table 1). The “CC” motifs for isoprenylation in the C-terminus of RAB-10(+), RAB-10(Q68L), and RAB-10(T23N) were deleted. Two colonies were picked from each transfection combination followed by stripping onto assay plates. The asterisk “*” indicates a strip colony shows positive results in both Leucine assay (colony strip) and β-Galactosidase assay (blue colony strip).
Figure 19B. continued from above. (A-D) Interaction of HUM-2, F52E1.13, and Y82E9BR.21 with various forms of RAB-10 by yeast two-hybrid assay. (A-B) β-Galactosidase assay, and (C-D) Leucine assay. (A, C) without galactose-induction, and (B, D) with galactose-induction. Yeast cells EGY48 previously transfected with Lac Z reporter plasmid pSH18-34 were cotransfected with combinations of prey plasmids (hum-2(I2, 1179-1387); f52e1.13(M17); or y82e9br.21(B4)) in pJG4-5 vector and bait plasmids (rab-10(+), wt; rab-10(Q68L), GTP-bound form; or rab-10(T23N), GDP-bound form) in pEG202 vector. Results of various combinations were summarized in Table 2A and strains with pairs of prey and bait plasmids were listed in Table 3. The prey plasmids I2 (hum-2), M17 (f52e1.13), and B4 (y82e9br.21) isolated from yeast colonies were originally identified from C. elegans cDNA library. Plasmid I2 only encodes HUM-2 protein partially (the region of amino acid 1179 to 1387). Plasmid M17 was only partially sequenced and plasmid B4 encodes the full length of Y82E9BR.21 protein (Figure 11 and Table 1). The “CC” motifs for isoprenylation in the C-terminus of RAB-10(+), RAB-10(Q68L), and RAB-10(T23N) were deleted. Two colonies were picked from each combination followed by stripping onto the assay plates. The asterisk “*” indicates a colony strip showed positive results in both Leucine assay (colony strip) and β-Galactosidase assay (blue colony strip).
Figure 20A. Phylogenetic relations among C. elegans RAB proteins. Sequences of 27 CeRABs are listed below based on the analysis made by Michael L. Nonet (Worm Breeder’s Gazette 17(2): 20 (April 1, 2002)): RAB-1 (Wormbase accession no. CE16905), RAB-2 (Wormbase accession no. CE10986), RAB-3A (Wormbase accession no. CE23555), RAB-5 (Wormbase accession no. CE09711), RAB-6.1 (Wormbase accession no. CE00234), RAB-6.2 (Wormbase accession no. CE07541), RAB-7 (Wormbase accession no. CE03777), RAB-8 (Wormbase accession no. CE30373), RAB-10 (Wormbase accession no. CE14114), RAB-11.1 (Wormbase accession no. CE11006), RAB-11.2 (Wormbase accession no. CE40145), RAB-14 (Wormbase accession no. CE11974), RAB-18B (Wormbase accession no. CE27340), RAB-19 (Wormbase accession no. CE24543), RAB-21 (Wormbase accession no. CE03587), RAB-27 (Wormbase accession no. CE23130), RAB-28 (Wormbase accession no. CE19030), RAB-30 (Wormbase accession no. CE19165), RAB-33 (Wormbase accession no. CE00992), RAB-35 (Wormbase accession no. CE24336), RAB-37B (Wormbase accession no. CE40139), RAB-39 (Wormbase accession no. CE30340), 4R79.2 (Wormbase accession no. CE19650), K02E10.1 (Wormbase accession no. CE39511), F11A5.4 (Wormbase accession no. CE15782), F11A5.3 (Wormbase accession no. CE15781), C56E6.2 (Wormbase accession no. CE30372), and yeast Sec4 (GenBank accession no. NP_116650.1). Sequences were analyzed and the amino acid alignment is listed in figure 11B. The phylogenetic tree was created using Clustal W 1.83 (EMBL-EBI, UK) in phylogram style where levels of inferred evolutionary change are proportional to the branch lengths of tree. According to the phylogenetic distances among various C. elegans RABs and yeast Sec4 proteins, C. elegans RAB-8, RAB-10, and yeast Sec4 proteins (shaded in gray) have the closest evolution relationships to one another. Ce, C. elegans; Yeast, Saccharomyces cerevisiae.
Figure 20B. Amino acid alignment of *C. elegans* RAB-5, RAB-7, RAB-8, RAB-10, and RAB-35. They are highly conserved in the PM (Phosphate-Magnicium binding)/G (guanine base binding) domains (bold in green), and RabF domains (RabF1-F4, bold in purple). The RAB CDR (Complementary-Determining Region, bold in yellow) and the switch I/II regions (bold in red) may be potential binding domains for various effectors (Valencia et al. 1991; Ostermeier and Brunger 1999; Pereira-Leal and Seabra 2000). The amino acids only similar or identical in RAB-8 and RAB-10 but not in other RABs are labeled bold in blue. The sequence alignment was created and refined using *Clustal W* 1.83 (*EMBL-EBI, UK*) followed by *Boxshade 3.21* (*Swiss Node, EMBnet*). Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray.
Figure 20C. Amino acid alignment of *C. elegans* RAB-8, RAB-10, and yeast Sec4p proteins. The *C. elegans* RAB-8, RAB-10, and yeast Sec4p proteins share more than 90% amino acid identity outside of the hypervariable C-terminal domain. They are highly conserved in the PM (Phosphate-Magnesium binding)/G (guanine base binding) domains (bold in green), and RabF domains (RabF1-F4, bold in purple). The RAB CDR (Complementary-Determining Region, bold in yellow) and the switch I/II regions (bold in red) may be potential binding domains for various effectors (Valencia et al. 1991; Ostermeier and Brunger 1999; Pereira-Leal and Seabra 2000). The sequence alignment was created and refined using *Clustal W 1.83* (EMBL-EBI, UK) followed by *Boxshade3.21* (Swiss Node, EMBnet). Identical amino acids are shaded in black; whereas similar amino acids are shaded in gray. Ce, *C. elegans*; Yeast, *Saccharomyces cerevisiae*. 
Figure 21A. The wild-type and GTP-bound, but not GDP-bound RAB-8 could also interact with RAB-10 interacting proteins. (A-D) Interaction of EHBP-1 or GCK-2 with various forms of RAB-8 by yeast two-hybrid assay. (A-B) β-Galactosidase assay, and (C-D) Leucine assay. (A, C) without galactose-induction, and (B, D) with galactose-induction. Yeast cells EGY48 previously transfected with Lac Z reporter plasmid pSH18-34 were cotransfected with combinations of prey plasmids (ehbp-1(Q4, 443-901); or gck-2(Q1)) in pJG4-5 vector and bait plasmids (rab-8(+), wt; rab-8(Q67L), GTP-bound form; or rab-8(T22N), GDP-bound form) in pEG202 vector. Results of various combinations were summarized in Table 2A and strains with pairs of prey and bait plasmids were listed in Table 3. The prey plasmids Q4 (ehbp-1) and Q1 (gck-2) isolated from yeast colonies were originally identified from C. elegans cDNA library. Plasmid Q4 only partially encodes EHBP-1 protein (the region of amino acid 443 to 901) and plasmid Q1 encodes the full length of GCK-2 protein (Figure 11 and Table 1). The “CNLL” motifs for isoprenylation in the C-terminus of RAB-8(+), RAB-8(Q67L), and RAB-8(T22N) were deleted (by Saumya Pant). Two colonies were picked from each combination followed by stripping onto the assay plates. The asterisk “*” indicates a colony strip shows positive results in both Leucine assay (colony strip) and β-Galactosidase assay (blue colony strip).
Figure 21B. continued from above. (A-D) Interaction of HUM-2, F52E1.13, and Y82E9BR.21 with various forms of RAB-8 by yeast two-hybrid assay. (A-B) β-Galactosidase assay, and (C-D) Leucine assay. (A, C) without galactose-induction, and (B, D) with galactose-induction. Yeast cells EGY48 previously transfected with Lac Z reporter plasmid pSH18-34 were cotransfected with combinations of prey plasmids (hum-2(I2, 1179-1387); f52e1.13(M17); or y82e9br.21(B4)) in pJG4-5 vector and bait plasmids (rab-8(+), wt; rab-8(Q67L), GTP-bound form; or rab-8(T22N), GDP-bound form) in pEG202 vector. Results of various combinations were summarized in Table 2A and strains with pairs of prey and bait plasmids were listed in Table 3. The prey plasmids I2 (hum-2), M17 (f52e1.13), and B4 (y82e9br.21) isolated from yeast colonies were originally identified from C. elegans cDNA library. Plasmid I2 only partially encodes HUM-2 protein (the region of amino acid 1179 to 1387). Plasmid M17 was only partially sequenced and plasmid B4 encodes the full length of Y82E9BR.21 protein (Figure 11 and Table 1). The “CNLL” motifs for isoprenylation in the C-terminus of RAB-8(+), RAB-8(Q67L), and RAB-8(T22N) were deleted. Two colonies were picked from each combination followed by stripping onto the assay plates. The asterisk “*” indicates a colony strip shows positive results in both Leucine assay (colony strip) and β-Galactosidase assay (blue colony strip).
Figure 22A. RAB-10 interacting proteins identified from yeast two-hybrid screens may be specific for RAB-8 and RAB-10. (A-D) Interaction of EHBP-1 or GCK-2 with various GTP-bound RABs by yeast two-hybrid assay. (A-B) β-Galactosidase assay, and (C-D) Leucine assay. (A, C) without galactose-induction, and (B, D) with galactose-induction. Yeast cells EGY48 previously transfected with Lac Z reporter plasmid pSH18-34 were cotransfected with combinations of prey plasmids (ehbp-1(Q4, 443-901); or gck-2(Q1)) in pJG4-5 vector and bait plasmids (rab-5(Q78L); rab-7(Q68L); rab-11(Q70L); or rab-35(Q69L)) in pEG202 vector (by Ken Sato and Miyuki Sato). Results of various combinations were summarized in Table 2A and strains with pairs of prey and bait plasmids were listed in Table 3. The prey plasmids Q4 (ehbp-1) and Q1 (gck-2) isolated from yeast colonies were originally identified from C. elegans cDNA library. Plasmid Q4 only partially encodes EHBP-1 protein (the region of amino acid 443 to 901) and plasmid Q1 encodes the full length of GCK-2 protein (Figure 11 and Table 1). The motifs for isoprenylation in the C-terminal tails of individual RAB were deleted (by Ken Sato and Miyuki Sato). Two colonies were picked from each combination followed by stripping onto the assay plates. The asterisk “*” indicates a colony strip shows positive results in both Leucine assay (colony strip) and β-Galactosidase assay (blue colony strip).
Figure 22B. continued from above. (A-D) Interaction of HUM-2, F52E1.13, and Y82E9BR.21 with various GTP-bound RABs by yeast two-hybrid assay. (A-B) β-Galactosidase assay, and (C-D) Leucine assay. (A, C) without galactose-induction, and (B, D) with galactose-induction. Yeast cells EGY48 previously transfected with Lac Z reporter plasmid pSH18-34 were cotransfected with combinations of prey plasmids (hum-2(I2, 1179-1387); f52e1.13(M17); or y82e9br.21(B4)) in pJG4-5 vector and bait plasmids (rab-5(Q78L); rab-7(Q68L); rab-11(Q70L); or rab-35(Q69L)) in pEG202 vector. Results of various combinations were summarized in Table 2A and strains with pairs of prey and bait plasmids were listed in Table 3. The prey plasmids I2 (hum-2), M17 (f52e1.13), and B4 (y82e9br.21) isolated from yeast colonies were originally identified from C. elegans cDNA library. Plasmid I2 only partially encodes HUM-2 protein (the region of amino acid 1179 to 1387). Plasmid M17 was only partially sequenced and plasmid B4 encodes the full length of Y82E9BR.21 protein (Figure 11 and Table 1). The isoprenylation motifs in the C-terminus of individual RABs were deleted. Two colonies were picked from each combination followed by stripping onto the assay plates. The asterisk “*” indicates a colony strip shows positive results in both Leucine assay (colony strip) and β-Galactosidase assay (blue colony strip).
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Figure 23. Three GAPs are not only specific for RAB-8 and RAB-10. (A-D) Interaction of RAB GAPs F20D1.2, ZK1248.10, or ARF-6 GAP CNT-1 with various GTP-bound RABs by yeast two-hybrid assay. (A-B) β-Galactosidase assay, and (C-D) Leucine assay. (A, C) without galactoase-induction, and (B, D) with galactoase-induction. Yeast cells EGY48 previously transfected with Lac Z reporter plasmid pSH18-34 were cotransfected with combinations of prey plasmids ( f20d1.2(C4); zk1248.10(K1); or cnt-1(N2)) in pJG4-5 vector and bait plasmids (rab-5(Q78L); rab-7(Q68L); rab-8(Q67L); rab-10(Q68L); rab-11(Q70L); or rab-35(Q69L)) in pEG202 vector. Results of various combinations were summarized in Table 2B and strains with pairs of prey and bait plasmids were listed in Table 3. The prey plasmids C4, K1, and N2 were only partially sequenced (Figure 11 and Table 1). The isoprenylation motifs in the C-terminus of individual RAB were deleted. Two colonies were picked from each combination followed by stripping onto the assay plates. The asterisk “*” indicates a colony strip shows positive results in both Leucine assay (colony strip) and β-Galactosidase assay (blue colony strip).
Figure 24A. RME-1 and LIN-10 do not interact with RAB-10-interacting proteins except Y82E9BR.21. (A-D) Interaction of EHBP-1 or GCK-2 with RME-1(EH only), RME-1(full length), or LIN-10 by yeast two-hybrid assay. (A-B) β-Galactosidase assay, and (C-D) Leucine assay. (A, C) without galactose-induction, and (B, D) with galactose-induction. Yeast cells EGY48 previously transfected with Lac Z reporter plasmid pSH18-34 were cotransfected with combinations of prey plasmids (ehbp-1(Q4, 443-901); or gck-2(Q1)) in pJG4-5 vector and bait plasmids (RME-1(EH domain only); RME-1(full-length); or LIN-10) in pEG202 vector (by Saumya Pant). Results of various combinations were summarized in Table 2A and strains with pairs of prey and bait plasmids were listed in Table 3. The prey plasmids Q4 (ehbp-1) and Q1 (gck-2) isolated from yeast colonies were originally identified from C. elegans cDNA library. Plasmid Q4 only partially encodes EHBP-1 protein (the region of amino acid 443 to 901) and plasmid Q1 encodes the full length of GCK-2 protein (Figure 11 and Table 1). Two colonies were picked from each combination followed by stripping onto the assay plates. The asterisk “*” indicates a colony strip shows positive results in both Leucine assay (colony strip) and β-Galactosidase assay (blue colony strip).
Figure 24B. continued from above. (A-D) Interaction of HUM-2, F52E1.13, and Y82E9BR.21 with RME-1(EH only), RME-1(full-length), or LIN-10 by yeast two-hybrid assay. (A-B) β-Galactosidase assay, and (C-D) Leucine assay. (A, C) without galactoase-induction, and (B, D) with galactoase-induction. Yeast cells EGY48 previously transfected with Lac Z reporter plasmid pSH18-34 were cotransfected with combinations of prey plasmids (hum-2(I2, 1179-1387); f52e1.13(M17); or y82e9br.21(B4)) in pJG4-5 vector and bait plasmids (rme-1(EH domain only); rme-1(+); or lin-10(+)) in pEG202 vector. Results of various combinations were summarized in Table 2A and strains with pairs of prey and bait plasmids were listed in Table 3. The prey plasmids I2 (hum-2), M17 (f52e1.13), or B4 (y82e9br.21) isolated from yeast colonies were originally identified from C. elegans cDNA library. Plasmid I2 only partially encodes HUM-2 protein (the region of amino acid 1179 to 1387). Plasmid M17 was only partially sequenced and plasmid B4 encodes the full length of Y82E9BR.21 protein (Figure 11 and Table 1). Two colonies were picked from each combination followed by stripping onto the assay plates. The asterisk ** indicates a colony strip shows positive results in both Leucine assay (colony strip) and β-Galactosidase assa
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Leu/β-gal/-Gal

