Analysis of candidate regulators of TBC-2 and endosome maturation in *Caenorhabditis elegans*

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Abstract

The Rab5 and Rab7 GTPases are key regulators of endosome to lysosome trafficking, whose activities are positively regulated by Rab Guanine nucleotide Exchange Factors and negatively regulated by GTPase Activating Proteins (GAP). In this thesis, the nematode *Caenorhabditis elegans* was used as a model system to study regulation of Rab GTPase-mediated endosomal trafficking, for simple genetics and easy visualization of organelles under Differential Interference Contrast (DIC) and confocal optics. It was previously demonstrated that TBC-2 functions as a RAB-5 GAP. Loss of *tbc-2* activity results in the formation of enlarged late endosomes in the intestine that require the activities of RAB-5, RAB-7 and components of the homotypic fusion and vacuole protein sorting (HOPS) complex. TBC-2 colocalizes with RAB-7 on late endosomes, and requires RAB-7 for membrane localization. My hypothesis is that RAB-7 recruits TBC-2 to late endosomes to inactivate RAB-5 and possibly RAB-7 itself, to facilitate early to late endosome maturation.

The *vh8* mutant was previously identified in the lab as a suppressor of the *tbc-2(-)* phenotype and displaces GFP::RAB-7 from vesicular membranes. It is possible that *vh8* encodes for a potential RAB-7 GEF. To investigate the molecular identity of *vh8*, I tested candidate genes by RNAi. While none of the candidates appear to be *vh8*, I did find that the HOPS core complex is required for the *tbc-2(-)* phenotype. ARL-8, and its human homolog arl8b, have recently been shown to be important for late endosome to lysosome trafficking. To investigate whether ARL-8 is required for TBC-2 mediated early to late endosome trafficking, I performed RNAi experiments. The results of which suggest that ARL-8 functions downstream of TBC-2. ARL-8 is required for the *tbc-2(-)* large late endosome phenotype, but is not required for membrane localization of either TBC-2 or RAB-7.

In mammalian cells, the Rac1 GTPase is able to bind Armus, a human homolog of TBC-2. That raises the possibility that RAB-7 recruits CED-10/Rac1 via TBC-2. However, I observe that in *tbc-2(tm2241)* mutant animals, CED-10 is still localized to vesicles. It suggests that CED-10 might be recruited to membrane via other mechanism. Active CED-10 is also crucial for phagocytosis and clearance of apoptotic cell corpses. CED-10 can be activated by the CED-5/CED-12 complex, which is proposed to be recruited and activated by $PI(3,5)P_2$. Work from this thesis suggests that the active state might not be a requirement for CED-10 localization, as *ced-5* RNAi does not change the localization of CED-10.

Résumé

Les GTPases Rab5 et Rab7 participent à la régulation du traffic des endosomes. L'activité de ces protéines est régulée positivement par les "Rab Guanine nucleotide Exchange Factors" (GEF) et régulée négativement par les "GTPase Activating Proteins" (GAP). Dans ce projet, le nematode Caenorhabditis elegans a été utilisé comme modèle pour étudier la régulation du traffic endosomal médié par les Rab GTPase car celui-ci est un bon modèle génétique et permet une visualisation des organites à l'aide de microscopie à constraste d'interférence différentielle et de microscopie confocale. Il a été démontré dans la passé que TBC-2 fonctionne en tant que GAP pour RAB-5. La perte de l'activité de tbc-2 résulte en la formation d'endosomes tardifs élargis dans les intestins des nématodes, qui requièrent normalement l'activité de RAB-5, RAB-7 et des constituants du complexe "homotypic fusion and vacuole protein sorting" (HOPS). TBC-2 se retrouve avec RAB-7 sur les endosomes tardifs et nécessite RAB-7 pour sa localisation sur la membrane. Notre hypothèse était que RAB-7 recrute TBC-2 aux endosomes tardifs pour inactiver RAB-5 et possiblement RAB-7, ce qui facilite la maturation des endosomes en endosomes tardifs.

Le mutant vh8 a préalablement été identifié dans le laboratoire en tant que suppresseur du phénotype tbc2(-) et cause le déplacement de GFP::RAB-7 des membranes vésiculaires. Il est possible que vh8 exprime un potentiel GEF de RAB-7. Pour trouver l'identité moléculaire de vh8, nous avons testé les gènes candidats à l'aide d'ARN d'interférence (ARNi). Malgré qu'aucun des candidats ne semblaient être vh8, nous avons découvert que le complexe central de HOPS est nécessaire au phénotype tbc-2(-). ARL-8 et son homolog humain arl8b ont récessement été démontrés comme étant importants dans le traffic des endosomess tardifis aux lysosomes. Pour vérifier si ARL-8 est requis dans le traffic des endosomes médiés par TBC-2. Nos travaux à l'aide d'ARNi suggèrent qu'ARL-8 fonctionne en aval de TBC-2. ARL-8 est nécessaire à l'apparition du phénotype tbc-2(-), mais n'est pas requis pour la localisation de TBC-2 et RAB-7 à la membrane.

Dans les cellules de mammifères, la GTPase Rac1 peut se lier à Armus, un homologue humain de TBC-2. Ceci suggère que RAB-7 pourrait recruter CED-10/RAC1 par l'entremise de TBC-2. Par contre, nous observons que chez les nématodes tbc-2(tm2241), CED-10 est toujours localisé sur les vésicules. Ceci suggère que CED-10 pourrait être recruté à la membrane par d'autres mécanismes. Un CED-10 activé est aussi nécessaire à la phagocytose et à la dégradation des corps cellulaires apoptotiques. CED-10 peut être activé par le complexe CED-5/CED-12, qui est possiblement recruté et activé par PI(3,5)P2. Nos travaux suggèrent par contre que l'activation de CED-10 n'est pas obligatoire à sa localisation, car l'ARNi de ced-5 ne change pas la localisation de CED-10.

List of Abbreviations

Arl: Arf-like protein

Arf: ADP-ribosylation factor

C.elegans: *Caenorhabditis elegans*

CORVET: Class-C CORe Vacuole/Endosome Tethering

DIC: Differential Interference Contrast

dsRNA: double-stranded RNA

EEA1: Early Endosome Antigen 1

ER: Endoplasmic Reticulum

GAP: GTPase-activating protein

GTT: Geranylgeranyl Transferase

GDI: Guanine Dissociation Inhibitor

GEF: Guanine nucleotide Exchange Factor

GDF: GDI Dissociation Factor

GTPases: Guanosine Tri-Phosphatases

Gyp:Gap Ypt/Rab Proteins

HOPS: homotypic fusion and Vacuole protein sorting

OSBP:human Oxysterol-Binding Protein

ORP1L: Oxysterol-binding Related Protein 1L

PH: Pleckstrin Homology

RILP: Rab7-Interacting Lysosomal Protein

REP: Rab Escort Protein

VPS:Vesicular Protein Sorting

Introduction

An overview of endosomal trafficking

Unlike prokaryotic cells, eukaryotic cells face the challenge of transporting materials from one membrane-enclosed organelle to another in a specific and regulated manner. It generally involves budding of a vesicular carrier, tethering, docking, and fusion with the recipient membrane. Extracellular macromolecules and plasma membrane proteins (the cargo) are endocytosed into the cell in vesicles. Many such vesicles fuse with the early endosome, which also receives vesicles from the Golgi and serves as a sorting station (Helenius et al., 1983). From the sorting endosomes, some cargo will go through the recycling pathway to be transported back to the plasma membrane. Other cargo will go to late endosomes then fuse with lysosomes for the content to be degraded (Saftig and Klumperman, 2009). The degradative pathway involves early-to-late endosome maturation, which is mediated by many small G proteins such as Rab family GTPases and Arf family GTPases.

Rab GTPases

Rabs are a group of GTP-binding proteins. There are 11 members in budding yeast and over 60 in humans (Pereira-Leal and Seabra, 2001; Seabra et al., 2002). Rab GTPases regulate intracellular trafficking by regulating budding, transporting, tethering and fusion of intracellular vesicles. As a molecular switch, Rab GTPases exist in either a GDP-bound inactive or GTP-bound activate state. In the GTP-bound active state, Rabs bind to different effector proteins to regulate

the abovementioned intracellular trafficking steps. The Rab Guanine nucleotide Exchange Factors (GEFs) activate Rabs by dissociating GDP, therefore allowing GTP, which is more abundant in the cytoplasm, to bind. The inactivation of Rab GTPases is catalyzed by the GTPase Activating Proteins (GAPs), which accelerates the hydrolysis of GTP. By regulating the Rab GTPase active state, Rab GEFs and GAPs play an important role in Rab membrane localization and interaction with effector proteins (Figure I1)(Barr and Lambright, 2010).

Rab GTPase localization

Rab GTPases are modified by a Rab Geranyl-geranyl Transferase (GGT), by attaching one or two prenyl groups to the carboxy terminal. When a Rab is in the cytosolic inactive state, the prenylated C-terminal is masked by GDP Dissociating Inhibitor (GDI) to prevent random insertion into the lipid bilayer. The GDI-associated Rab is shuttle to a specific membrane by a Rab Escort Protein (REP). At the target membrane, GDI displacing factor (GDF) catalyzes the dissociation of GDI from Rab, exposing the prenylated C-terminal, therefore allowing association between the target membrane and the Rab. Once on the membrane, the Rab GTPase can be activated by its GEF, giving the associated membrane a distinct identity (Figure I1). For example, active Rab5 and Rab7 localize to early and late endosomes respectively and Rab11 localizes to the recycling endosomes (Figure I2) (Stenmark, 2009). Moreover, studies suggest that there can be multiple Rabs occupying distinct micro-domains on the same endosomal compartment. Rab4, which regulates rapid recycling from early endosomes, Rab5 and Rab11 have been found on the same endosome, each

occupying a distinct micro-domain that do not intermix (Sonnichsen et al., 2000). Rab5 and Rab7 also transiently coexist on the same vesicle as Rab5-positive early endosomes mature into Rab7-positive late endosomes (Rink et al., 2005; Vonderheit and Helenius, 2005).

The concerted recruitment of Rab GEFs and effectors are required for targeting Rabs to specific micro-domains and maintaining its active state. For example, Rabex5 activates Rab5, promoting its membrane localization. Active Rab5 recruits Rabadaptin5, one of the Rab5 effectors, to increase the GEF activity of Rabex5, therefore to keep Rab5 in an active state and allow downstream signaling. This positive feedback mechanism is suggested to be the general mechanism amongst all Rab GTPases (Grosshans et al., 2006).

Rab conversion model

Because of the distinct micro-domain localization and specificity of the trafficking compartment, Rab GTPases must function in a coordinated fashion to couple trafficking steps, to ensure continuous compartment transition. The current model of Rab cooperation in Rab conversion is that an activated Rab GTPase recruits the GEF of a downstream Rab GTPase. The GEF will activate the Rab GTPase, which in turn will recruit the GAP of the upstream Rab GTPase. The GAP will inactivate the upstream Rab GTPase and displace it from the vesicle (Rink et al., 2005; Rivera-Molina and Novick, 2009). The sequential activation and inactivation of Rab GTPases is mediated by the unique GEF and GAP cascade to ensure a directional flow of the cargo (Figure I3).

The Rab conversion model was first described in yeast during exocytosis. In yeast, Ypt31p/Ypt32p are the two redundant homologues of Rab11, which are involved in Golgi transport steps. During early to late Golgi transport in yeast, Ypt1p positive vesicles convert to Ypt32p positive vesicles, which then convert to Sec4p positive vesicles. During the conversion, Ypt1p recruits the Ypt32p GEF, TRAPPII, to activate Ypt32p (Yamasaki et al., 2009). Then the activated Ypt32p recruits Sec2p, a Sec4p GEF, to secretory vesicles (Ortiz et al., 2002). Sec2p then activates the Rab GTPase Sec4p (Walch-Solimena et al., 1997), which in turn activates trans-Golgi transport to the plasma membrane. However, once a Rab GTPase is activated, it should induce the removal of the upstream Rab GTPase to complete the conversion. Ypt32p, once activated, recruits Gyp1p, which is a GAP for Ypt1p. Once inactive, Ypt1p is removed from the vesicle, completing the Ypt1p to Ypt32p conversion (Rivera-Molina and Novick, 2009).

The Rab cascade exists in mammalian cells as a mechanism of early to late endosome maturation. Rab5 positive vesicles move away from the cell periphery and fuse with each other. Those vesicles lose Rab5 and acquire Rab7. Vps39 is a component of the homotypic fusion and vacuole protein sorting (HOPS) complex, it can bind to both GTP-bound Rab5 and Rab7, and is an important regulator of Rab5 to Rab7 conversion. In yeast, Vps39p was demonstrated to be both an effector and GEF for Ypt7p, the yeast homolog of Rab7. In the Rab conversion model, it is proposed that active Rab5 recruits Vps39, which recruits and activates Rab7. Although Vps39 regulates Rab conversion, its role as a Rab7 GEF has been questioned (see below). A mathematics model supports that the Rab5 to

Rab7 conversion is operated by a "cut-out switch" controlled by Rab7. In this model, Rab5 activates Rab7 until Rab7 activity reaches a threshold, then it inactivates Rab5 through a negative feedback loop (Del Conte-Zerial et al., 2008). It was hypothesized that Rab7 recruits a Rab5 GAP to create a negative feedback that results in the transition from Rab5 to Rab7. As discussed later, *C. elegans* TBC-2 fills this role suggesting one of its mammalian homologs does the same.

Rab7 GEFs

Until recently mammalian Vps39 subunit of the HOPS complex has been presumed to function as a Rab7 GEF based on a study in yeast showing that Vps39p was a GEF for Ypt7p, the Rab7 homolog (Wurmser et al., 2000). Vps39 family proteins were shown to regulate endocytic trafficking in zebrafish and mammalian cells, however, the GEF activity was not directly tested (Caplan et al., 2001; Schonthaler et al., 2008). Ccz1p/Mon1p, but not Vps39p, have now been demonstrated to possess Ypt7p GEF activity (Nordmann et al., 2010). That brings to question what the Rab7 GEF really is in metazoans. Work in *C. elegans* show that VPS-39 is not required for RAB-7 membrane localization, a hallmark of RAB-7 activation, suggesting it is either not a RAB-7 GEF or functions redundantly with an unidentified RAB-7 GEF (Chotard et al., 2010). In mammalian cells and *C. elegans*, SAND-1, the Mon1p homolog, forms a complex with CCZ-1 and both regulate Rab5 to Rab7 conversion (Cabrera and Ungermann, 2010). It is shown that Sand1/Mon1-Ccz1 complex is able to displace Rabex5, a Rab5 GEF, and recruit Rab7, probably through interaction with HOPS complex (Kinchen and Ravichandran, 2010; Poteryaev et al., 2010). Therefore, HOPS

might still be important for Rab conversion. The Sand1/Mon1-Ccz1 has not yet been demonstrated to be a Rab7 GEF in metazoans, but as with Rab5, there could be multiple GEFs for Rab7.

Rab7 Effectors

HOPS complex includes a VPS-C class core consisting of VPS11,VPS16, VPS33 and VPS18, plus two accessory components, VPS39 and VPS41 (Sato et al., 2000; Seals et al., 2000; Wurmser et al., 2000). In yeast, Vps41 is the Ypt7 effector that is required for proper homotypic fusion between vacuoles (Cabrera et al., 2009). In addition to VPS/HOPS complex there are other Rab7 Effectors. Two such effectors are Rab-Interacting Lysosomal Protein (RILP) and a member of the human Oxysterol Binding Protein (OSBP), ORP1L. These two effectors promote endosomal trafficking by regulating transport of the vesicles along microtubules. The movement of vesicles in the cell requires two components: motor protein and a track towards the targeted compartment. RILP, together with ORP1L, bind to dynactin-dynein motor to mediate minus-end movement of late endosomes and lysosomes towards the perinuclear region of the cell (Johansson et al., 2007). Interaction between ORP1L and the ER protein VAP stabilize the perinuclear localization (Rocha et al., 2009). In *C. elegans*, both OBR-1, the closest homolog of ORP1L, and C32A3.3, a RILP-related protein, lack the Rab7 binding motif, but have the FFAT motif that is important for VAP binding (Loewen et al., 2003). The difference in homology suggest that in *C. elegans*, RAB-7 either doesn't couple to dynein to control vesicle motility or there are other proteins that couples RAB-7 to dynein

Rab GAPS

GAPs limit Rab activity either temporally or spatially (Nottingham and Pfeffer, 2009; Zerial and McBride, 2001). A catalytic TBC (Tre-2/Cde16/Bud2) domain is shared by most Rab GAPs characterized to date (Neuwald, 1997). The GAP activity depends on an arginine finger that interfaces the Rab nucleotide binding pocket to stimulate GTP hydrolysis, while the glutamine finger substitutes for the glutamine in the DXXGQ motif in Rab GTPases (Albert and Gallwitz, 1999; Albert et al., 1999; Barr and Lambright, 2010; Pan et al., 2006). TBC domain proteins RN-Tre and Rab GAP-5 can catalyze hydrolysis of GTP by Rab5 in vitro. When overexpressed, they can disrupt endocytosis in cell culture (Barr and Lambright, 2010; Lanzetti et al., 2000). There may be multiple GAPs that act on a single Rab to regulate its function in a specific compartment at a specific time. However, Rab GTPases have an intrinsic ability to hydrolyze GTP at a reasonable rate, suggesting that GAPs may not be an absolute requirement for proper regulation of endocytic trafficking (Barr and Lambright, 2010). However, as mentioned above, Rab GAPs serve an important role during Rab conversion to ensure the directional flow of the cargo.

TBC-2 and homologs

C. elegans TBC-2 is a unique TBC domain RAB-5 GAP that has four conserved domains: a pleckstrin homology (PH) domain, a coiled-coil domain, a TBC-2 homologous region (THR) and the TBC catalytic domain (Figure I4A). Loss of TBC-2 results in enlarged vesicles accumulating in the intestine, identical

to that seen with expression of constitutively active RAB-5 (Figure I5). RAB-5 is required for *tbc-2(-)* phenotype. The mutated transgene GFP::TBC-2 R689K fails to rescue the *tbc-2(-)* phenotype (Figure I6E). It shows that the arginine finger in the TBC domain of TBC-2, which is important for it's GAP activity, is required for TBC-2 function. An in vitro GAP assay shows that the TBC domain of TBC-2 has the strongest GAP activity on RAB-5 (Chotard et al., 2010). *tbc-2(-)* also causes a defect in phagocytosis of apoptotic cell corpses that is likely caused by RAB-5 over-activation (Li et al., 2009). TBC-2, although weakly, also catalyze hydrolysis of GTP on RAB-7. Moreover, TBC-2 colocalizes with RAB-7, but not RAB-5, on the late endosomal membrane in intestinal cells (Figure I6). Furthermore, TBC-2 localization to vesicular membranes requires RAB-7 (Figure I7), suggesting that TBC-2 is recruited by RAB-7 (Chotard et al., 2010). However, a direct physical interaction between TBC-2 and RAB-7 has not been detected. The recruitment of TBC-2 by RAB-7 is likely to be indirect. It is not known if membrane localization is required for TBC-2 function or what proteins might bridge an interaction between TBC-2 and RAB-7.

TBC-2 shows high homology with the human TBC1D2/PARIS-1/Armus and TBC1D2B (Figure I4A and B). TBC1D2/PARIS-1 was identified as a prostate tumor antigen and is highly expressed in some prostate cancer cell lines (Zhou et al., 2002). Armus is a TBC1D2 isoform and differs by three polymorphic sites. Armus has been shown to be a Rac1 effector that inactivates Rab7 to regulate E-cadherin degradation (Frasa et al., 2010). It suggested that in *C.*

elegans, TBC-2 might function similarly with CED-10, a Rac1 homolog, to inactivate RAB-7.

Other small GTPases that controls endosomal trafficking

In addition to Rab GTPases, Arf and Rho family small GTPases are also participate in intracellular trafficking. The Arf (ADP-ribosylation factor) family consists of three groups: Arf, Sar, and Arl (Arf like) GTPases (Goldberg, 1998). All members in the Arf group localized to the Golgi, except active Arf6 that is also present on the plasma membrane, to control specific coated-vesicle membrane dynamics and transport of those vesicles to the Golgi (Kahn, 2009). The Sar group is the most divergent in sequence amongst the three groups (Li et al., 2004). One member in yeast, Sar1, has been shown to have ER localization, where it binds to a component of COPII-coated vesicle (Bi et al., 2002). Members of the Arl group are evolutionarily conserved, but little is known about their roles in intracellular trafficking. Arl1 is targeted to Trans-Golgi Network and mediates vesicle tethering. A recent study shows that ARL-8 mediates late endosome to lysosome fusion in *C. elegans* (Nakae et al., 2010). In mammalian cell lines, one of the two human Arl8 isoforms, Arl8b, binds to Vps41, a Rab7 effector, and recruits the HOPS complex to the lysosome. Phagosome-lysosome fusion is also impaired in Arl8b depleted cells (Garg et al., 2011). It is tempting to propose that Arl8 might form a complex with Rab7 or TBC-2, regulating Rab5 to Rab7 conversion.

Phosphoinositides and membrane trafficking

Phosphoinositides (PI) are phosphorylated intermediates of phosphatidylinositol (PtdIns) that was initially discovered as precursors of soluble or membrane-bound second messengers for signal transduction. Phosphoinositides are a minor constituent of the phospholipid bilayers and are turned-over continuously (Vicinanza et al., 2008). There are seven species of phosphoinositides, each with distinct regulatory functions (Cantley, 2002). PI metabolism is temporally and spatially controlled by its kinases and phosphatases. Due to the distinct distribution of these enzymes and their substrate specificity, a heterogeneous distribution of the PIs is generated through the cell. (De Matteis and Godi, 2004).