Leu/β-gal/+Gal

C: Prey

D: Prey

Leu/-Gal

Leu/+Gal
Figure 25. Full-length EHBP-1 does not interact with RAB-8 or RAB-10 and HUM-2 interacts with RAB-11 via a region distinct from that interacting with RAB-10. (A-D) Interaction of full-length EHBP-1 or C-terminal half of HUM-2 with various GTP-bound RABs and RME-1 by yeast two-hybrid assay. (A-B) β-Galactosidase assay, and (C-D) Leucine assay. (A, C) without galactose-induction, and (B, D) with galactose-induction. Yeast cells EGY48 previously transfected with Lac Z reporter plasmid pSH18-34 were cotransfected with combinations of prey plasmids (ehbp-1(1-901); or hum-2(899-1839)) in pJG4-5 vector and bait plasmids (rab-5(Q78L); rab-7(Q68L); rab-8(Q67L); rab-10(Q68L); rab-11(Q70L); rab-35(Q69L); rme-1(EH only); or rme-1(+) in pEG202 vector. Results of various combinations were summarized in Table 2A and strains with pairs of prey and bait plasmids were listed in Table 3. The prey plasmid ehbp-1(1-901) encodes the full length of EHBP-1 protein and plasmid hum-2(899-1839) encodes the C-terminal half of full-length HUM-2 protein (Figure 11 and Table 1). The isoprenylation motifs in the C-terminus of individual RAB were deleted. Two colonies were picked from each combination followed by stripping onto the assay plates. The asterisk “*” indicates a colony strip shows positive results in both Leucine assay (colony strip) and β-Galactosidase assay (blue colony strip).
Figure 26. Diagrams of targeting sites in each gene by their corresponding RNAi clones.

(A) **ehbp-1**, **sjj_F25B3.1**.  
(B) **gck-2**, **sjj_ZC404.9**. (C) **f52e1.13**, **sjj_F52E1.13**. (D) **y82e9br.21**, **L4440-Y82E9BR.21** (home-made construct with full-length cDNA coding sequence of the **y82e9br.21** gene).  
(E) **f20d1.2**, **sjj_F20D1.2**.  
Clone number with initial "**sjj**" means that it was from AJ RNAi library.  
Diagram was adapted from the Wormbase website and modified manually.
Figure 27. Subcellular localization of RME-1 is affected by hum-2 mutants. Confocal images in a wild-type background are shown in the top focal plane (A) and in the middle focal plane (C). Confocal images in a hum-2(ok596) background are shown in the top focal plane (B) and in the middle focal plane (D). Arrowheads indicate punctate, tubular endosomes labeled by GFP-RME-1 in the area close to basal membranes (A-B), or apical membranes (C-D). Arrows indicate punctate, tubular endosomes labeled by GFP-RME-1 in the cortical area of apical membranes or medial cytoplasm (C-D). Quantification of endosome number and size as visualized by the GFP-RME-1 is shown in E and F. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixl^2) box positioned at random). The asterisk “*” means it is statistic significance (p<0.001) compared to wild type animals. Bar, 10 μm.
Figure 28. Subcellular localization of RAB-10 is affected by hum-2 mutants. Confocal images in a wild-type background are shown in the top focal plane (A-B) and in the middle focal plane (E-F). Confocal images in a hum-2(ok596) background are shown in the top focal plane (C-D) and in the middle focal plane (G-H). Images with higher magnification are shown in B, D, F, and H. Arrowheads indicate punctate endosomes labeled by GFP-RAB-10 in the area close to basal membranes (A-D), or apical membranes and medial cytoplasm (E-H). Arrows indicate the "foggy" local gathering of GFP-RAB-10-labeled small vesicles in the cortical area. Quantification of endosome number or size as visualized by the GFP-RAB-10 is shown in I and J. Error bars represent standard deviations from the mean. The asterisk (*) means it is statistically significant (p<0.001) compared to wild-type animals. Bar, 10 μm.
Figure 29. HUM-2 is broadly expressed in *C. elegans*. Expression of a HUM-2-GFP transgene driven by the hum-2 promoter is shown in A, hypodermis (arrows); in B, body wall muscle, arrows indicate the muscle myofilament and arrowheads indicate the dense body; in C, intestine, arrows indicate cytoplasmic puncta along the apical membrane; and in D, pharynx (arrows) and nerve ring (arrowhead). Blue color, intestinal autofluorescent lysosomal granules. Bar, 10 μm.
Figure 30. Depletion of EHBP-1, but not GCK-2, F52E1.13, Y82E9BR.21, or F20D1.2, affects the subcellular localization of RME-1. Confocal images are shown in A, L4440 RNAi control vector; in B, L4440-gck-2; in C, L4440-ehbp-1; in D, L4440-f52e1.13; in E, L4440-y82e9br.21; and in F, L4440-f20d1.2. Arrowheads indicate GFP-RME-1 labeling in the cortical area of basal membranes; whereas arrows indicate GFP-RME-1 labeling on the basolateral membranes. Blue color, intestinal autofluorescent lysosomal granules. Quantification of endosome number or size as visualized by the GFP-RME-1 is shown in G and H. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixel) box positioned at random). The asterisk (*) means it is statistic significance (p<0.001) compared to wild type animals. Bar, 10 μm.
Figure 31. Depletion of EHBP-1, but not GCK-2, F52E1.13, Y82E9BR.21, or F20D1.2, affects the subcellular localization of RAB-10.