PI(3)P and PI(3,5)P_2 are enriched on early and late endosomes respectively (Cantley, 2002). PI(3)P is generated by phosphorylating at the 3-position of the inositol head group by a class III PI 3-kinase, such as Vps34. Vps34 is the only class III PI 3-Kinase in yeast. It interacts with the protein kinase Vps15, which is required for membrane targeting of the complex (Schu et al., 1993; Stack et al., 1995). Moreover, Rab5 binds to Vps34/Vps15 complex and is required for the membrane recruitment of the complex (Christoforidis et al., 1999; Murray et al., 2002). Rab5 then can recruit FYVE domain containing effectors, which will bind to PI(3)P, such as early endosomal antigen 1(EEA1) (Simonsen et al., 1998). Vps34 generated PI(3)P is essential for protein sorting and autophagy in yeast .

Synthesis of $PI(3,5)P_2$ requires the precursor PI(3)P (Dove et al., 1997). In yeast, Fab1 was identified as the kinase responsible for $PI(3,5)P_2$ synthesis (Cooke et al., 1998). Fab1 binds to PI(3)P via its FYVE domain (Gary et al., 1998). The mammalian homolog PIKfyve binds to PIs in a similar fashion and is shown to be involved in endosome/lysosome trafficking (Ikonomov et al., 2001; Sbrissa et al., 1999). Fab1, in order to have its enzymatic activity, forms a complex with a scaffold protein Vac14 (Jin et al., 2008). Vac14 binds to a $PI(3,5)P_2$ specific phosphatase Fig4. Fig4 is required for Fab1-Vac14 complex formation, thus coupling $PI(3,5)P_2$ synthesis with its turnover (Botelho et al., 2008; Duex et al., 2006; Sbrissa et al., 2008). Increasing number of cases show that unique phosphinositides interact with the Arf and Rab family GTPases to direct organelle-specific trafficking. In addition, phosphoinositides can also control membrane-cytoskeleton interactions and signal transduction at the plasma membrane that are important for maintaining membrane dynamics and trafficking (Mayinger, 2012).

CED-10 in endocytic and phagocytic pathways in *C. elegans*

A recent study suggests that Rac1 might have a novel role in regulating endosomal trafficking. Rac1 can inactivate Rab7 via direct physical interaction with TBC1D2/Armus, which is a bona fide GAP for Rab7 (Frasa et al., 2010). CED-10 is the Rac1 homolog in *C. elegans*. It raises the question if CED-10 can bridge the cross talk between TBC-2 and RAB-7 in nematodes. Consistent physical interaction was observed between TBC-2 and CED-10 in GST pull-down assays, suggest the interaction is evolutionarily conserved (Sun et al., 2012).

Moreover, CED-10 is required for efficient recruitment of TBC-2 to endosomal membranes. Loss of CED-10 also results in disruption of endocytic recycling. However, overexpressing TBC-2 greatly rescues the CED-10 phenotype, suggest TBC-2 is the major, possibly the only, effector of CED-10 in regulating endosomal trafficking.

In *C. elegans*, over 20 genes participate in three partial redundant pathways that might contribute to the activation of CED-10 (Kinchen, 2010). In one of the three pathways, the Rho small GTPase GEF, UNC-73 (Trio), activates the small GTPase MIG-2(RhoG) (deBakker et al., 2004). MIG-2 recruits the CED-5(DOCK180)-CED-12(Elmo) bipartite GEF complex to activate CED-10. The GEF complex is stabilized by adaptor protein CED-2 (CrkII) (Akakura et al., 2005; Gumienny et al., 2001). Phosphoinositides are crucial regulators of cytoskeletal dynamics and intracellular trafficking (Simonsen et al., 2001). The mammalian ortholog of CED-5, DOCK180 is predicted to bind $PI(3,4,5)P_3$ and $PI(3,5)P_2$ via its DHR1 domain (Cote et al., 2005). The PH domain of CED-12 also has the ability to bind $PI(3,5)P_2$. It has been proposed that the recruitment of CED-5-CED-12 GEF complex is mediated through binding of $PI(3,5)P_2$ (Neukomm et al., 2011).

Once a cell is committed to death, a neighboring cell or specialized phagocytotic cell will recognize, internalize and degrade the cell corpse to ensure that harmful intracellular contents will not be released into the surrounding tissue (Savill and Fadok, 2000). CED-10 is important for the engulfment of apoptotic cell corpses. Once CED-10 is activated, it leads to an extensive re-organization of

the cytoskeleton that initiates the internalization of the cell corpse (Kiyokawa et al., 1998; Reddien and Horvitz, 2000). Even the CED-5-CED-12-CED-10 pathway is well characterized in phagocytosis, its role, including the requirement of phosphoinositides in this pathway, is still unclear in endocytic processes.

The *C. elegans* intestine as a model for endocytic trafficking

C. elegans is a nematode that was originally used as model organism for study in development and cell lineage. The animal is transparent, allowing easy observation of some intracellular organelles under the Differential Interference Contrast (DIC) optics and visualization of fluorescence fusion proteins to characterize protein functions (Chalfie et al., 1994). The intestine is one of the largest organs in *C. elegans* and its function involves food digestion and fat storage (McGhee, 2007). There are 20 polarized cells in the intestine, arranged in 9 rings. The apical side of intestinal cells has a microvilli brush border that forms part of the lumen. The basal side is covered with lamina, which provides mechanical integrity of the intestine. Given the function of the intestine, it is expected that endocytosis is highly active, since nutrients must be taken into the cell and transported to the rest of the animal. Even though endosomal trafficking is most studied in oocytes and coelomocytes, a number of genes that are involved in vesicular trafficking, when mutated, also show change in morphology of the intestinal cells. Such genes include *rab-10, tat-1, rme-1, ppk-3* and *tbc-2* (Chen et al., 2006; Chotard et al., 2010; Grant et al., 2001; Nicot et al., 2006; Ruaud et al., 2009). The intestine contains numerous membrane bound vesicles and vacuoles, as well as autofluorescent gut granules, which are visible under the DIC optics

(McGhee, 2007). The high level of traffic and good visibility of the vesicles makes *C. elegans* intestinal cells a good model to study endosomal trafficking.

Rationale and objectives

Vesicular trafficking is an important cellular process in transporting cargo between membrane-bound organelles. The vesicular budding, transporting, tethering, and fusion steps of trafficking are regulated by Rab GTPases. A process of Rab5 to Rab7 conversion regulates early to late endosome maturation. As a negative regulator of Rab GTPases, Rab GAPs could be potential therapeutic targets for drug development. Previous studies have shown that *C. elegans* TBC-2 has GAP activity towards RAB-5 and possibly also RAB-7 during endosomal maturation. However, it is still not clear how TBC-2 is recruited onto vesicular membranes, which other Rab GTPases TBC-2 regulates, and what functional relationship between TBC-2 and other proteins.

The goal of my study is to further identify and characterize proteins that function closely with TBC-2 in regulation of early to late endosome maturation. Work in the thesis is focused on identification of potential genetic TBC-2 interactors and RAB-7 regulators participating in TBC-2 mediated endocytic regulations.

Material and Method

C. elegans alleles and general methods

C. elegans culture and genetics were performed according to standard protocols, using the *E. coli* strain HB101 as a food source (Brenner, 1974). All strains used in this work are derived from *C. elegans* var Bristol strain N2. Strain GE2158 was used in deficiency test. Specific genes and alleles are described on Wormbase (www.wormbase.org) or otherwise stated. N2 and GE2158 strains are available from the Caenorhabditis Genetic Centre (CGC, University of Minnesota, Minneapolis, MN, USA). *tbc-2(tm2241)* strain was a gift from Dr. Mitani (National Bioresource project, Tokyo Women's Medical Univeristy School of Medicine, Tokyo, Japan) *obr-1(xh16)* was a gift from Dr. Takao Inoue (Tokyo University, Tokyo, Japan). YB388, *arl-8(tm2508)/nT1; Ex[ARL-8::GFP]* was kindly provided by Dr. Toshiyaki Kadata (Tokyo University, Tokyo, Japan). *pwIs170 [Pvha-6::GFP::rab-7 + Cb-unc-119(+)], pwIs429[Pvha-6::mCherry::rab-7],* and *pwIs731[Pvha-6::GFP::CED-10]* were kindly provided by Dr. Barth Grant (Rutgers University, New Jersey, USA), *vhIs12[Pvha6::GFP::tbc-2 + Cb-unc-119(+)],* and *vhIs23[Pvha6::GFP::tbc-2 R689K+cb-unc-119(+)]* were generated previously in lab by Dr. Laetitia Chotard (McGill University, Quebec ,Canada). *tbc-2(tm2241); CED-10::GFP (pwIs731)* was generated previously in lab by Dr. Christian Rocheleau (McGill University, Quebec ,Canada). *obr-1(xh16); tbc-2(tm2241); GFP::RAB-7(pwIs170)* and *CED-10::GFP(pwIs731);mCherry::RAB-7(pwIs429)* were generated in this study.

Deficiency Mapping

Wild type N2 fourth larval stage (L4) males were mated with *vh8/hT2; GFP::RAB-7* L4 hermaphrodites at a ratio of 5:1. The *hT2* balancer is marked with *myo-2::GFP* which is expressed strongly in the pharynx. The resulting *vh8/+; GFP::RAB-7* male progeny were then picked under the epifluorescent dissecting scope (Leica) to mate with *tDf2/qC1 dpy-13 glp-1(GE2158)* hermaphrodites. The progeny were allowed to grow to L4 stage and only those with a male appearance were picked for scoring. The criteria of a transformer phenotype were set to be the presence of oocytes in male gonads and a partially formed male tail.

RNA interference

RNAi by feeding was performed as previously described by Kamath et al. (Kamath and Ahringer, 2003). The RNA feeding clones *vps-16*(III-3K20), *vps-33.1*(III-4L17), *obr-1*(III-5P10), *C32A3.3*(III-1F24), *rab-7*(II-8G13), *ced-5*(IV-5F11), and *vps-34*(I-2F20) are from the Ahringer library (www.geneservices.co.uk). Clones were verified by DNA sequencing. Double stranded RNA(dsRNA) production was induced by 0.5mM IPTG. L4 animals were placed on plates containing feeding bacteria expressing dsRNA of each RNAi clone or empty L4440 vector in control. Progeny born 48-72 hours after plating were scored at L3 or L4 stage.

Microscopy and Phenotype Analysis

Live animals were mounted on 1% agarose pad with 100mM levamisol in water as described in wormbook (www.wormbook.org). L3, L4 and young adult animals were analyzed. Axio Zeiss A1 Imager compound microscope (Carl Zeiss, Oberkochen, Germany) was used to obtain DIC and epifluorescent images, and images were captured using an Axio Cam MRm camera and AxioVision software (Carl Zeiss) and processed using Adobe Photoshop CS2 software (Adobe, California, USA). For Confocal microscopic analysis, the animals were prepared in the same way. Animals were analyzed using a Zeiss LSM-510 Meta laser scanning microscope (Carl Zeiss Microimaging, Oberkochen, Germany) with 63X oil immersion objective lens in a single or multitrack mode by using a single or dual excitation (488 nm for GFP and/or 543 nm for mCherry). Images were captured using LSM Image software and Zen2009 software (Carl Zeiss, Oberkochen, Germany) and processed using ImageJ software (National Institute of Mental Health, Maryland, USA). Spectral fingerprinting function was used to eliminate background caused by non-specific autofluorescence in intestine as previously described (Chen et al., 2006).