Confocal images are shown in A, L4440 RNAi control vector; in B, L4440-gck-2; in C, L4440-ehbp-1; in D, L4440-f52e1.13; in E, L4440-y82e9br.21; and in F, L4440-f20d1.2. Arrowheads indicate GFP-RAB-10-labeled endosomes; whereas arrows indicate cytosolic diffusion of GFP-RAB-10 in animals treated with ehbp-1(-) RNAi (C). Quantification of endosome number or size as visualized by the GFP-RAB-10 is shown in G and H. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixel²) box positioned at random). The asterisk (***) means it is statistically significant (p<0.001) compared to wild type animals. Bar, 5 μm.
Figure 32. Tissue and intestine-specific expression of GCK-2 in *C. elegans*. (A-C) Epi-fluorescence images of tissue-specific expression of a GCK-2-GFP transgene driven by the *gck-2* promoter. (A) Pharynx (arrows) and nerve ring (arrowhead). (B) Ventral nerve cord (arrows) and cell body (arrowheads). (C) Intestine, labeling of GCK-2-GFP on apical membranes (arrows) and medial cytoplasmic puncta (arrowheads). (D-F) Epi-fluorescence images of expression of a GFP-GCK-2 transgene driven by the intestine-specific promoter *vha-6*. (D) Image of top focal plane shows labeling of GFP-GCK-2 on the lateral membranes (arrows) and intestinal vacuoles (arrowheads). (E) Image of middle focal plane shows labeling of GFP-GCK-2 on the lateral membranes (arrows) and intestinal vacuoles (arrowheads). (F) Nomarski image of an intestinal vacuole observed in animals with overexpression of GFP-GCK-2. Blue color, intestinal autofluorescent lysosomal granules. Arrow indicates the intestinal vacuole. Bar, 10 μm.
The effect of Eph-RNAi on the intracellular expression of GFP-EHBP-1.

% of GFP-EHBP-1 positive animals (%)
Figure 33. Efficient knockdown of ehbp-1 by RNAi feeding. (A and D) Nomarski images of the intestines in the wild-type (L4440) and ehbp-1 RNAi (L4440-ehbp-1(-)) animals. (B and E) Epi-fluorescent images of GFP-EHBP-1 expression in intestines of the wild-type (L4440) and ehbp-1 RNAi (L4440-ehbp-1(-)) animals. Arrowheads indicate the intestinal expression of GFP-EHBP-1. (C and F) Epi-fluorescent images of intestinal autofluorescent lysosomal granules in the wild-type (L4440) and ehbp-1 RNAi (L4440-ehbp-1(-)) animals. Arrowsheads indicate the intestinal expression of GFP-EHBP-1. For each group, 100 animals with normal locomotion (UNC-119(+), GFP-EHBP-1(+)) were examined for intestinal expression of GFP-EHBP-1-positive expression is shown in Fig. 33C and F. Quantification analysis of percentage of animals with GFP-EHBP-1-positive expression is shown in Fig. 33I.
Figure 34. *ehbp-1* (RNAi) animals display *rab-10*-like endocytosis defects in the *C. elegans* intestine. (A-B) Nomarski images of the intestine in wild type (*L4440*) and *ehbp-1* RNAi (*L4440-ehbp-1(-)) animals. No large transparent vacuoles were observed in the intestines of wild type animals (A). Very large transparent vacuoles were found in the intestines of *ehbp-1* RNAi animals (B). Arrows indicate the positions of vacuoles. (C-D) Intestinal endocytosis of the basolateral recycling marker ssGFP in the wild-type and *ehbp-1* RNAi animals. In wild type animals, very little secreted GFP accumulates in the intestinal endosomes because of efficient recycling back to the body cavity (C). The *L4440-ehbp-1(-)-specific* vacuoles accumulated basolaterally endocytosed GFP (D, arrows). A *cup-4(arm494)* mutation was also included in the strain shown in C and D to impair coelomocyte function and to increase steady-state levels of secreted GFP in the body cavity. (E-J) Confocal images of the worm intestine expressing GFP-tagged endocytic transmembrane cargo markers in wild type and *ehbp-1* RNAi animals. In wild type animals (*L4440* only), the basolateral membranes and basolateral endocytic compartments are labeled by hTAC-GFP (E and G, arrows) and hTfR-GFP (human transferrin receptor; I). In *ehbp-1* RNAi animals, loss of labeling by hTAC-GFP on basolateral membranes (F and H) but both transmembrane cargo markers accumulate in the enlarged endosomes: hTAC-GFP (H), hTfR-GFP (J), respectively. Arrows indicate the basolateral membranes in wild type animals (E and G), missing of labeling on basolateral membranes (F and H) and enlarged endosomes in *ehbp-1* RNAi worms (H and J), respectively. (K-L) Endocytosis of YP170-GFP by the oocytes of adult hermaphrodites. In wild type animals, YP170-GFP is secreted by intestine and endocytosed efficiently by oocytes (K). In the *ehbp-1* RNAi animals, the secretion of YP170-GFP by the intestine and endocytosis of YP170-GFP by oocytes are normal (L). Arrows indicate the YP170-GFP accumulation in the oocytes and embryos. “OO”, oocyte; “SP”, spermatheca; “Emb”, embryo. (M-N) Endocytosis of the fluid-phase marker ssGFP by coelomocytes in wild type and *ehbp-1* RNAi animals. In wild type animals, ssGFP is secreted by muscle cells into body cavity and mostly is endocytosed by coelomocytes (M). In *ehbp-1* RNAi worms, the secretion of GFP by muscle and the endocytosis of GFP by coelomocytes are normal (N). Arrows indicate the intracellular vesicles. Bar, 10 μm.
Figure 35. *ehbp-1* RNAi results in a dramatic increase in RAB-5-positive early endosomes and with a loss of RME-1-positive recycling endosomes. Confocal images in a wild-type background are shown for GFP-RAB-5 (A), GFP-RAB-7 (C), GFP-RAB-8 (E and G), GFP-RAB-10 (I and K), GFP-RAB-11 (M), and GFP-RME-1 (O and Q). Confocal images in the *ehbp-1* RNAi animals are shown for GFP-RAB-5 (B), GFP-RAB-7 (D), GFP-RAB-8 (F and H), GFP-RAB-10 (J and L), GFP-RAB-11 (N), and GFP-RME-1 (P and R). Arrowheads indicate punctate, tubular, or endosomes labeled by GFP-RAB-5, GFP-RAB-7, GFP-RAB-8, GFP-RAB-10, GFP-RAB-11, and GFP-RME-1 in the apical, cytosolic, lateral, or basolateral compartments. Arrows indicate enlarged intestinal endosomes (vacuoles) labeled by GFP-RAB-5 (B), GFP-RAB-7 (D), and GFP-RAB-8 (F and H). Quantification of endosome number and size as visualized by the markers is shown in S and T. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixel²) box positioned at random). The asterisk “∗” indicates comparison between *ehbp-1* RNAi and control animals (*L4440* only) is statistically significant (p<0.001). Bar, 10µm.
Figure 36. *ehbp-1* RNAi-induced intestinal vacuoles labeled by transmembrane cargo hTAC-GFP are enlarged RAB-5-positive early endosomes. Arrows indicate enlarged intestinal vacuoles labeled by both hTAC-GFP and RFP-RAB-5. Blue color, intestinal autofluorescent lysosomal granules. Bar, 10 μm.
Figure 37. Loss of EHBP-1 function affects the subcellular localization of ACT-5-GFP-labeled compartments in the intestine.

(A and D) Confocal images of subcellular localization of ACT-5-GFP-labeled compartments in the intestine of wild type (L4440 only) animals. (B-F) Confocal images of subcellular localization of ACT-5-GFP-labeled compartments in the intestine of ehbp-1 RNAi animals. Higher magnification of images on the ACT-5-GFP-labeled intestinal vacuoles in ehbp-1 RNAi animals are shown in C and F. Arrows indicate the ACT-5-GFP-labeled apical, basolateral membranes (A and D, B and E) and intestinal vacuoles without actin-fiber accumulation (C and F). Arrowheads indicates cytoplasmic puncta (A and D). Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixl2) box positioned at random). The asterisk "*" indicates the comparison between ehbp-1 RNAi and control animals is statistically significant (p<0.001).
Figure 38. Loss of ehbp-1 function decreases the accumulation of ACT-5 on the enlarged endosomes of rab-10 mutants. (A and D) Nomarski images of the intestine in the control (L4440) and control (L4440-ehbp-1(-)) animals in rab-10 (q373) mutant background. ehbp-1 RNAi had no affection on the formation of endosomes in rab-10 mutants. Arrows indicate the location of intestinal vacuoles found in rab-10 mutants. (B) and (E) Confocal images of the ACT-5-GFP-labeled cytoplasmic puncta and small vesicles. Bar, 10 μm. Images with higher magnification are shown in C and F. Arrows indicate the ACT-5-GFP-labeled apical, basolateral membranes, and vacuoles with accumulated actin fibers. Arrowheads indicate cytoplasmic puncta and small vesicles. Bar, 1 μm. Quantification of endosomes number and size as visualized by the ACT-5-GFP is shown in I and J. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 pixel box positioned at random). The asterisk "*" indicates the comparison between ehbp-1 RNAi and control (100 %) animals is statistically significant (p<0.001).
Figure 39. EHBP-1 is broadly expressed in *C. elegans*. (A-F) Confocal images of expression of the EHBP-1-GFP transgene driven by the *ehbp-1* promoter. Tissue-specific expression was observed in A, pharynx (arrows), nerve ring and ventral nerve cord (arrowheads); B, myofilament of body-wall muscle (arrows); C, seam cells (arrow); D, intestine (Top); E, intestine (Middle); F, intestine (Bottom). Blue color, intestinal autofluorescent lysosomal granules. Bar, 10 μm.

(A) Middle cross-sections of the intestine, where arrows indicate the basal intestinal membranes and arrowheads the apical intestinal membranes. (B) Subcellular localization of GFP-EHBP-1 was shown in a GFP-EHBP-1 transgene driven by the intestine-specific *vha-6* promoter. Subcellular localization of GFP-EHBP-1 was shown in G, middle cross-sections of the intestine, where arrowheads indicate the apical intestinal membranes and arrows indicate the basal intestinal membranes. (C) Blue color, intestinal autofluorescent lysosomal granules. Bar, 10 μm.
Figure 40. EHBP-1 is associated with endosomes but not Golgi in the intestine.
Images of the “Top” focal plane show basolateral membranes, whereas images of the “Middle” focal plane show the intestine in cross-section. (A1-B3) Epi-fluorescence images of colocalization of GFP-EHBP-1 with early endosomal marker RFP-RAB-5. Colocalization with both GFP-EHBP-1 and RFP-RAB-5 in puncta or partially in fiber-like structures in the cortical area close to basolateral membranes (A1-A3) or in apical or lateral membranes (B1-B3) is indicated by arrowheads, whereas it labeled by either GFP-EHBP-1 or RFP-RAB-5 only but not by both markers are indicated by arrows. (C1-D3) Epi-fluorescence images of colocalization of GFP-EHBP-1 with endosomal marker RFP-RAB-8. Arrowheads indicate puncta and fiber-like structures labeled by both GFP-EHBP-1 and RFP-RAB-8. (E1-F3) Epi-fluorescence images of colocalization of GFP-EHBP-1 with marker RFP-RAB-10. Colocalization with both GFP-EHBP-1 and RFP-RAB-10 in the cortical area near the basal or lateral membranes (E1-E3), or in the apical or lateral PM (F1-F3) is indicated by arrowheads. (G1-H3) Lack of colocalization of GFP-EHBP-1 and RFP-RAB-11. Arrows indicate puncta labeled only by GFP-EHBP-1 or RFP-RAB-11. (I1-J3) Epi-fluorescence images of colocalization of GFP-EHBP-1 with basolateral recycling marker RFP-RME-1. Arrowheads indicate puncta and fiber-like structures labeled by both GFP-EHBP-1 and RFP-RME-1, whereas labeled by either GFP-EHBP-1 or RFP-RME-1 only but not by both markers are indicated by arrows. (K1-K3) Lack of colocalization of GFP-EHBP-1 and TGN marker MANS-RFP. Arrows indicate puncta or fiber-like structures labeled only by GFP-EHBP-1 but not by MANS-RFP. “L” indicates the position of the intestinal lumen. Blue color, intestinal autofluorescent lysosomal granules. Bar, 10 µm.
Figure 4. Overexpression of GFP-EHBP-1 induces tubulation of endosomes. Bar, 10 µm.

Blue color, intestinal autofluorescence; lysosomal granules. Arrowheads indicate colocalized fibers and puncta. Expression of RFP-RAB-8 alone is shown in A, whereas it co-expressed with GFP-EHBP-1 is shown in B. The co-expressed GFP-EHBP-1 is shown in C and the merged image is shown in D. Arrowheads indicate colocalized fibers and puncta. Expression of RFP-RAB-10 alone is shown in E, whereas it co-expressed with GFP-EHBP-1 is shown in F. The co-expressed GFP-EHBP-1 is shown in G and the merged image is shown in H. Arrowheads indicate colocalized fibers and puncta. Blue color, intestinal autofluorescence.
EHBP-1-GFP
(ehbp-1p; A-B)

rab-10 (q373);
EHBP-1-GFP
(ehbp-1p; C-D)

GFP-EHBP-1
(vha-6p; E-F)

rab-10 (q373);
GFP-EHBP-1
(vha-6p; G-L)
Figure 42. EHBP-1 labels the enlarged endosomes of *rab-10* mutants. (A-D) Accumulation of EHBP-1-GFP-labeled tubule/fiber-like structures on the limiting membranes of intestinal vacuoles in adult *rab-10* (q373) mutant animals. Expression of an EHBP-1-GFP transgene driven by the *ehbp-1* promoter in the wild type background is shown in A or B; whereas expression in the *rab-10* (q373) mutant background is shown in C or D. Arrows indicate the EHBP-1-GFP-labeled apical or basolateral membranes (A-B), or accumulation of tubule/fiber-like on the limiting membranes of intestinal vacuoles (C-D). Arrowheads indicate EHBP-1-GFP-labeled puncta along basolateral membranes or in the cytoplasm. (E-L) Accumulation of GFP-EHBP-1-labeled puncta and tubule/fiber-like structures on the limiting membranes of intestinal vacuoles in *rab-10* (q373) mutants. Expression of a GFP-EHBP-1 transgene driven by the *vha-6* promoter in the wild-type background is shown in E and F, whereas expression in the *rab-10* (q373) mutant background is shown in G to L. Arrows indicate GFP-EHBP-1-labeled apical and basolateral membranes in F, tubule/fiber-like structures labeled by GFP-EHBP-1 in E, G, and H, and large accumulation of GFP-EHBP-1 in lateral membranes in K. Arrowheads indicate GFP-EHBP-1-labeled puncta along the basolateral membranes or in the cortical area close to it (E and F), small vesicles or vacuoles with local gathering of puncta in the cortical area near basal membranes (G-J) or in the medial cytoplasm (K-L). “L” indicates the position of the intestinal lumen. Blue color, intestinal autofluorescent lysosomal granules. Bar, 10 µm.
Figure 43. Loss of RME-1 function disrupts formation of GFP-EHBP-1-labeled tubules and changes the subcellular localization of GFP-EHBP-1.

(A-C) Epi-fluorescence images of intestines in the rme-1(b1045) mutant animals with an EHBP-1-GFP transgene driven by the ehbp-1 promoter. Image taken from the cortical area close to the basal membrane is shown in A (Top), image with higher magnification is shown in B, and image taken from a cross-section of the intestine is shown in C (Middle). Arrows indicate accumulated GFP-labeled EHBP-1-labeled tubular puncta on the limiting membranes of intestinal vacuoles (A and C), and on the basolateral membranes (D and F). Arrowheads indicate the accumulation of cytosolic GFP-labeled EHBP-1-labeled tubules (A and C). L arrows indicate accumulated GFP-labeled EHBP-1-labeled tubular puncta on the limiting membranes of intestinal vacuoles (A and C), and on the basolateral membranes (D and F). Arrowheads indicate the accumulation of cytosolic GFP-labeled EHBP-1-labeled tubules (A and C). L indicates the position of the intestinal lumen. Blue color, intestinal autofluorescent lysosomal granules. Bar, 10 µm.

(D-F) Epi-fluorescence images of intestines in the rme-1(b1045) mutant animals with a GFP-EHBP-1 transgene driven by the intestine-specific vha-6 promoter. Image taken from the cortical area close to basal membrane is shown in D (Top), image with higher magnification is shown in E, and image taken from a cross-section of the intestine is shown in F (Middle). Arrows indicate accumulated GFP-labeled EHBP-1-labeled tubular puncta on the limiting membranes of intestinal vacuoles (A and C), and on the basolateral membranes (D and F). Arrowheads indicate the accumulation of cytosolic GFP-labeled EHBP-1-labeled tubules (A, B, D, and F).
Figure 44. A working model of EHB-1/RAB-10/HUM-2 in endocytic recycling.

Vesicle Movement
Endocytic Recycling

Membranes of Endosomes
Early

EHB-1
EHP-1

GTP

HUM-2

RAB-10

GDP

Cytosol

ACT-5

GNEF
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*Scale bars in panels B, D, F, and H are 10 μm.*
Figure 45. *rme-1* mutants do not cause accumulation early endosomes labeled by GFP-RAB-5, GFP-7. Confocal images in wild-type background are shown for GFP-RAB-5 (A), GFP-RAB-7 (C), GFP-RAB-10 (E), or GFP-RAB-11 (G). Confocal images in *rme-1(b1045)* background are shown for GFP-RAB-5 (B), GFP-RAB-7 (D), GFP-RAB10 (F), or GFP-RAB-11 (H). Arrowheads indicate punctate endosomes labeled by GFP-RAB-5, GFP-RAB-7, GFP-RAB-10, and GFP-RAB-11 in the apical, cytosolic, lateral, or basolateral compartments. Arrows indicate enlarged intestinal endosomes (vacuoles) not labeled by GFP-RAB-5 (B) or GFP-RAB-7 (D). Bar, 10 μm.
Figure 46. *rab-10* mutant-specific enlarged intestinal vacuoles labeled by transmembrane cargo hTfR-GFP or LMP-1-GFP are also RFP-RAB-5-positive endosomes. (A-F) Epi-fluorescence images of colocalization of RFP-RAB-5 and hTfR-GFP are shown on the limiting membranes of intestinal vacuoles in *rab-10(q373)* mutants. (G-L) Epi-fluorescence images of colocalization of RFP-RAB-5 and LMP-1-GFP are shown on the limiting membranes of intestinal vacuoles in *rab-10(q373)* mutants. Images in higher magnification are shown in D-F and J-L. Arrows indicate enlarged intestinal vacuoles labeled by RFP-RAB-5/hTfR-GFP or RFP-RAB-5/LMP-1-GFP. Blue color indicates autofluorescent intestinal granules. Bar, 10 µm.
Figure 47. Rab-10 mutants accumulate abundant endosomes labeled by GFP-RAB-8.

(A-E) Confocal images of GFP-RAB-8 in a wild-type or gum-1 mutant animals. Images of GFP-RAB-8 taken from "Top" or "Middle" focal planes in the wild-type intestine are shown in A or C; whereas images of GFP-RAB-8 taken from "Top" or "Middle" focal planes in the mutant intestine are shown in B or D. (E) Confocal image of intestinal vacuole labeled by GFP-RAB-8. Arrowheads indicate puncta or small vesicles labeled by GFP-RAB-8. Arrows indicate the intestinal vacuoles labeled by GFP-RAB-8.

Quantification of endosome number and size as visualized by the GFP-RAB-8 is shown in E and F. Error bars represent standard deviations from the mean in each of 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixl²) box positioned at random. The asterisk (*) indicates comparison between wild-type and mutant animals is statistically significant (p<0.001).

Bar, 10 μm.
Figure 48. *rme-1* mutants also cause accumulation of GFP-RAB-8-labeled subcellular compartments. (A-D) Confocal images of GFP-RAB-8 in a wild-type or *rme-1(b1045)* mutant animals. Images of GFP-RAB-8 taken from “Top” or “Middle” focal planes in the wild-type intestine are shown in A or C; whereas images of GFP-RAB-8 taken from “Top” or “Middle” focal planes in the *rme-1(b1045)* mutant intestine are shown in B or D. Arrowheads indicate puncta, small vesicles, or intestinal patches labeled by GFP-RAB-8. Quantification of endosome number and size as visualized by the GFP-RAB-8 is shown in E and F. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixl²) box positioned at random). The asterisk (*) indicates the comparison between wild-type and mutant animals is statistically significant (p<0.001). Bar, 10 μm.
**Figure 49.** RAB-5, RAB-7, and RAB-11 but not MANS can colocalize to a compartment near the basal plasma membranes of intestinal cells. (A-F) Epi-fluorescent images of colocalization of GFP-RAB-5 with early/late endosomal marker RFP-RAB-7. Puncta labeled by both GFP-RAB-5 and RFP-RAB-7 are indicated by arrowheads; whereas puncta labeled by GFP-RAB-5 or RFP-RAB-7 only but not both markers are indicated by arrows. (G-L) Epi-fluorescent images of colocalization of GFP-RAB-5 with apical recycling endosome/Golgi marker RFP-RAB-11. Puncta labeled by both GFP-RAB-5 and RFP-RAB-11 are indicated by arrowheads; whereas puncta labeled by GFP-RAB-5 or RFP-RAB-7 only but not both markers are indicated by arrows. (M-R) Epi-fluorescent images of colocalization of GFP-RAB-5 with Golgi marker MANS-RFP. Arrowheads indicate puncta labeled only by GFP-RAB-5 or MANS-RFP. Images of the “Top” focal plane show basolateral membranes; whereas images of the “Middle” focal plane show the intestine in cross-section. Blue color, intestinal autofluorescent granules. “L” indicates the position of the intestinal lumen. Bar, 10 µm.
Figure 50. RAB-8 is associated with endosomes and Golgi in a similar manner of RAB-10. (A-F) Epi-fluorescent images of colocalization of GFP-RAB-8 with early endosomal marker RFP-RAB-5. Puncta labeled by both GFP-RAB-8 and RFP-RAB-5 are indicated by arrowheads; whereas puncta labeled by GFP-RAB-8 or RFP-RAB-5 only but not both markers are indicated by arrows. (G-L) Epi-fluorescent images of colocalization of GFP-RAB-10 with RFP-RAB-8. Puncta labeled by both GFP-RAB-10 and RFP-RAB-8 are indicated by arrowheads; whereas puncta labeled by GFP-RAB-10 or RFP-RAB-8 only but not both markers are indicated by arrows. (M-R) Epi-fluorescent images of colocalization of GFP-RAB-8 with Golgi marker MANS-RFP. Arrowheads indicate puncta labeled by both GFP-RAB-8 and RFP-markers; whereas arrows indicate puncta labeled only by GFP-RAB-8 or RFP-markers but not by both. Images of the “Top” focal plane show the areas near the basal membranes; whereas images of the “Middle” focal plane show the intestine in cross-section. “L” indicates the position of the intestinal lumen. Blue color, intestinal autofluorescent granules. Bar, 10 µm.
Figure 51. GFP or RFP-tagged RAB endosomal markers have similar subcellular localizations. (A-C) Epi-fluorescent images of colocalization of GFP-tagged with RFP-tagged RAB-5. Puncta labeled by both GFP- and RFP-tagged RAB-5 are indicated by arrowheads. (D-F) Epi-fluorescent images of colocalization of GFP-tagged with RFP-tagged RAB-10. Puncta labeled by both GFP- and RFP-tagged RAB-10 are indicated by arrowheads. Blue color, intestinal autofluorescent granules. Bar, 10 µm.
Figure 52. Intestinal subcellular localization of Golgi marker MANS-GFP is not affected by rab-10 mutants.