Result

vh8 is not a mutation in the *obr-1* or *C32A3.3* genes

The *vh8* mutation was previously identified in the lab as a strong suppressor of the *tbc-2(-)* phenotype during a mutagenesis screening of 4500 haploid genomes (K. Bhende and C. Rocheleau, unpublished data). *vh8; tbc-2(-)* homozygous animals have smaller intestinal vesicles, as compared to *vh8/+;tbc-2(-)* heterozygous animals (Figure R1A), or *tbc-2(-)* alone (Figure I5B). In addition to suppressing the *tbc-2(-)* large endosome phenotype, GFP::RAB-7 is displaced from the vesicle membranes in *vh8; tbc-2 ;GFP::RAB-7* animals, suggesting that the gene mutated by *vh8* is required for recruitment of RAB-7 to endosomes. Chromosomal mapping showed that *vh8* is located on chromosome III. *vh8* homozygous animals are also sterile and have a transformer phenotype: an XX hermaphrodite with male phenotypes such as a male tail and male gonad. The *tra-1(e1099)* mutant failed to complement the transformer phenotype of *vh8*, but *tra-1(e1099)* did not suppress the *tbc-2(-)* phenotype (A. Chavolovski and C. Rocheleau, unpublished data). The above data suggest that *vh8*, being a suppressor allele of *tbc-2(-)* phenotype, is linked to a second mutation in *tra-1*.

There are several genes that encode for putative endocytic regulators in vicinity of *tra-1* that are good candidates for being the gene carrying the *vh8* mutation. *obr-1* and *C32A3.3* encode proteins with homology to the Rab7 effectors ORP1L and RILP respectively. I performed RNAi experiments to knock down the two abovementioned genes individually in *tbc-2(tm2241); GFP::RAB-7*

animals to determine if either had phenotypes similar to *vh8*. Scoring under DIC optics, the RNAi of either gene failed to suppress of *tbc-2(-)* phenotype (Figure R2 G-J). The RNAi experiment suggests that OBR-1 or C32A3.3 are not required for the *tbc-2(-)* phenotype. However, a negative RNAi result is not informative as RNAi is not always effective at knocking down gene activity. To further test the requirements for OBR-1, I constructed a *obr-1(xh16); tbc-2(tm2241); GFP::RAB-7* double mutant. *xh16* is a strong loss of function mutation that resulted from a deletion in the *obr-1* locus (Kobuna et al., 2010). The double mutant also shows no suppression of *tbc-2(-)* phenotype (Figure R2 K and L). The results suggest that *vh8* is not a mutation in *obr-1* or *C32A3.3*.

Core components of the HOPS complex are required for the *tbc-2(tm2241)* phenotype

Other candidate genes for *vh8* are *vps-16* and *vps-33.1.* They both locate on chromosome III close to *tra-1* and encode for two core components of the HOPS complex that are also present in the CORVET complex. It has been shown previously that VPS-39 and VPS-41, two components that are unique to the HOPS complex are required for the *tbc-2(-)* phenotype, but do not displace GFP::RAB-7 from the endosomal membranes (Chotard et al., 2010). To further investigate requirement of the core components of the HOPS complex, I performed RNAi experiments of *vps-16* and *vps-33.1* in the *tbc-2(-); GFP::RAB-7* strain. The results show that both *vps-16* and *vps-33.1* are required for *tbc-2(-)* large vesicle phenotype. However, neither RNAi displaces GFP::RAB-7 from the endosomal membrane to the same degree as seen in *vh8/vh8* mutant (Figure R1A),

indicating that *vh8* is not likely to be a mutation in either *vps-16* or *vps-33.1* (Figure R2 C-F). These results indicate that the core components of the HOPS complex are required for *tbc-2(-)* phenotype.

vh8 **complements the** ***tDf2*** **chromosomal deficiency**

To further refine the position of *vh8*, I performed deficiency mapping using the *tDf2* deficiency allele, which deletes a portion of chromosome III from map unit position 5.48 to 8.35. Since *tDf2* deficiency deletes *tra-1* gene from chromosome III, all *vh8/tDf2* mutant animals should have the same transformer phenotype as *vh8* mutants. If *vh8* is inside *tDf2*, the *vh8/tDf2* transformer animals should have same GFP::RAB-7 displacement as seen in *vh8;GFP::RAB-7* animals (Figure R1C). If *vh8* were outside of the deficiency, it should complement *tDf2*, therefore resulting a wild-type GFP::RAB-7 localization in the intestine in *vh8/tDf2* transformer animals. To construct the *vh8/tDf2; GFP::RAB-7* mutants, *vh8/hT2; GFP::RAB-7* hermaphrodites were crossed with wild-type males. The resulting *vh8/+;GFP::RAB-7* males were then crossed with GE2185 *tDf2/qC1 dpy-19(e1259) glp-1(q339)III* hermaphrodites. The transformer animals from the progeny were picked and examined under the DIC optics. In contrast to diffused GFP::RAB-7 seen in *vh8; GFP::RAB-7* animals, *vh8/tDf2; GFP::RAB-7* animals show wild-type localization of GFP::RAB-7 also seen in *vh8/+; GFP::RAB-7* (Figure R1 B-D). The fact that *tDf2* complements GFP::RAB-7 localization phenotype in *vh8* animals suggest that *vh8* is located outside of the region 5.48 to 8.35 on chromosome III.

ARL-8 is require for the *tbc-2(-)* phenotype, but not for membrane localization of RAB-7 or TBC-2

In search for additional regulators of TBC-2 in endosomal trafficking, I performed *arl-8(RNAi)* on *tbc-2; GFP::RAB-7* and *tbc-2; GFP::TBC-2 R689K* strains and examined the intestinal vesicle phenotype using DIC optics. Wild-type ARL-8 localizes to vesicles in coelomocytes and intestinal cells (Nakae et al., 2010). In coelomocytes ARL-8 has been shown to colocalize with RAB-7 and LMP-1 on late endosomes and lysosomes, respectively. In *arl-8* mutants, endocytosed macromolecules cannot be trafficked to lysosomes properly. Therefore ARL-8 likely functions closely with RAB-7 and TBC-2. I found that an *arl-8* mutant results in supernumerary vesicles in the intestine, similar to loss of *rab-7* (Figure R3 A and B). Also similar to *rab-7(RNAi), arl-8(RNAi)* suppresses the *tbc-2(-)* large intestinal vesicle phenotype (Figure R3 C-F).

It was previously shown that RAB-7 is required for TBC-2 membrane localization (Chotard et al., 2010). However, the direct physical interaction between RAB-7 and TBC-2 was never discovered. To test if ARL-8 plays a role in TBC-2 recruitment by RAB-7, I examined the localization of GFP::TBC-2 and GFP::RAB-7 in *arl-8(RNAi)* animals using the confocal microscopy. The results show that in while *arl-8(RNAi)* can efficiently suppress the *tbc-2(-)* phenotype, membrane localization of GFP::RAB-7 and GFP::TBC-2 R689K, was not affected (Figure R3 C-F). These results suggest that ARL-8 is required for the large late endosome phenotype of *tbc-2* mutants, but is not required for membrane recruitment of TBC-2 or RAB-7.

CED-10 localization does not require *rab-7* or *tbc-2*

Literature suggests that mammalian Rac1 protein can bind Rab7 and Armus/TBC1D2, which is the TBC-2 homolog in human (Sun et al., 2005) (Frasa et al., 2010). GST pull-down experiments done in the lab shows that TBC-2 can physically interact with all three *C. elegans* Rac proteins: CED-10/Rac1, RAC-2 and MIG-2 (Sun et al. 2012; F. Karbassi and C. Rocheleau, unpublished data). That raises the possibility that RAB-7 recruits TBC-2 onto maturing endosomal membrane via one or more Rac proteins. To test this hypothesis for CED-10, I used mutant strains to test if TBC-2 is required for GFP::CED-10 localization, and *rab-7* RNAi on both GFP::CED-10 and *tbc-2* GFP::CED-10 strains to test if RAB-7 is required for GFP::CED-10 localization. In wild type worms, GFP::CED-10 localized to vesicular structures in the intestine in addition to cell junctions and the apical membrane. In *tbc-2(-)* mutant animals, localization of GFP::CED-10 on apical membrane and cell junction was not affected. Interestingly, GFP::CED-10 is localized to enlarged vesicles in *tbc-2(-)* animals (Figure R4 A and B). Loss of RAB-7 by RNAi mediated knock-down in both wild-type and the *tbc-2* mutant does not change GFP::CED-10 localization (Figure R4 C and D) . Transgenic animals carrying both CED-10::GFP and mCherry::RAB-7 are examined under the confocal microscope. Preliminary data suggest no colocalization of CED-10::GFP and RAB-7::mCherry (Figure R3 I-K). These data suggest that CED-10 is unlikely to bridge an interaction between TBC-2 and RAB-7.

CED-12/CED-5 was shown to be a bipartite GEF complex required for CED-10 activation (Brugnera et al., 2002). Literature suggests that the recruitment or activation of the complex may be mediated by phosphoinositol-(3,5)-biphosphate ($PI(3,5)P_2$) (Neukomm et al., 2011). The synthesis of $PI(3,5)P_2$ requires the precursor phosphoinositides-3-phosphate (PI(3)P), which is synthesized by Class III PI3-kinase VPS34 (Dove et al., 1997; Vanhaesebroeck et al., 2001). To determine if CED-10 localization is affected by its active state, I performed RNAi mediated knock down of *ced-5* and *vps-34* on CED-10::GFP in wild-type and *tbc-2(-)* mutant strain and examined the animals under the confocal microscope. CED-10::GFP still localized to vesicles in both *vps-34(RNAi)* and *ced-5(RNAi)* animals in both wild-type and the *tbc-2(-)* background (Figure R4 E-H). The result suggests that CED-5 and VPS-34 might not be required for GFP::CED-10 localization.

Discussion

The purpose of this project is to identify additional genes that function with TBC-2 to regulate early to late endosome maturation. Here I further mapped the *vh8* mutation, a suppressor of the *tbc-2(-)* phenotype and in the process identified core components of the HOPS complex as also being required for the *tbc-2(-)* phenotype. ARL-8 and CED-10, which are important regulators in phagocytic pathways, were tested for their roles in endocytic pathway. I found that *arl-8* was required for the *tbc-2(-)* phenotype, but not for the localization of TBC-2 or RAB-7 proteins. I found that CED-10 localized to the large late endosomes of *tbc-2(-)* intestinal cells and this localization was independent of *ced-5, rab-7* and *vps-34*. .

The *vh8* mutation was identified as a suppressor of the *tbc-2(-)* phenotype (K. Bhende and C. Rocheleau, unpublished). Moreover, in *vh8* mutants, GFP::RAB-7 is displaced from vesicle membranes. Loss of RAB-7 from membranes suggests that RAB-7 cannot be activated properly in *vh8* mutants. It is tempting to hypothesize that *vh8* is a loss of function mutation allele of a RAB-7 GEF. Ccz1p/Mon1p complex has been demonstrated to be the Ypt7p GEF in yeast (Nordmann et al., 2010). In *C. elegans*, a homologous complex CCZ-1/SAND-1 has been shown to be able to displace a RAB-5 GEF and to recruit RAB-7 (Cabrera and Ungermann, 2010), raising the possibility that CCZ-1/SAND-1 complex can be the RAB-7 GEF. However, neither *sand-1* nor *ccz-1* is on the same chromosome as *vh8* and thus are not candidate genes. It is possible that there might be multiple RAB-7 GEFs in *C. elegans*, one of such could be

encoded by *vh8*. I tested several candidate genes on chromosome III by RNAi, but none were required for RAB-7 membrane localization, thus not likely the gene mutated in *vh8*.