(A-C) Confocal images of MANS-GFP in the wild-type or rab-10(q373) mutant animals. Images of MANS-GFP taken from the wild-type intestine are shown in A; whereas images of MANS-GFP taken from the rab-10(q373) mutant intestine are shown in B. Nomarski images of MANS-GFP in the wild-type or rab-10(q373) mutant animals are shown in C. Arrowheads indicate the positions of intestinal vacuoles labeled by MANS-GFP in A and B. Arrows indicate the intestinal vacuoles not labeled by MANS-GFP in B and the position of intestinal vacuoles in C. Quantification of puncta number and size as visualized by the MANS-GFP is shown in D and E. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixel²) box positioned at random). Bar, 10 μm.
Figure 53. Intestinal subcellular localization of apical transmembrane cargo OPT-2-GFP is affected by rab-10 mutants.

(A-F) Confocal images of OPT-2-GFP in the intestine of a wild-type or rab-10 (q373) mutant animal. Confocal images of OPT-2-GFP taken from the wild-type intestine are shown in A and D; whereas images of OPT-2-GFP taken from the rab-10 (q373) mutant intestine are shown in B and E, C and F. Images with higher magnification are shown in D and E. Nomarski image indicating the positions of intestinal vacuoles is shown in C. Arrowheads indicate intestinal large patches or ring-like structures labeled by OPT-2-GFP; whereas arrows indicate the positions of intestinal vacuoles in C and OPT-2-GFP-labeled vacuoles in F, respectively.

Quantification of puncta and foci number and size as visualized by the OPT-2-GFP is shown in G and H. Error bars represent standard deviations from the mean (n = 24 each; 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixel$^2$) box positioned at random). The asterisk (*) indicates the comparison between wild-type and mutant animals is statistically significant (p<0.001). Bar, 10 μm.
Figure 54. Negative control: intestinal expression of GFP shows cytosolic diffusion and no labeling on the limiting membrane of intestinal vacuoles in *rab-10* or *rme-1* mutants. (A-D) Confocal images of intestinal GFP expression taken from the wild-type, *rab-10(q373)*, or *rme-1(b1045)* mutants. Arrows show GFP-labeling in the areas near basal membranes or nucleus of wild-type intestines in A and B; whereas intestinal vacuoles without GFP-labeling on the limiting surfaces in *rab-10(q373)* or *rme-1(b1045)* mutants are shown in C and D. Bar, 10 μm.
Figure 55. The actin-cytoskeleton may be involved in the basolateral endocytic recycling in the *C. elegans* intestine. (A-H) *rab-10(q373)* mutant or *rme-1(b1045)* mutant causes accumulation of endosomes labeled by ACT-5-GFP. Confocal images of ACT-5-GFP in a wild-type background are shown in A and E; in the *rme-1(b1045)* mutant background are shown in B and F; in the *rab-10(q373)* mutant background are shown in C and G; and in the *rme-1(b1045)* mutant background are shown in D and H. Arrows show apical and lateral labeling of ACT-5-GFP in a wild-type background. Arrows show apical and lateral labeling of ACT-5-GFP in *rme-1(b1045)* mutant background. Arrows show apical and lateral labeling of ACT-5-GFP in *rab-10(q373)* mutant background. Arrowheads show ACT-5-GFP-labeled cytosolic puncta in A and E, show accumulation of ACT-5-GFP-labeled cytosolic puncta in *rab-10(q373)* mutant in B and F, C and G, and show labeled puncta in *rme-1(b1045)* mutant in D and H. Accumulation of endosomes labeled by ACT-5-GFP is shown in A and E. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 pixl^2^ box). The asterisk (*) indicates the comparison between wild-type and mutant animals is statistically significant (p<0.001). Bar, 10 μm.
Figure 56. Different effects on the subcellular localization of syndapin-GFP are observed in the intestine of rab-10 and rme-1 mutants. Confocal images of Syndapin-GFP (SDNP-1-GFP) in a wild-type background are shown in A and D, in rab-10(q373) mutant are shown in B and E, and in rme-1(b1045) mutant are shown in C and F. Arrowheads show basal and lateral labeling of SDNP-1-GFP-labeled patches or puncta adjacent to small vacuoles in rme-1(b1045) mutant in C, or labeling onto the limiting surfaces of intestinal vacuoles for rab-10(q373) mutant in E. Quantification of endosomes number and size as visualized by the surface of intestinal vacuoles for rab-10(q373) or rme-1(b1045) mutants are shown in G and H. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixel$^2$) box positioned at random). The asterisk (*) indicates the comparison between wild-type and mutant animals is statistically significant (p<0.001). Bar, 10 μm.
Figure 57. **Diagram of the deletion site in the arf-6(tm1447) null mutant.** Gene body of *arf-6* is labeled in purple. A 2263bp deletion region in the *arf-6(tm1447)* mutant is labeled in red. Diagram was adapted from the Wormbase website and modified manually.
Figure 58. Basolateral recycling endosomes labeled by GFP-RME-1 and Syndapin-GFP are affected by arf-6 mutants. (A-D) Confocal images of GFP-RME-1 in the wild-type or arf-6(tm1447) mutant animals. Images of GFP-RME-1 taken from wild-type intestine are shown in A and C; whereas images of GFP-RME-1 taken from the arf-6(tm1447) mutant intestine are shown in B and D. (E-H) Confocal images of Syndapin-GFP in wild-type or arf-6(tm1447) mutant animals. Images of Syndapin-GFP taken from wild-type intestine are shown in E and G; whereas images of Syndapin-GFP taken from the arf-6(tm1447) mutant intestine are shown in F and H. Arrowheads indicate intestinal tubular puncta labeled by GFP-RME-1 or Syndapin-GFP in the cortical areas of basal membranes; whereas arrows indicate the GFP-tagged labeling of GFP-RME-1 or Syndapin-GFP in apical membranes. Quantification of puncta number and size as visualized by the GFP-RME-1 or Syndapin-GFP is shown in I and J. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixel²) box positioned at random). The asterisk (*) indicates the comparison between wild type and mutant animals is statistically significant (p<0.001). Bar, 10 μm.
Figure 59. The subcellular localization of GFP-RAB-10 is not affected in arf-6 mutants. (A-D) Confocal images of GFP-RAB-10 in wild-type or arf-6(tm1447) mutant animals. Images of GFP-RAB-10 taken from wild-type intestine are shown in A and C; whereas images of GFP-RAB-10 taken from arf-6(tm1447) mutant intestines are shown in B and D. Arrowheads indicate intestinal puncta labeled by GFP-RAB-10 in the cortical areas of basal membranes (A-B) or in the medial cytoplasm (C-D). Quantification of puncta number and size as visualized by GFP-RAB-10 is shown in E and F. Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixl^2) box positioned at random). Bar, 10 μm.
Figure 60. The subcellular localization of ACT-5-GFP is affected in arf-6 mutants. (A-F) Confocal images of ACT-5-GFP in wild-type or arf-6(tm1447) mutant animals. Images of ACT-5-GFP taken from the wild-type intestine are shown in A and D; whereas images of ACT-5-GFP taken from arf-6(tm1447) mutants are shown in B and E, C and F. Arrows indicate labeling of ACT-5-GFP in lateral membranes (A, C, D), in enriched labeling on lateral membrane (B), in apical plasma membranes (D), and in cytosolic small vacuoles (F). Arrowheads indicate ACT-5-GFP-labeled cytosolic puncta (A, F), and accumulation of ACT-5-GFP. Quantification of endosomes number and size as visualized by labeled fibers on the surfaces of small intestinal vacuoles (C and F). The asterisk (*) indicates the comparison between wild type and mutant animals is statistically significant (p<0.001). Bar, 10 μm.
Figure 61. LIN-10 is broadly expressed in *C. elegans*. (A-J) Epi-fluorescent images of expression of *lin-10p-LIN-10-GFP* transgene. Expression of the LIN-10-GFP transgene driven by the *lin-10* promoter (7 kb upstream of *lin-10* gene) is indicated in A. Puncta in pharynx (arrows) and nerve ring (arrowheads), in B, puncta in the ventral nerve cord (arrowheads), in C, body-wall muscle where the dense body along the muscle myofilament is indicated (arrowheads), in D, cell body of tail touch neuron (arrows) and cytosolic puncta (arrowheads), in E, spermatheca (arrowheads), in F, puncta in hypodermis (arrows), in G, coelomocyte where intracellular vesicles and puncta are indicated (arrows and arrowheads), in H, vulva (arrows), and in I and J, intestine (top and middle focal planes), where arrows indicate intestinal autofluorescent granules and puncta are indicated cytoplasmic puncta labeled by LIN-10-GFP. (K-L) Epi-fluorescent images of expression of *vha-6p-LIN-10-GFP* transgene in the intestine. Expression of LIN-10-GFP in the cortical areas of basal plasma membranes is shown in K (Top), whereas expression in apical plasma membranes and medial cytoplasm is shown in L (Middle). Arrowheads indicate cytoplasmic puncta in K and L, whereas arrows indicate the LIN-10-GFP labeling on apical membranes. Blue color indicates the intestinal autofluorescent granules. Bar, 10 μm.
Figure 62. *lin-10* mutation affects the subcellular localization of GFP-RME-1 in the *C. elegans* intestine. Confocal images in wild-type background are shown for ssGFP (A), GFP-RAB-10 (C and E), and GFP-RME-1 (G and I); whereas images in the *lin-10*(ok596) mutant background are shown for ssGFP (B), GFP-RAB-10 (D and F), and GFP-RME-1 (H and J). Arrowheads indicate ssGFP-labeled intracellular vesicles or punctate endosomes labeled by GFP-RAB-10 or GFP-RME-1 in cytosolic, lateral, or basolateral compartments. Arrows indicate the GFP-RME-1-labeling in the apical plasma membranes (I) or the medial cortical area close to apical membranes (J). Quantification of endosomes number and size as visualized by the markers is shown in K and L (GFP-RAB-10) or M and N (GFP-RME-1). Error bars represent standard deviations from the mean (n = 24 each, 8 animals of each genotype sampled in three different regions of each intestine defined by a 100 x 100 (pixl^2) box positioned at random). The asterisk "*" indicates statistically significant (p<0.001) compared to control animals. Bar, 5 μm (A-B), and 10 μm (C-J).
Figure 63. LIN-10 is associated with both early endosomes and Golgi in the *C. elegans* intestine. (A-F) Epi-fluorescent images of colocalization of LIN-10-GFP with early endosomal marker RFP-RAB-5. Puncta labeled by both LIN-10-GFP and RFP-RAB-5 are indicated by arrowheads, whereas puncta labeled by either LIN-10-GFP or RFP-RAB-5 only but not both markers are indicated by arrows. (G-L) Epi-fluorescent images of colocalization of LIN-10-GFP with RFP-RAB-10. Puncta labeled by both LIN-10-GFP and RFP-RAB-10 are indicated by arrowheads, whereas puncta labeled by either LIN-10-GFP or RFP-RAB-10 only but not by both markers, or apical membranes without colocalization are indicated by arrows. Images of the “Top” focal plane show areas near basal membranes, whereas images of the “Middle” focal plane show the intestine in cross-section. “L” indicates the position of intestine lumen. Blue color indicates the intestinal autofluorescent granules. Bar, 10 μm.
TABLES
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Table 1. Summary of subgroup of plasmids identified in yeast two-hybrid screens, corresponding proteins, and regions of each corresponding protein encoded in each prey plasmid. Underline “__” indicates plasmids were sequenced. “*” indicates plasmids with different XhoI/EcoRI digesting patterns but encoding same regions of corresponding protein. “**” indicates plasmids were only partially sequenced (number represents the position in amino acid). Plasmids used to test binding specificity with various RABs were shown in red. N.A., not available.
Table 2. Summary of results in various combinations of bait and prey plasmids by yeast two-hybrid assays. (A) Summary of interactions between RAB-10-interacting proteins (preys) and various RABs, RME-1, RME-1(EH), and LIN-10 (baits). (B) Summary of interactions between various GAPs (preys) and various GTP-bound RABs (baits). “+” indicates that both picked strips showed positive results in Leucine and β-Galactosidase assays. “+*” indicates that only one strip showed positive results in both assays; whereas a strip showed only positive result in either one of assays would not be concluded as a positive strip. N.A., not available.

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Table 3. Summary of yeast strain collections with pairs of prey and bait plasmids used to test binding specificity in yeast two-hybrid assays. Number represents a yeast strain transfected with various combinations of bait and prey plasmids.
Table 4. List of *C. elegans* strains used in each chapter

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RT731  rab-10(dx2); pwls69[vha-6-GFP-rab-11, Cbunc-119(+)]
RT733  rab-10(dx2); pwls83[vha-6-GFP-rhab10(+), Cbunc-119(+)]
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RT1078  rme-1(b1045); pwIs170[vha-6-GFP-rab-7, Cbunc-119(+)]
RT1080  rme-1(b1045); pwIs396[act-5p-act-5-GFP, Cbunc-119(+)]
RT1087  unc-119(ed3); pwIs414[vha-6-mcRFP-rab-10, Cbunc-119(+)]
RT1102  unc-119(ed3); pwIs428[vha-6-mcRFP-rab-11, Cbunc-119(+)]
RT1176  dpy-5(e61); pwIs410[vha-6-mcRFP-rab-10, Cbunc-119(+)]
RT1177  dpy-5(e61); pwIs414[vha-6-mcRFP-rab-10, Cbunc-119(+)]
RT1178  dpy-5(e61); pwIs421[vha-6-mcRFP-rab-10, Cbunc-119(+)]
RT1239  unc-119(ed3); pwIs480[vha-6-mcRFP-rab-5, Cbunc-119(+)]
RT1240  pwIs206[vha-6-GFP-rab-10, Cbunc-119(+)]; pwIs480[vha-6-RFP-rab-5, Cbunc-119(+)]
RT1241  pwIs206[vha-6-GFP-rab-10, Cbunc-119(+)]; pwIs428[vha-6-RFP-rab-11, Cbunc-119(+)]
RT1242  unc-119(ed3); pwIs481[vha-6-mans-GFP, Cbunc-119(+)]
RT1243  pwIs481[vha-6-mans-GFP, Cbunc-119(+)]; pwIs414[vha-6-mcRFP-rab-10, Cbunc-119(+)]
RT1268  pwIs414[vha-6-mcRFP-rab-10, Cbunc-119(+)]; pwIs87[vha-6-GFP-rme-1-D, Cbunc-119(+)]
RT1269  pwIs414[vha-6-mcRFP-rab-10, Cbunc-119(+)]; pwIs170[vha-6-GFP-rab-7, Cbunc-119(+)]
RT1270  pwIs414[vha-6-mcRFP-rab-10, Cbunc-119(+)]; pwIs72[vha-6-GFP-rab-5, Cbunc-119(+)]
RT1309  unc-119(ed3); pwIs500[vha-6-mcRFP-rab-8, Cbunc-119(+)]
RT1315  unc-119(ed3); pwIs503[vha-6-mans-GFP, Cbunc-119(+)]
RT1377  rab-10(q373); pwIs503[vha-6-mans-GFP, Cbunc-119(+)]
RT1420  unc-119(ed3); pwEx102[vha-6-mcRFP, Cbunc-119(+)]
RT1521  pwIs206[vha-6-GFP-rab-10, Cbunc-119(+)]; pwIs414[vha-6-mcRFP-
Strains not used but related to Chapter 2, Appendix-1, and Appendix-2