The RNAi screening show that two core components of the HOPS complex are required for the *tbc-2(-)* phenotype. It is consistent with previous data that the HOPS complex might be required for Rab5 to Rab7 transition (Poteryaev et al., 2010). The HOPS complex has previously been thought to be the Rab7 GEF until recently (Wurmser et al., 2000). VPS-16 and VPS-33.1 are the two core components of the HOPS complex (Sato et al., 2000). My results show that *vps-16(RNAi)* and *vps-33.1(RNAi)* suppress the *tbc-2(-)* phenotype, but RAB-7 is still on membranes, suggesting that RAB-7 remains active in absence of the HOPS complex. It is consistent with the idea that the HOPS complex is not a RAB-7 GEF in *C. elegans*. The suppression of *tbc-2(-)* phenotype in *vps-16* and *vps-33.1* RNAi experiments can serve as a control for induction of dsRNA expression and consequent knock-down of the corresponding gene expression.

OBR-1 and C32A3.3 are *C. elegans* homologs of two mammalian Rab7 effectors, ORP1L and RILP. ORP1L and RILP regulate vesicle motility (Johansson et al., 2007). It was previously proposed that the *tbc-2(-)* phenotype could be caused by too much RAB-7 activity, leading to excess OBR-1 and C32A3.3 binding to the dynactin-dynein motor, resulting in the accumulation and the subsequent fusion of vesicles. My data from the RNAi experiments show that *tbc-2(-)* phenotype was not suppressed in the *obr-1* and *C32A3.3* RNAi experiments. Unfortunately, in those two RNAi experiment, there is no other

visible phenotype produced, therefore we cannot confirm the knock down of respective gene expression. No conclusion can be drawn from those experiments. A RT-PCR could be performed on treated animal to quantitatively confirm the knock down of particular gene expression.

ARL-8 and its human homolog Arl8b were recently found to regulate late endosome to lysosome trafficking. ARL-8 localizes with RAB-7 on late endosomes suggesting that it could function closely with TBC-2. I found that *arl-8* RNAi suppresses the *tbc-2(-)* phenotype, but does not affect RAB-7 or TBC-2 localization to endosome membranes. Therefore, ARL-8 could function downstream of TBC-2 or RAB-7. In an *arl-8(-)* mutant, endocytosed cargo is trapped in the late endosome, failing to be trafficked to lysosomes (Nakae et al., 2010). Based on ARL-8 localization and suppression of *tbc-2(-)* phenotype by *arl-8* RNAi, it is tempting to guess that ARL-8 could be participating in the Rab cascade. RAB-7 or TBC-2 might recruit an ARL-8 GEF and active ARL-8 might recruit a RAB-7 GAP, driving the late endosome fusion with lysosome. The fact that Arl8b binds to Vps41 in mammalian cells suggests that the HOPS complex could facilitate this process (Garg et al., 2011).

Armus has been shown to be the effector of Rac1 in mammalian cells that physically binds to Rac1. The physical interactions also exist between TBC-2, homolog of Armus, and CED-10, homolog of Rac1 (Sun et al., 2012). That raises the possibility that RAB-7 could recruit TBC-2 to the membrane via CED-10. Consistent with such a hypothesis, *ced-10* was recently shown to be required for membrane localization of TBC-2 (Sun et al., 2012) and I found TBC-2 is not

required for CED-10 membrane localization. Therefore CED-10 might function upstream of TBC-2. Moreover, I found that CED-10 localizes to the enlarged vesicles in *tbc-2(-)* animals. It was previously shown that both RAB-7 and catalytically inactive TBC-2 localized on the enlarged late endosomes in the *tbc-2(-)* intestinal cells. It is possible that CED-10 is colocalized with RAB-7 or TBC-2 on these vesicles. However, my results show that in wild-type animals, CED-10 does not significantly colocalize with RAB-7. It could be that CED-10 localizes to membranes regardless of its active state, but to different endosomal compartments in wild-type and *tbc-2(-)* mutants. It is also possible that the increased Rab GTPase activity in *tbc-2(-)* animals results in increased recruitment of CED-10 to endosomes. To make the data more quantitative, fluorescent intensity profiling can be done across the vesicle on each channel to confirm the localization of CED-10 on membrane.

CED-10 is activated by the bipartite CED-5/CED12 GEF complex in the phagocytic pathway in *C. elegans* (Gumienny et al., 2001). The complex is proposed to be recruited by $PI(3,5)P_2$, which requires VPS-34 generated PI(3)P (Vanhaesebroeck et al., 2001; Zou et al., 2009). Therefore, knockdown of either CED-5 or VPS-34 should alter the active state of CED-10 and presumably displace it from the vesicular membrane. However, my data suggest that CED-10 is still localized on endosomes in the intestinal cells of *ced-5(RNAi)* and *vps-34(RNAi)* animals. Vps34 in yeast is considered as the only PI3 kinase that generates PI3P (Schu et al., 1993). However, there might be additional PI3P kinase in *C. elegans* that function redundantly with VPS-34. There are two other

pathways that regulate CED-10 activity in *C. elegans* phagocytic regulation. One pathway involves ABL-1 regulating remodeling of the cytoskeleton through ABI-1, potentially through CED-10 (Hurwitz et al., 2009). Other pathway consists of CED-7 and CED-1 binding to adaptor protein CED-6, relaying the signal to CED-10 (Liu and Hengartner, 1998; Wu and Horvitz, 1998; Zhou et al., 2001). It is possible such redundant pathways also exist in endocytic regulation to compensate the loss of CED-5/CED-12 complex.

In conclusion, my work demonstrates that core components of HOPS complex and ARL-8 are required for *tbc-2(-)* phenotype. My data support the model that RAB-7 might recruit TBC-2 via CED-10, but not ARL-8. It has recently been demonstrated that TBC-2 and CED-10 function together during endosome recycling (Sun et al., 2012), the role of CED-10 in the early to late endosome maturation is still not clear.

Future directions

To further characterize *vh8*, a 3-point mapping can be conducted using visible maker such as Dpy and Unc. At the same time, more candidates can be tested using RNAi. Once *vh8* is mapped to a reasonable region, whole genomic sequencing can be conducted to reveal the location of the lesion.

In order to further study the mechanism of function of ARL-8, it would be helpful to first generate a transgenic line expressing ARL-8::GFP specifically in the intestine for a clearer visualization of ARL-8 during endocytic trafficking. To test if ARL-8 participates in the Rab cascade, RNAi experiment or mutant strains can be used to test if RAB-7 or TBC-2 is required for ARL-8 activation. RAB-7 and TBC-2 expression in can be knocked-down in animals expressing ARL-8::GFP fusion protein using RNAi techniques. Since active and inactive Arl8b localize to different cellular structures (Bagshaw et al., 2006), the difference in active state can be easily assessed under the confocal microscope. Biochemical analysis can be conducted at the same time. Use the constitutively active and inactive GST fusion construct of ARL-8 to test if TBC-2 binds to ARL-8 in a nucleotide sensitive manner. Even though ARL-8 does not seem to regulate TBC-2 membrane localization, a physical binding might still exist between TBC-2 and ARL-8 as ARL-8 could be activated and recruited to membranes by TBC-2.

To test if CED-10 participates in the Rab cascade, first we need to investigate if CED-10 localizes on the same vesicles in the presence and absence of TBC-2. We will make the *tbc-2(tm2241)*; GFP::CED-10; mCherry::RAB-7

transgenic animal and examine the localization of CED-10::GFP and mCherry::RAB-7 under the confocal microscope. To test if the active state of CED-10 alters the localization, the transgenic lines of the inactive and active CED-10 expressing in the *ced-10(-)* mutant should be examined. In wild-type animals, CED-10 localizes to puncta in the cytoplasm of intestinal cells. The identity of the vesicles should be assessed by expressing different endosomal markers in GFP::CED-10 animals and to check for colocalization. To test the previous hypothesis that PI(3,5)P mediates the recruitment of CED-5/CED-12 to membrane, *vps-34* should be knocked down in CED-5::GFP and CED-12::GFP strains. Testing if the CED-5/CED-12 complex changes its localization in *vps-34(-)* animals in response to its altered active state will help to define the mechanism of PPIn regulation of the complex, whether by localized activation or physical recruitment.

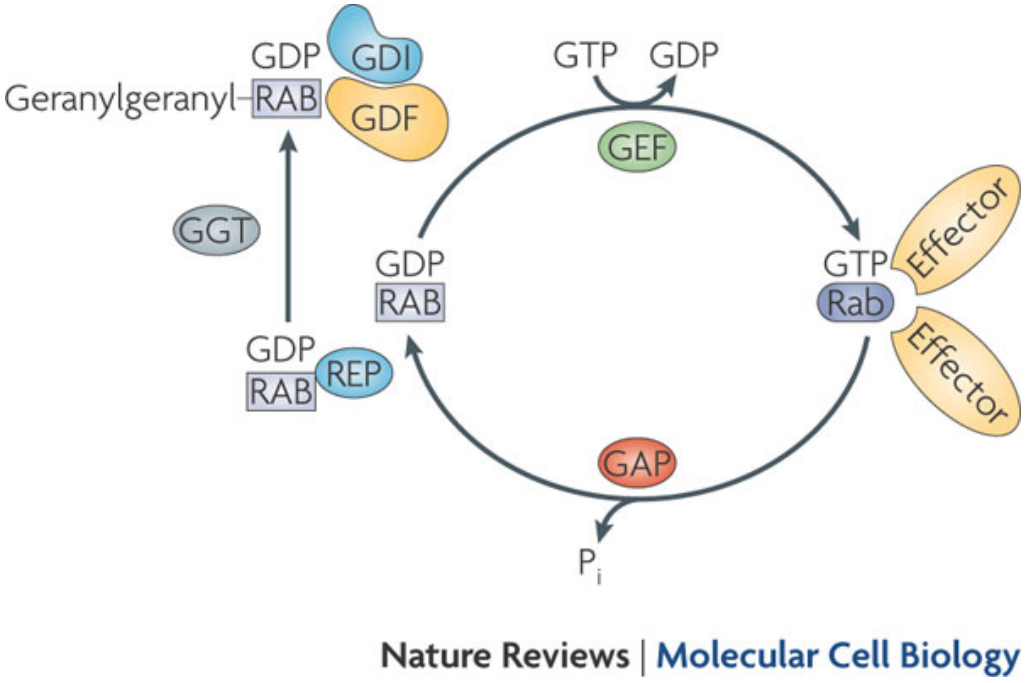

Figure I1. Regulation of Rab GTPases. The active state of Rabs are regulated by GEFs, which displace GDP and promote GTP binding, therefore activating the Rab. GTP-bound active Rabs can bind to effector proteins. The inactive state is promoted by GAPs, which catalyzes the hydrolysis of GTP. The inactive Rabs are stabilized in the cytoplasm by REPs (Rab escort proteins). Geranylgeranyl transferase adds a geranylgeranyl tail to GDP bound Rab, preparing it for future membrane association. The geranylgeranyl-Rab will be stabilized by GDI and GDF, until it is ready to be activated again. (From Stenmark. H, 2009)