RT8  
  rab-10(q373); bls46[rme-1p-gfp-rme-1, rol-6(su1006)]
RT29  
  rab-10(dx2); pgExT23H2.5
RT43  
  rab-10(dx2); bls46[rme-1p-gfp-rme-1, rol-6(su1006)]
RT144  
  unc-119(ed3); pwls17[arf-6p-arf-6-GFP, Cbunc-119(+)]
RT199  
  rab-10(dx2); unc-119(ed3)
RT200  
  rab-10(dx2); bls34[rme-8p-GFP-rme-8, rol-6(su1006)]
RT225  
  unc-119(ed3); pwls94[vha-6-GFP-snpx-1, Cbunc-119(+)]
RT233  
  rab-10(dx2); bls5[chcp-GFP-chc, rol-6(su1006)]
RT243  
  unc-119(ed3); pwEx24[vha-6-GFP-rab-10, Cbunc-119(+)]
RT244-246  
  unc-119(ed3); pwEx(25-27)[mtl-2-GFP-rab-10, Cbunc-119(+)]
RT254-255  
  unc-119(ed3); pwEx(28-29)[vha-6-GFP-rab-10, Cbunc-119(+)]
RT282-283  
  unc-119(ed3); pwls65-66 [rab-10p(1.2kb)-GFP-stop, Cbunc-119(+)]
RT284-290  
  unc-119(ed3); pwEx(33-39)[vha-6-GFP-rab-10, Cbunc-119(+)]
RT291-294  
  unc-119(ed3); pwEx(40-43)[rab-10p(1.2kb)-GFP-stop, Cbunc-119(+)]
RT299  
  rab-10(dq373); unc-119(ed3)
RT309  
  unc-119(ed3); pwls67[vha-6-GFP-rab-5, Cbunc-119(+)]
RT312  
  unc-119(ed3); pwEx44[vha-6-GFP-rab-5, Cbunc-119(+)]
RT313  
  unc-119(ed3); pwEx45[vha-6-GFP-rab-7, Cbunc-119(+)]
RT315  
  unc-119(ed3); pwEx46[vha-6-GFP-rab-5, Cbunc-119(+)]
RT316-317  
  unc-119(ed3); pwEx(47-48)[vha-6-GFP-rab-7, Cbunc-119(+)]
RT318-319  
  unc-119(ed3); pwEx(49-50)[vha-6-GFP-rab-11, Cbunc-119(+)]
RT326  
  unc-119(ed3); pwEx54[vha-6-GFP-rab-5, Cbunc-119(+)]
RT328  
  unc-119(ed3); pwEx55[vha-6-GFP-rab-8, Cbunc-119(+)]
RT408  
  unc-119(ed3); pwls116[rme-2p-rme-2-GFP, Cbunc-119(+)]
RT520-522  
  unc-119(ed3); pwls(203-205)[vha-6-GFP-rab-7, Cbunc-119(+)]
RT523-524  
  unc-119(ed3); pwEx(72-73)[vha-6-GFP-rab-7, Cbunc-119(+)]
RT526-527  
  unc-119(ed3); pwls(207-208)[vha-6-GFP-rab-10, Cbunc-119(+)]
RT529  
  unc-119(ed3); pwls210[vha-6-GFP-rab-10(Q68L), Cbunc-119(+)]
RT531  
  unc-119(ed3); pwls212[vha-6-GFP-rab-10(T23N), Cbunc-119(+)]
RT535-536  
  unc-119(ed3); pwEx(74-75)[rab-10p-GFP-rab-10, Cbunc-119(+)]
RT537  
  rab-10(q373); pwls17[arf-6p-arf-6-GFP, Cbunc-119(+)]
RT538  
  rab-10(q373); pwls213[vha-6-GFP-stop, Cbunc-119(+)]
RT539  rab-10(q373); pwIs94[vha-6-GFP-snx-1, Cbunc-119(+)]
RT547  dpy-5(e61); pwIs94[vha-6-GFP-snx-1, Cbunc-119(+)]
RT562  dpy-5(e61); pwIsX [vha-6-GFP-mAA1, Cbunc-119(+)]
RT566-568 unc-119(ed3); pwIs227-229[vha-6-mRFP-rab-10, Cbunc-119(+)]
RT607  dpy-5(e61); pwIs227[vha-6-mRFP-rab-10, Cbunc-119(+)]
RT609  dpy-5(e61); pwIs108[vha-6-GFP-mrme-1, Cbunc-119(+)]
RT610  pwIs227[vha-6-mRFP-rab-10, Cbunc-119(+)]; pwIs206[vha-6-GFP-rab-10, Cbunc-119(+)]
RT612  unc-119(ed3); pwIs250[vha-6-mRFP-rab-5, Cbunc-119(+)]
RT615  unc-119(ed3); pwIs253[vha-6-mRFP-rab-7, Cbunc-119(+)]
RT626  dpy-5(e61); pwIs17[arf-6p-arf-6-GFP, Cbunc-119(+)]
RT638  pwIs72[vha-6-GFP-rab-5, Cbunc-119(+)]; pwIs249[vha-6-mRFP-rab-5, Cbunc-119(+)]
RT639  pwIs206[vha-6-GFP-rab-10, Cbunc-119(+)]; pwIs249[vha-6-mRFP-rab-5, Cbunc-119(+)]
RT652  rab-10(q373); pwIsX [vha-6-GFP-mAA1, Cbunc-119(+)]
RT658-662 unc-119(ed3); pwIs262-266[rab-10p-GFP-stop, Cbunc-119(+)]
RT663  unc-119(ed3); pwEx78[vha-6-mRFP-stop, Cbunc-119(+)]
RT688  unc-119(ed3); pwIs281[pie-1p-cav-1-GFP, Cbunc-119(+)]
RT698  pwIs249[vha-6-mRFP-rab-5, Cbunc-119(+)]; pwIs50[lmp-1p-lmp-1-GFP, Cbunc-119(+)]
RT700  pwIs249[vha-6-mRFP-rab-5, Cbunc-119(+)]+/; pwIs112[vha-6-hTAC-GFP, Cbunc-119(+)]
RT701  pwIs206[vha-6-GFP-rab-10, Cbunc-119(+)]; pwIs253[vha-6-mRFP-rab-7, Cbunc-119(+)]
RT704  rab-10(q373); pwIs108[vha-6-GFP-mrme-1, Cbunc-119(+)]
RT705(RT997) rab-10(q373); pwIs209[vha-6-GFP-rab-10(Q68L), Cbunc-119(+)]
RT706  rab-10(q373); pwIs83[vha-6-GFP-hrab-10(+), Cbunc-119(+)]
RT707(RT975) rab-10(q373); pwIs211[vha-6-GFP-rab-10(T23N), Cbunc-119(+)]
RT708  dpy-5(e61); dyn-1(ky51)
RT709  rme-1(b1045); pwIs90[vha-6-hTfR-GFP, Cbunc-119(+)]; pwIs249[vha-6-mRFP-rab-5, Cbunc-119(+)]
RT710  rme-1(b1045); pwIs50[lmp-1p-lmp-1-GFP, Cbunc-119(+)]; pwIs249[vha-6-mRFP-rab-5, Cbunc-119(+)]
RT712  rme-1(b1045); pwIs112[vha-6-hTAC-GFP, Cbunc-119(+)]; pwIs249[vha-6-mRFP-rab-5, Cbunc-119(+)]+/+
RT713  rab-10(q373); rme-1(b1045); pwIs50[lmp-1p-lmp-1-GFP, Cbunc-119(+)]; pwIs249[vha-6-mRFP-rab-5, Cbunc-119(+)]
RT714-720 lmp-1(nr2045); pwIs206[vha-6-GFP-rab-10, Cbunc-119(+)](1-7)
RT732  rab-10(dx2); pwIsX [vha-6-GFP-mAA1, Cbunc-119(+)]
RT735  rab-10(dx2); pwIs28 [rme-1p-idimerRFP-rme-1, Cbunc-119(+)]
RT736  rab-10(q373); rme-1(b1045); pwIs50[lmp-1p-lmp-1-GFP, Cbunc-119(+)]
RT737  rab-10(q373); rme-1(b1045)
RT738  dpy-5(e61); rme-1(b1045); cdIs5[myo3p-ssdsRed2]
RT739  rab-10(dx2); pwIs108[vha-6-GFP-mrme-1, Cbunc-119(+)]
RT741  pwIs215[rab-10p-GFP-rab-10, Cbunc-119(+)]; odIs6[glr-1p-mRFP-stop]
RT742  rab-10(q373); dyn-1(ky51)
RT745  rme-1(b1045); pwIs108[vha-6-GFP-mrme-1, Cbunc-119(+)]
RT747  rme-1(b1045); pwIs83[vha-6-GFP-hrab10, Cbunc-119(+)]
RT749  rme-1(b1045); pwIs83[vha-6-GFP-hrab10, Cbunc-119(+)]; cdIs5[myo3p-ssdsRed2]
RT750  rab-10(q373); odIs6[glr-1p-mRFP-stop]
RT751-755 unc-119(ed3); pwIs(293-297)[vha-6-mRFP-rab-10, Cbunc-119(+)]
RT756-758 unc-119(ed3); pwIs(298-300)[vha-6-mRFP-rab-11, Cbunc-119(+)]
RT759-762 unc-119(ed3); pwIs(301-304)[vha-6-GFP-hTACLI, Cbunc-119(+)]
RT763-766 unc-119(ed3); pwIs(305-308)[vha-6-GFP-sec-3, Cbunc-119(+)]
RT767-771 unc-119(ed3); pwIs(309-313)[vha-6-mRFP-stop, Cbunc-119(+)]
RT779  rab-10(dx2); rme-1(b1045)
RT784  rme-1(b1045); pwIs94[vha-6-GFP-snx-1, Cbunc-119(+)]
RT785  rme-1(b1045); pwIsX[vha-6-GFP-mAA1, Cbunc-119(+)]
RT786  rme-1(b1045); pwIs214[rab-10p-GFP-rab-10, Cbunc-119(+)]
RT829  rab-10(dx2); rme-1(b1045); pwIs50[imp-1p-imp-1-GFP, Cbunc-119(+)]
RT863  rab-10(q373); pwIs306[vha-6-GFP-sec-3, Cbunc-119(+)]
RT866  rme-1(b1045); cdIs5[myo3p-ssdsRed2]; pwIs306[vha-6-GFP-sec-3, Cbunc-119(+)]
RT879  rme-1(b1045); cdIs5[myo3p-ssdsRed2]; pwIs76[SDNP-1p-Syndapin-I-GFP, Cbunc-119(+)]
RT883  rme-1(b1045); cdIs5[myo3p-ssdsRed2]; pwIs87[vha-6-GFP-rme-1-D, Cbunc-119(+)]
RT884  rme-1(b1045); cdIs5[myo3p-ssdsRed2]; pwIs314[act-5p-act-5-GFP, Cbunc-119(+)]
RT931-935 unc-119(ed3); pwIs(358-362)[rab-5p-mRFP-rab-5, Cbunc-119(+)]
RT936-939 unc-119(ed3); pwIs(363-366)[rab-8p-mRFP-rab-8, Cbunc-119(+)]
RT985  pwIs214[rab-10p-GFP-rab-10, Cbunc-119(+)]; pwIs361[rab-5p-mRFP-rab-5, Cbunc-119(+)]
RT986  pwIs214[rab-10p-GFP-rab-10, Cbunc-119(+)]; pwIs363[rab-8p-mRFP-rab-8, Cbunc-119(+)]
RT987  pwIs214[rab-10p-GFP-rab-10, Cbunc-119(+)]; pwIs364[rab-8p-mRFP-rab-8, Cbunc-119(+)]
RT988  pwIs216[vha-6-mRFP-rme-1-F, Cbunc-119(+)]; pwIs90[vha-6-hTfR-GFP, Cbunc-119(+)]
RT989  pwIs216[vha-6-mRFP-rme-1-F, Cbunc-119(+)]; pwIs209[vha-6-GFP-rab-10(Q68L), Cbunc-119(+)]
RT990  pwIs216[vha-6-mRFP-rme-1-F, Cbunc-119(+)]; pwIs211[vha-6-GFP-rab-10(T23N), Cbunc-119(+)]
RT997  rab-10(q373); pwIs209[vha-6-GFP-rab-10(Q68L), Cbunc-119(+)]/
RT999  pwIs216[vha-6-mRFP-rme-1-F, Cbunc-119(+)]; pwIs112[vha-6-hTACLI-GFP, Cbunc-119(+)]
RT1012  pwIs216[vha-6-mRFP-rme-1-F, Cbunc-119(+)]; pwIs76[SDYNp-1p-Syndapin-I-GFP, Cbunc-119(+)]
RT1014  pwIs216[vha-6-mRFP-rme-1-F, Cbunc-119(+)]; pwIs50[imp-1p-imp-1-GFP, Cbunc-119(+)]
RT1015 pwIs209[vha-6-GFP-rab-10(Q68L), Cbunc-119(+)];
RT1021 rab-10(dx2); pwIs72[vha-6-GFP-rab-5, Cbunc-119(+)]; pwIs253[vha-6-mRFP-rab-7, Cbunc-119(+)]
RT1062-1065 unc-119(ed3); pwIs(405-408)[pie-1p-mcRFP-rab-10, Cbunc-119(+)]
RT1082-1095 unc-119(ed3); pwIs(409-422)[vha-6-mcRFP-rab-10, Cbunc-119(+)]
RT11244 unc-119(ed3); pwIs482[vha-6-mans-GFP, Cbunc-119(+)]
RT11249 unc-119(ed3); pwIs487[vha-6-mcRFP-rab-5, Cbunc-119(+)]
RT11253 rab-10(dx2); pwIs410[vha-6-mcRFP-rab-10, Cbunc-119(+)]
RT11254 rab-10(dx2); pwIs421[vha-6-mcRFP-rab-10, Cbunc-119(+)]
RT11260-1262 unc-119(ed3); pwIs(493-495)[vha-6-mcRFP-rab-5, Cbunc-119(+)]
RT11263-1264 unc-119(ed3); pwIs(496-497)[rab-10p-mcRFP-rab-10, Cbunc-119(+)]
RT11265-1266 unc-119(ed3); pwIs(498-499)[vha-6-mans-GFP, Cbunc-119(+)]
RT11375 rab-10(q373); pwIs116[rme-2p-rme-2-GFP, Cbunc-119(+)]
RT11376 rab-10(q373); pwls281[pie-1p-cav-1-GFP, Cbunc-119(+)]
RT11382 rab-10(dx2); pwls503[vha-6-mans-GFP, Cbunc-119(+)]
RT11383 rab-10(dx2); pwIs481[vha-6-mans-GFP, Cbunc-119(+)]
RT11421 rab-10(dx2); pwIs116[rme-2p-rme-2-GFP, Cbunc-119(+)]
RT11422 rab-10(dx2); pwIs281[pie-1p-cav-1-GFP, Cbunc-119(+)]
RT11425 rab-10(q373); pwIs481[vha-6-mans-GFP, Cbunc-119(+)]
RT11426 rab-10(q373); pwls490[hum-2p-hum-2-GFP, Cbunc-119(+)]
RT11427 pwIs112[vha-6-hTAC-GFP, Cbunc-119(+)]/+; pwls480[vha-6-mcRFP-rab-5, Cbunc-119(+)]/
RT11428 rab-10(q373); pwIs112[vha-6-hTAC-GFP, Cbunc-119(+)]; pwEx102[vha-6-mans-mcRFP, Cbunc-119(+)]
RT11344 pwls112[vha-6-hTAC-GFP, Cbunc-119(+)]; pwEx102[vha-6-mans-mcRFP, Cbunc-119(+)]
RT11446 unc-119(ed3); pwls564[vha-6-mans-mcRFP, Cbunc-119(+)]
RT11524 pwls396[opt-2p-opt-2-GFP, Cbunc-119(+)]; pwls480[vha-6-mcRFP-rab-5, Cbunc-119(+)]
RT11525 pwls396[opt-2p-opt-2-GFP, Cbunc-119(+)]; pwls428[vha-6-mcRFP-rab-11, Cbunc-119(+)]
RT11526 pwls396[opt-2p-opt-2-GFP, Cbunc-119(+)]; pwEx102[vha-6-mans-mcRFP, Cbunc-119(+)]
RT11527 rab-10(q373); pwls396[opt-2p-opt-2-GFP, Cbunc-119(+)]; pwls480[vha-6-mcRFP-rab-5, Cbunc-119(+)]
RT11528 rab-10(q373); pwls396[opt-2p-opt-2-GFP, Cbunc-119(+)]; pwls428[vha-6-mcRFP-rab-11, Cbunc-119(+)]
RT11529 rab-10(q373); pwls396[opt-2p-opt-2-GFP, Cbunc-119(+)]; pwEx102[vha-6-mans-mcRFP, Cbunc-119(+)]

Strains used in Chapter 3

HUM-2-related:

RT1026 dpy-11(e224); arIs37[myo3p-ssGFP, dpy-20(+)]
RT1040 dpy-11(e224); pwIs206[vha-6-GFP-rab-10, Cbunc-119(+)]
RT1041  dpy-11(e224); bls1[vit-2-GFP]  
RT1042  dpy-11(e224); pws87[vha-6-GFP-rme-1-D, Cbunc-119(+)]  
RT1044  hum-2(ok596); pws87[vha-6-GFP-rme-1, Cbunc-119(+)]  
RT1045  hum-2(ok596); pws206[vha-6-GFP-rab-10, Cbunc-119(+)]  
RT1046  hum-2(ok596); bts1[vit-2-GFP]  
RT1047  hum-2(ok596); arls37[myo3p-ssGFP, dpy-20(+)]  
RT1250-1251  unc-119(ed3); pws87(488-489)[hum-2p-hum-2-GFP, Cbunc-119(+)]  
RT1252  unc-119(ed3); pwEx97[hum-2p-hum-2-GFP, Cbunc-119(+)]  
RT1255-1257  unc-119(ed3); pws87(490-492)[hum-2p-hum-2-GFP, Cbunc-119(+)]  
RT1258-1259  unc-119(ed3); pwEx98-99[hum-2p-hum-2-GFP, Cbunc-119(+)]  
RT1271  dpy-11(e224); pws50[imp-1p-imp-1-GFP, Cbunc-119(+)]  
RT1272  dpy-11(e224); pws170[vha-6-GFP-rab-7, Cbunc-119(+)]  
RT1273  dpy-11(e224); pws[vha-6-GFP-opt-2, Cbunc-119(+)]  
RT1274  dpy-11(e224); pws531[vha-6-lin-10-GFP, Cbunc-119(+)]  
RT1275  dpy-11(e224); nulls25[glr-1p-act-1-GFP, Cbunc-119(+)]  
RT1276  dpy-11(e224); pws69[vha-6-GFP-rab-11, Cbunc-119(+)]  
RT1277  dpy-11(e224); pws576[SDNPp-syndapin-GFP, Cbunc-119(+)]  
RT1289  dpy-11(e224); pws90[vha-6-hTfR-GFP, Cbunc-119(+)]  
RT1295  dpy-11(e224); pws112[vha-6-hTAC-GFP, Cbunc-119(+)]  
RT1316  dpy-11(e224); pws314[act-5p-act-5-GFP, Cbunc-119(+)]  
RT1317  dpy-11(e224); pws72[vha-6-GFP-rab-5, Cbunc-119(+)]  
RT1424  hum-2(ok596); nulls25[glr-1p-act-1-GFP, Cbunc-119(+)]

**GCK-2-related**

RT1141-1147  unc-119(ed3); pws87(461-467)[gck-2p-gck-2-GFP, Cbunc-119(+)]  
RT1163-1164  unc-119(ed3); pws578(470-471)[gck-2p-gck-2-GFP, Cbunc-119(+)]  
RT1165-1168  unc-119(ed3); pwEx93-96[act-5p-act-5-GFP, Cbunc-119(+)]  

**Strains used in Chapter 4**

RT1125  unc-119(ed3); pws451[ehbp-1p-ehbp-1-GFP, Cbunc-119(+)]  
RT1292  rab-10(q373); pws451[ehbp-1p-ehbp-1-GFP, Cbunc-119(+)]  
RT1309  unc-119(ed3); pws500[vha-6-mcRFP-rab-8, Cbunc-119(+)]  
RT1314  rme-1(b1045); pws451[ehbp-1p-ehbp-1-GFP, Cbunc-119(+)]  
RT1394  unc-119(ed3); pws542[vha-6-mcRFP-rme-1-F, Cbunc-119(+)]  
RT1420  unc-119(ed3); pwEx102[vha-6-mans-mcRFP, Cbunc-119(+)]  
RT1448  unc-119(ed3); pwEx103[vha-6-GFP-ehbp-1, Cbunc-119(+)]  
RT1449  unc-119(ed3); pwEx104[vha-6-GFP-ehbp-1, Cbunc-119(+)]  
RT1478  pws414[vha-6-mcRFP-rab-10, Cbunc-119(+)]; pwEx104[vha-6-GFP-ehbp-1, Cbunc-119(+)]  
RT1479  pws414[vha-6-mcRFP-rab-10, Cbunc-119(+)]; pwEx103[vha-6-GFP-ehbp-1, Cbunc-119(+)]  
RT1480  pwEx104[vha-6-GFP-ehbp-1, Cbunc-119(+)]; pws480[vha-6-mcRFP-rab-5, Cbunc-119(+)]  
RT1483  pwEx104[vha-6-GFP-ehbp-1, Cbunc-119(+)]; pws428[vha-6-mcRFP-
Strains not used but related to Chapter 4

RT1121-1131  
unc-119(ed3); pwIs(447-456)[ehbp-1p-ehbp-1-GFP, Cbunc-119(+)]

RT1280  
pwIs451[ehbp-1p-ehbp-1-GFP, Cbunc-119(+)]; pwIs28[rme-1p-mRFP-rme-1]

RT1443  
pwIs451[ehbp-1p-ehbp-1-GFP, Cbunc-119(+)]; pwIs414[vha-6-mcRFP-rab-10, Cbunc-119(+)]

RT1445  
pwIs451[ehbp-1p-ehbp-1-GFP, Cbunc-119(+)]; pwIs480[vha-6-mcRFP-rab-5, Cbunc-119(+)]

RT1481  
pwEx104[vha-6-GFP-ehbp-1, Cbunc-119(+)]; pwIs542[vha-6-mcRFP-rme-1-F, CbUnc-119(+)]

RT1482  
pwEx103[vha-6-GFP-ehbp-1, Cbunc-119(+)]; pwIs542[vha-6-mcRFP-rme-1-F, CbUnc-119(+)]

RT1538  
rab-10(q373); pwEx104[vha-6-GFP-ehbp-1, Cbunc-119(+)]; pwEx102[vha-6-mans-mcRFP, Cbunc-119(+)]

RT1539  
rab-10(q373); pwEx104[vha-6-GFP-ehbp-1, Cbunc-119(+)]; pwIs480[vha-6-mcRFP-rab-5, Cbunc-119(+)]

Strains used for ARF-6 in Appendix-3

RT859  
dpy-4(e1166); pwIs50[lmp-1p-lmp-1-GFP, Cbunc-119(+)]

RT865  
dpy-4(e1166); pwIs206[vha-6-GFP-rab-10, Cbunc-119(+)]

RT867  
dpy-4(e1166); pwIs314[act-5p-act-5-GFP, Cbunc-119(+)]

RT868  
dpy-4(e1166); pwIs306[vha6-GFP-sec3, Cbunc-119(+)]

RT869  
dpy-4(e1166); pwIs87[vha-6-GFP-rme-1, Cbunc-119(+)]

RT885  
arf-6(tm1447); pwIs306[vha6-GFP-sec3, Cbunc-119(+)]

RT886  
arf-6(tm1447); pwIs314[act-5-GFP, Cbunc-119(+)]

RT887  
arf-6(tm1447); pwIs206[vha-6-GFP-rab-10, Cbunc-119(+)]

RT888  
arf-6(tm1447); pwIs76[syndapin-GFP, Cbunc-119(+)]

RT889  
arf-6(tm1447); pwIs87[vha-6-GFP-rme-1, Cbunc-119(+)]

RT1277  
dpy-4(e1166); pwIs69[vha-6-GFP-rab-11, Cbunc-119(+)]

RT1278  
dpy-4(e1166); pwIs112[vha-6-hTAC-GFP, Cbunc-119(+)]

RT1279  
dpy-4(e1166); pwIs90[vha-6-hTfR-GFP, Cbunc-119(+)]

RT1281  
dpy-4(e1166); pwIs170[vha-6-GFP-rab-7, Cbunc-119(+)]

RT1282  
dpy-4(e1166); pwIs531[vha-6-lin-10-GFP, Cbunc-119(+)]

RT1283  
dpy-4(e1166); pwIs50[lmp-1p-lmp-1-GFP, Cbunc-119(+)]
RT1284  dpy-4(e1166); nuls25[glr-1p-glr-1-GFP, Cbunc-119(+)]
RT1285  dpy-4(e1166); arls37[myo3p-ssGFP, dpy-20(+)]
RT1286  dpy-4(e1166); pwls72[vha-6-GFP-rab-5, Cbunc-119(+)]
RT1287  dpy-4(e1166); pwls396 [vha-6-opt-2-GFP, Cbunc-119(+)]
RT1319  dpy-4(e1166); bls1[vit-2-GFP]

Strains used for LIN-10 in Appendix-4

RT1148-1149  unc-119(ed3); pwls(468-469)[lin-10p-lin-10-GFP, Cbunc-119(+)]
RT1150-1152  unc-119(ed3); pwEx(90-92)[lin-10p-lin-10-GFP, Cbunc-119(+)]
RT1153  lin-10(n1508); arls37[myo3p-ssGFP, dpy-20(+)]
RT1154  lin-10(n1508); pwls87[vha-6-GFP-rme-1-D, Cbunc-119(+)]
RT1161  lin-10(n1508); pwls206[vha-6-GFP-rab-10, Cbunc-119(+)]
RT1169  lin-10(n1508); bls1[vit-2-GFP, rol-6(su1006)]
RT1179  pwls410[vha-6-mcRFP-rab-10, Cbunc-119(+)]; pwEx92[lin-10p-lin-10-GFP, Cbunc-119(+)]
RT1290  lin-10(n1508); pwls112[vha-6-hTAC-GFP, Cbunc-119(+)]
RT1291  lin-10(n1508); pwls72[vha-6-GFP-rab-5, Cbunc-119(+)]
RT1293  rab-10(q375); pwls531[vha-6-lin-10-GFP, Cbunc-119(+)]
RT1294  lin-10(n1508); pwls50[lmp-1p-lmp-1-GFP, Cbunc-119(+)]
RT1313  lin-10(n1508); pwls351[vha-6-lin-10-GFP, Cbunc-119(+)]
RT1381  unc-119(ed3); pwls531[vha-6-lin-10-GFP, Cbunc-119(+)]
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Doctor of Philosophy, Cell and Developmental Biology Program, Rutgers University, Piscataway N.J. Thesis Advisor: Dr. Barth D. Grant.
Thesis title: “Functional Analysis of RAB-10 and its Interacting Partner EHB-P1 during Endocytosis in the Caenorhabditis elegans Intestine”.

09/1994-10/1997
Master of Science, Medical Microbiology & Immunology, University of Alberta, Edmonton, Alberta, Canada. Thesis Advisor: Dr. Lung-Ji Chang.
Thesis title: “The Differential Apoptotic Effects of Vpr and Vpx of Human and Simian Immunodeficiency Virus in Mammalian Cells”.

09/1989-05/1991
Master of Science, Biochemistry, College of Medicine, National Taiwan University, Taipei Taiwan. Thesis Advisor: Dr. Jung-Yaw Lin.
Thesis title: ”Inhibition of Angiotensin-I Converting Enzyme by the compounds of the Xanthones and Flavonoids: in vitro & in vivo”.

09/1985-05/1989
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Working Experiences
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Project title: ’Investigation of chemokine receptor on which HIV-1 influences during mother-child vertical transmission’.

08/1993-06/1994
Research Assistant, Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei Taiwan. Research Advisor: Dr. Jung-Yaw Lin.
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Patent

Publications (selected)