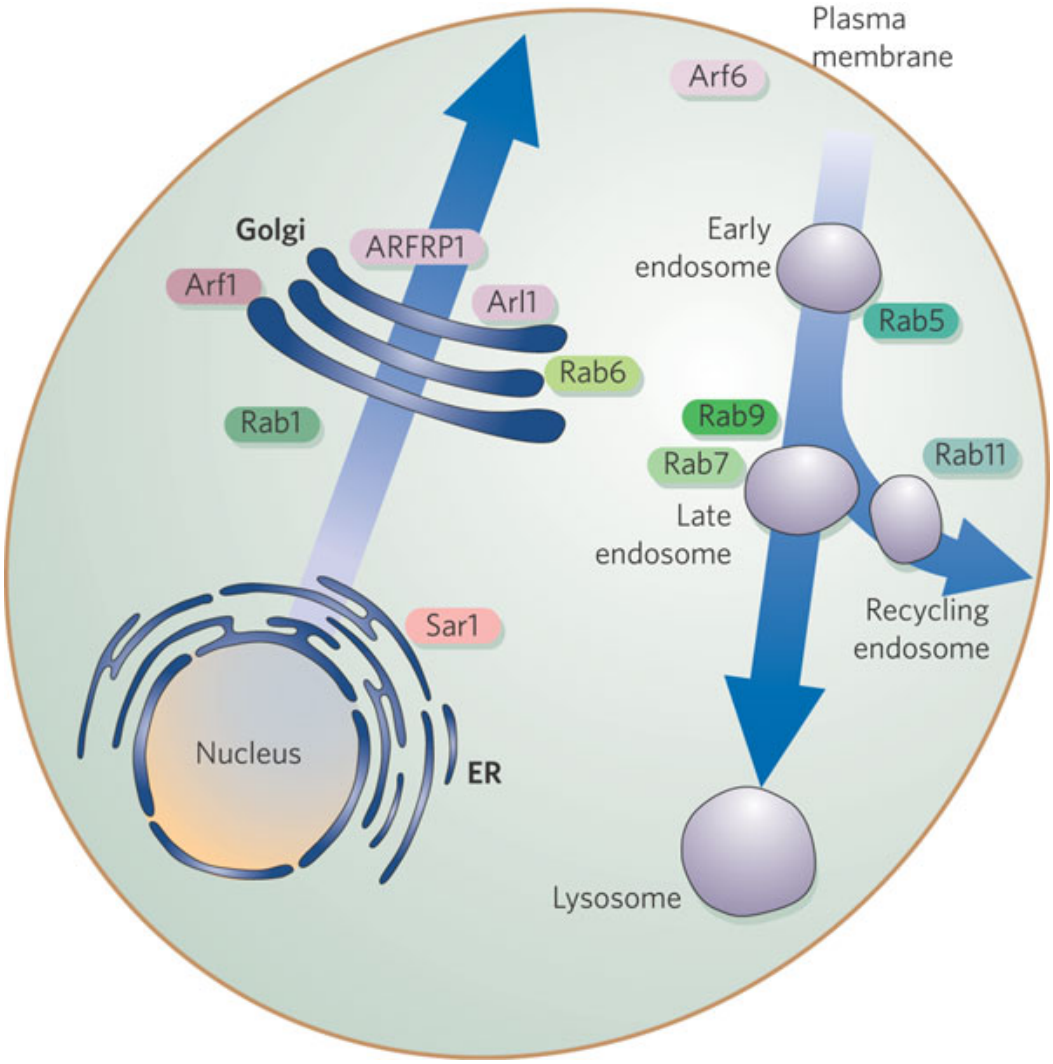

Figure I2. Localization of Rab and Arf GTPases. Arf (purple) and Rab (green) family GTPases are localized to membrane compartments. While some are found at multiple locations, most reside on only one organelle. The diagram shows only some of the better characterized GTPases, however many more exist. (From Behnia and Munro, 2005)

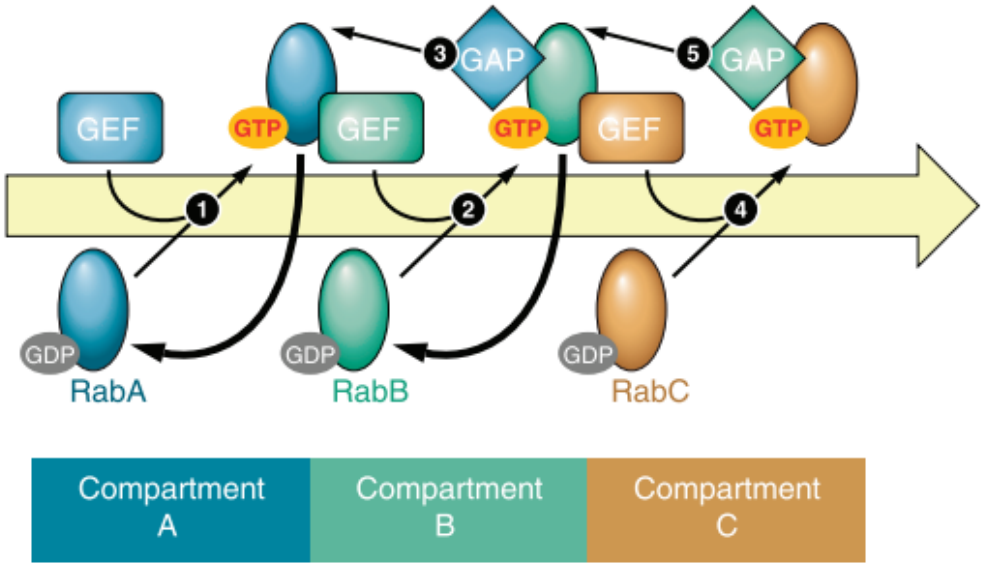

Figure I3. The Rab GEF and GAP cascade. RabA is activated by its GEF and localizes to a compartment (1). Once localized, active RabA will recuit the GEF of RabB (2). Activated RabB in turn recruits the GAP for RabA (3) to inactivate RabA, as well as recruit the GEF for RabC (4). Active RabC now can recruit the GAP of RabB (5). Here, the specific localization of an active Rab plays a role in restricting the action of the downstream Rab to a defined region. (From Hutagalung and Novick, 2011)

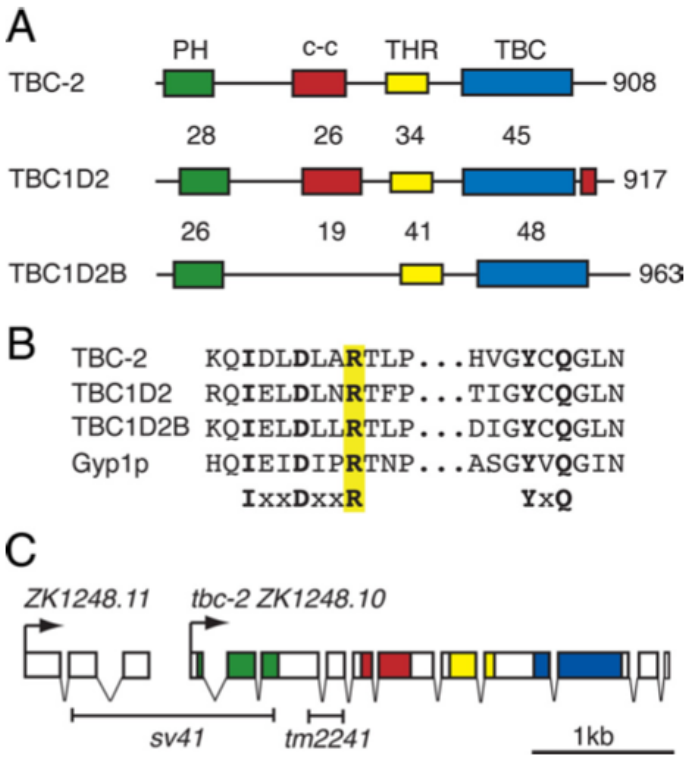

Figure I4. TBC-2: protein homology, TBC domain structure, and deletion alleles. (A) Schematic diagram of *C. elegans* TBC-2 and its human homologs TBC1D2 and TBC1D2B. The PH domain is represented in green, coiled-coil (c-c) domain in red, TBC homology region (THR) in yellow and TBC catalytic domain in blue. TBC1D2B lacks the coiled-coil domain. The percentage amino acid identity between TBC-2 and the two human homologues is shown above each domain. (B) Alignment of the TBC catalytic motifs IXXDXXR and YXQ from *C. elegans* TBC-2, human TBC1D2, and TBC1D2B and yeast Gyp1p. The conserved arginine finger is highlighted in yellow (R) and other residues required for activity in bold. (C) Genomic structure of *tbc-2 ZK1248.10* and the upstream gene *ZK1248.11*. The regions encoding the different domains in TBC-2 are highlighted in corresponding colors. The regions deleted by the deletion alleles *sv41* and *tm2241* are shown at the bottom. *tm2241* allele deletes intron 3, resulting in a frameshift and a premature stop codon. (From Chotard, et al. 2010)

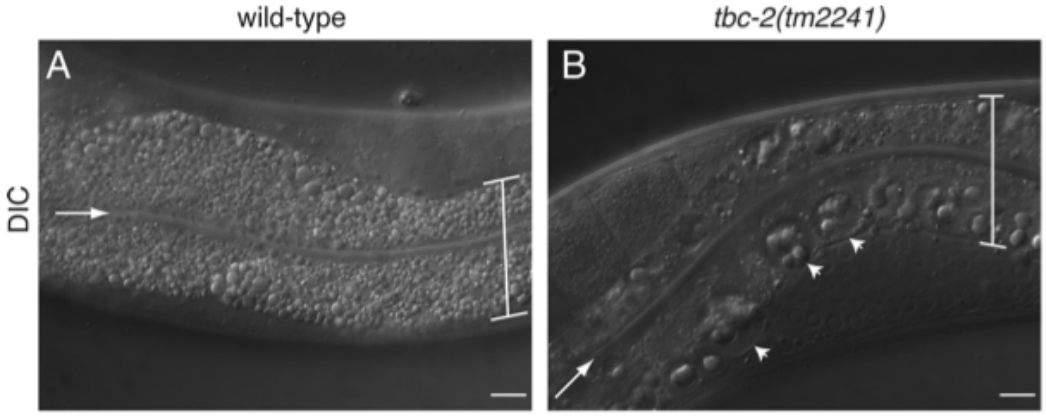

Figure I5. *tbc-2(-)* intestinal phenotypes. DIC images of the intestine of wild-type (A) and *tbc-2(tm2241)* (B) animals. Intestinal lumen is marked with a arrow and the boundaries of the intestine is marked using a bracket. Representative large vesicles in *tbc-2(tm2241)* animals are marked with arrowheads. (From Chotard, et al. 2010)

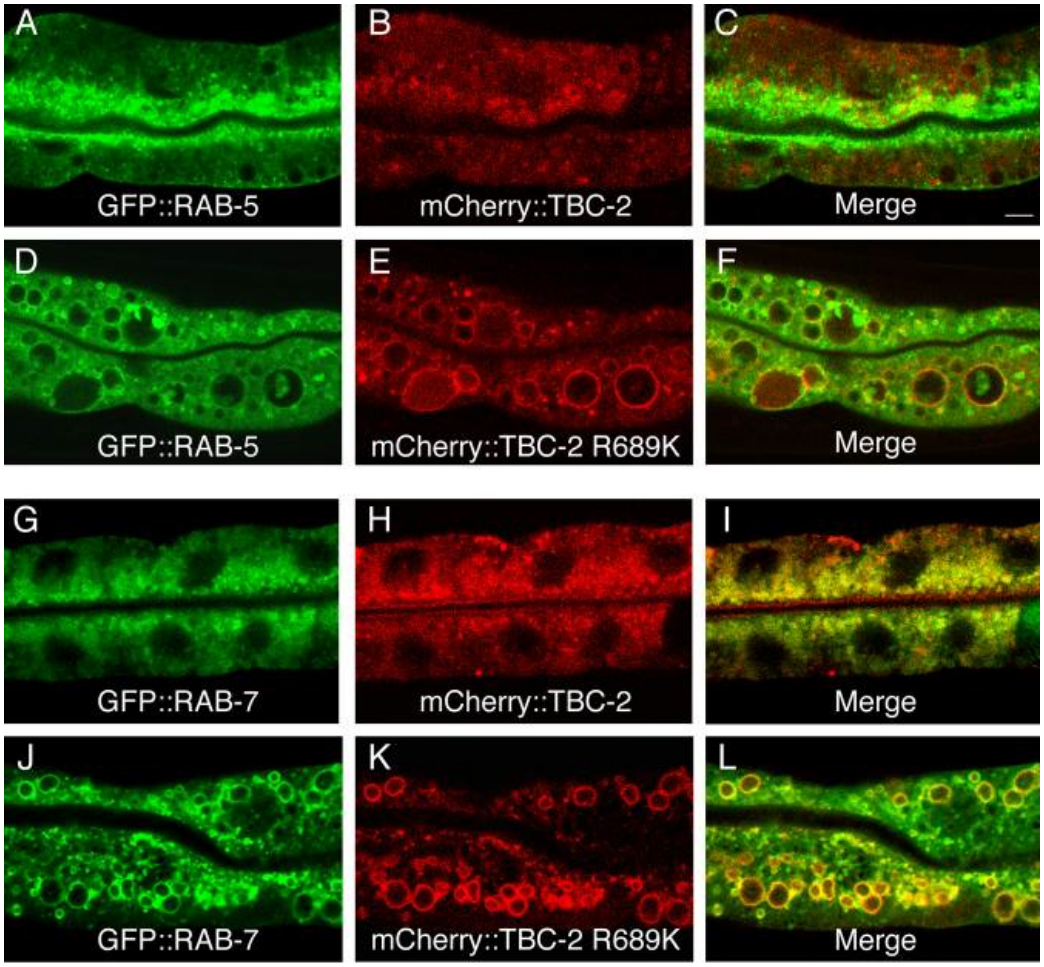

Figure I6. TBC-2 colocalizes with RAB-7 but not RAB-5. Confocal images of the intestine of *tbc-2(tm2241)* animals expressing GFP or mCherry fusion proteins. Each marker is labeled on the corresponding images. TBC-2 R689K is the catalytic inactive form of TBC-2 (From Chotard, et al. 2010)

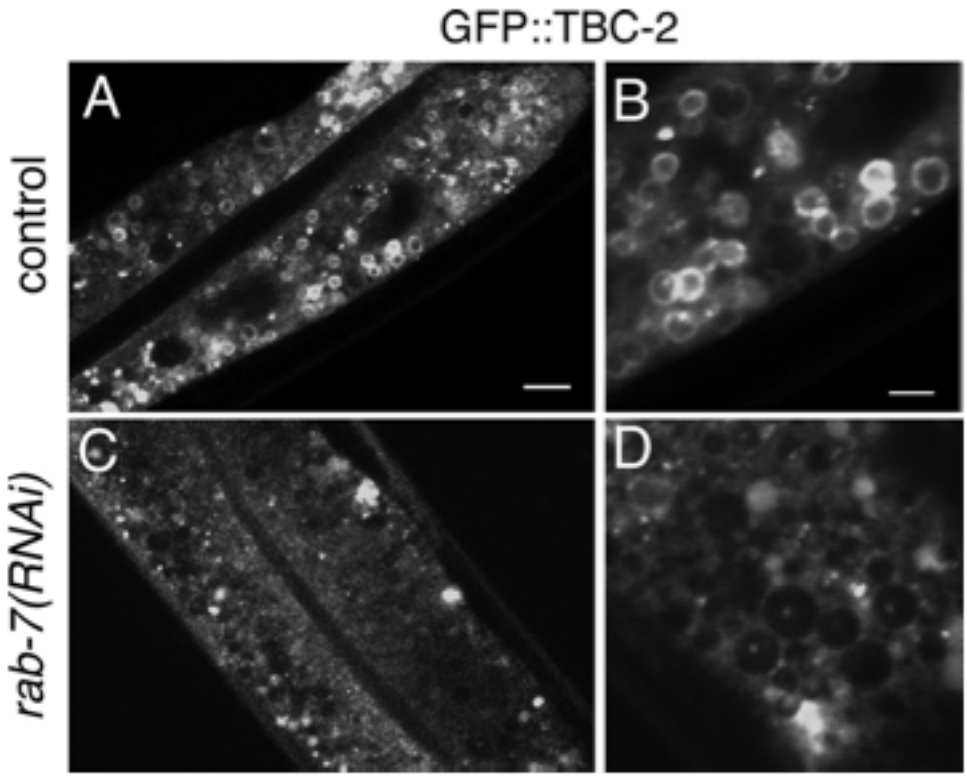

Figure I7. **RAB-7 is required for TBC-2 localization to membranes.** Confocal images of *tbc-2(tm2241); GFP::TBC-2* animals fed empty L4440 vector (A and B) or dsRNA targeting *rab-7* (C and D). B and D are higher magnification images of A and C respectively. (From Chotard, et al. 2010)

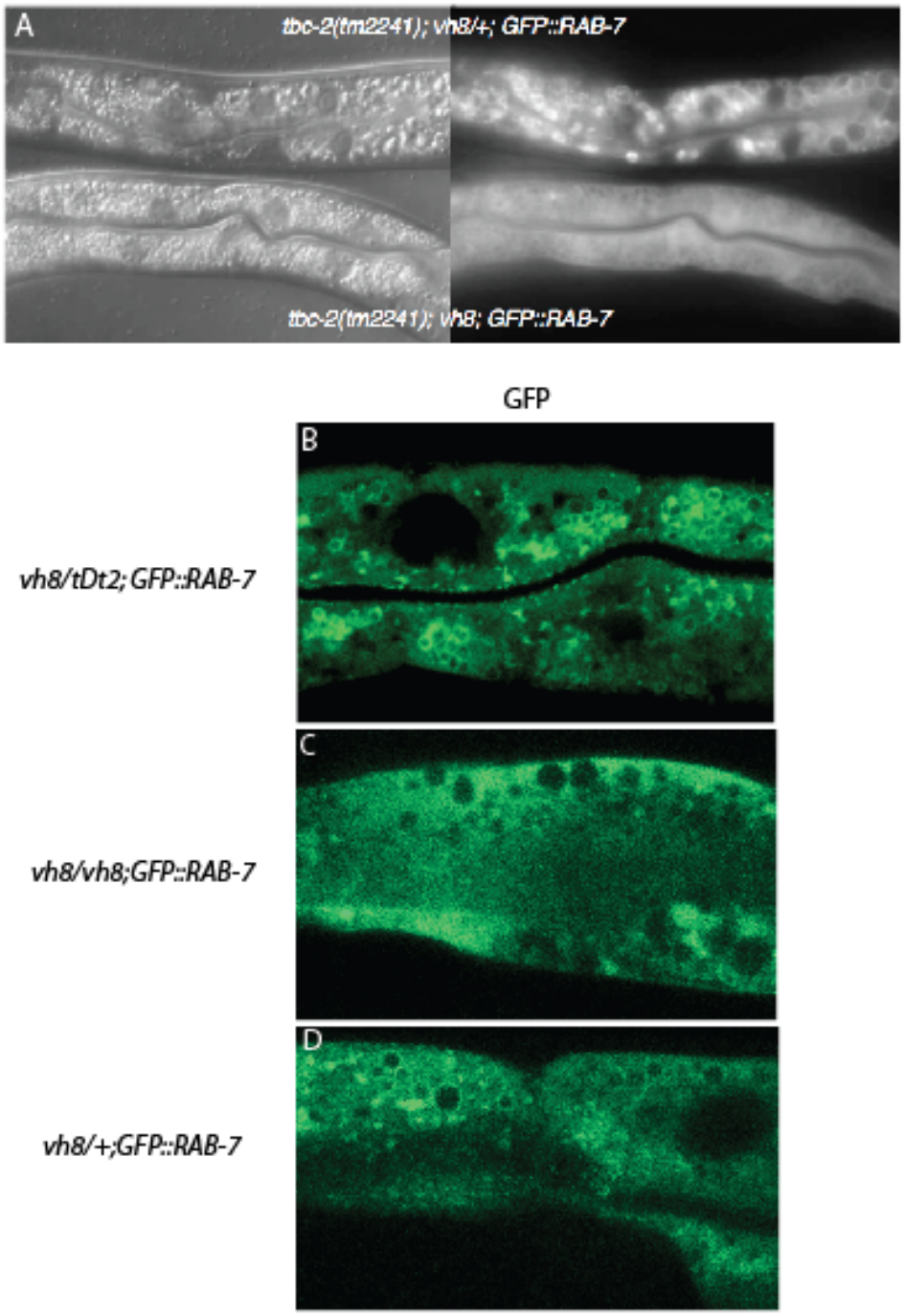

Figure R1. *vh8* phenotypes. DIC (A, left) and epiflouresent (A, right) images of a *tbc-2(tm2241); vh8/+; GFP::RAB-7* animal (A, top) and a *tbc-2(tm2241); vh8; GFP::RAB-7* animal (A, bottom) (From A. Chavlovski and C. Rocheleau, unpublished data). The *tDf2* deficiency complements the *vh8* allele. Confocal images of *vh8/tDf2; GFP::RAB-7* (B), *vh8; GFP::RAB-7* animals (C), and *vh8/+; GFP::RAB-7* (D).

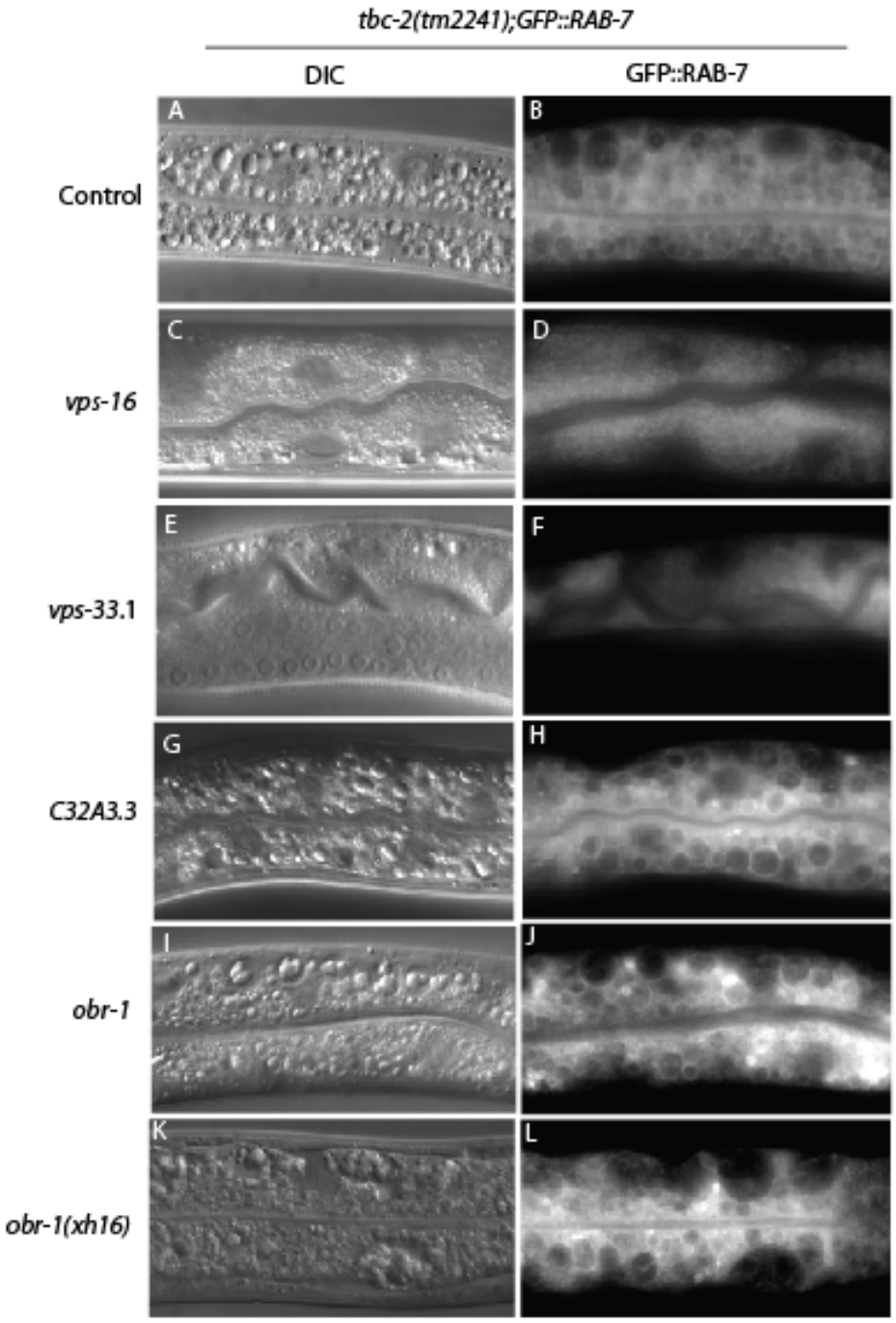

Figure R2. Core components of the HOPS complex are required for *tbc-2(-)* phenotype. DIC images (A, C, E, G, I, and K) and epiflourescent images (B, D, F, H, J, and L) of the intestine of *tbc-2(tm2241)* animals expressing GFP::RAB-7 in different RNAi conditions (A-J) and the *obr-1(xh16)* mutant background (K and L). The animals were fed dsRNA correspond to *vps-16* (C and D), *vps-33.1* (E and F), *C32A3.3* (G and H), *obr-1* (I and J) and the empty L4440 vector (A and B).

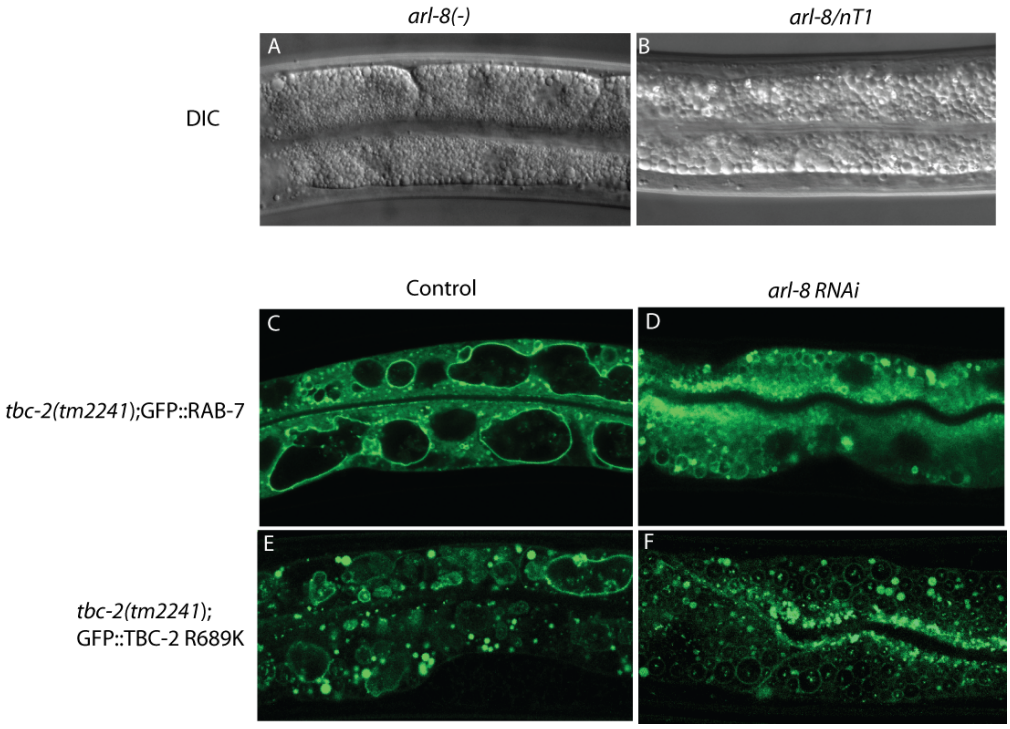

Figure R3. *arl-8* mutant phenotypes in the intestine. DIC images of the intestine of *arl-8(-)* mutant animal (A) and *arl-8/nT1* balanced animal (B). *arl-8* RNAi suppresses the *tbc-2(-)* phenotype, but *arl-8* is not required for TBC-2 or RAB-7 membrane localization. Confocal images of intestine of *tbc-2(tm2241); GFP::RAB-7 (*C and D) and *tbc-2(tm2241); GFP::TBC-2 R689K* (E and F) fed with empty L4440 vector control (C and E) or dsRNA correspond to *arl-8* (D and F).

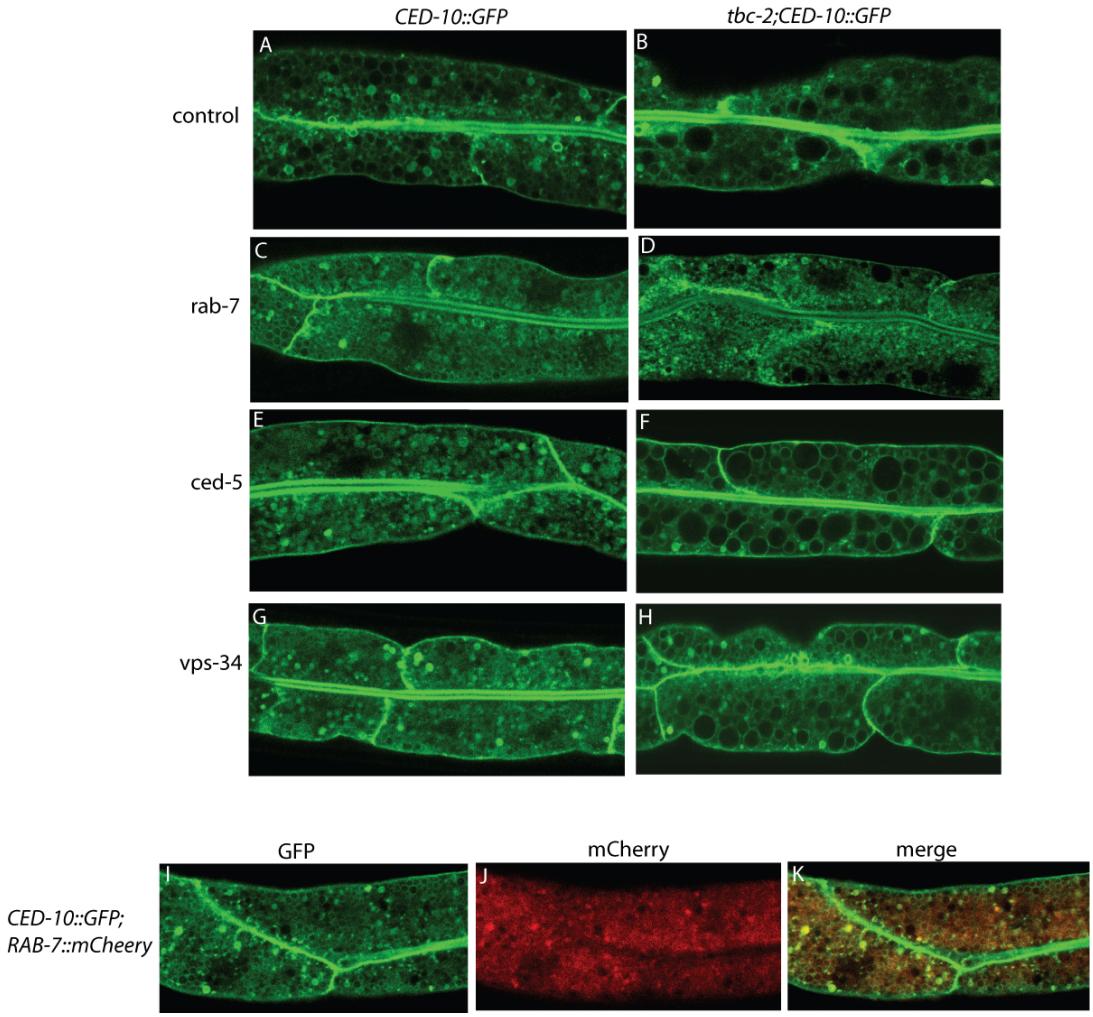

Figure R4. RAB-7, CED-5 AND VPS-34 are not required for CED-10 localization. Confocal images of the intestine of wild-type (A, C, E, and G) and *tbc-2(tm2241)* mutant animals (B, D, F, and H) expressing GFP::CED-10 in different RNAi backgounds. The animals were fed the empty L4440 vector (A and B) and dsRNA correspond to *rab-7* (C and D), *ced-5* (E and F), and *vps-34* (G and H). RAB-7 does not colocalize with CED-10. Confocal images of wild-type animals expressing mCherry::RAB-7 (I) and GFP::CED-10 (J), the merged image in (K).

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