IDENTIFICATION AND CHARACTERIZATION OF COMPONENTS OF RAB-6-MEDIATED TRAFFICKING IN *CAENORHABDITUS ELEGANS*

by

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ABSTRACT OF THE THESIS

Identification and Characterization of Components of RAB-6-mediated Trafficking in Caenorhabditus elegans

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Membrane trafficking in neurons is an important mechanism used to regulate signaling. Controlling the abundance of AMPA-type receptors at the synapse is important for synaptic plasticity and influences processes involved in learning, memory formation, and motor control (Greger and Esteban, 2007; Shepherd and Huganir, 2007). In *Caenorhabditis elegans*, recycling of the AMPA receptor subunit GLR-1 has been demonstrated to be one method by which synaptic signaling mechanisms can be controlled. Rab GTPases 6.1 and 6.2 have been profiled for their role in trafficking of GLR-1 in neurons. RAB-6.2 plays a role in the retrograde trafficking of the AMPA-type glutamate receptor GLR-1 (Zhang et al., 2012). Activated Rab GTPases regulate membrane trafficking by recruiting multiple effectors, including proteins that modify phospholipid membrane composition, motor proteins that tether membranes to the cytoskeleton, and scaffolding proteins that bind to specific proteins within membranes (Stenmark, 2009). In order to understand RAB-6.2 function, we used a yeast two-hybrid

approach to screen for candidate effector molecules. Herein, we discuss the screen performed and detail candidates of interest. We identified one particularly compelling candidate, a phosphoinositol-5-phosphatase named SAC-2, which we hypothesize to be involved in vesicle uncoating and/or in modifying membrane phospholipids. SAC-2::GFP is localized to punctate structures along the ventral nerve cord and in the neuron soma. SAC-2::GFP localization to puncta in the soma is enhanced in *rab-6.2(ok2254)* null mutant animals, which is opposite to our expectation for RAB-6.2 retrograde cargo or effector molecules. Additionally, we assessed the overlap in functions between RAB-6.1 and RAB-6.2, and delineating their pathways. In agreement with previous data from our lab, our findings suggest that the two RAB-6 isoforms perform similar trafficking functions, but do so independently of each other.

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Introduction

Proper regulation of the activity and abundance of post-synaptic membrane receptors is critical for the effective transduction of neurological signals. The AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) - type glutamate receptors (AMPARs) are involved in a diverse set of functions including learning, memory, and synaptic plasticity, but the precise mechanism by which their localization and synaptic abundance is regulated has yet to be fully elucidated (Greger and Esteban, 2007; Shepherd and Huganir, 2007). In *Caenorhabditis elegans*, recycling of the AMPAR subunit GLR-1 is one method by which synaptic transmission and signaling can be controlled. GLR-1 is found in the command interneurons, where it participates in the reception of synaptic input from nose-touch mechanosensory neurons and plays a role in the overall coordination of motor behavior (Chang and Rongo, 2005; Hart et al., 1995; Maricq et al., 1995). In addition to integrating mechanosensory information, GLR-1 and the command interneurons also mediate a spontaneous reversal behavior in the nematode that is used to control long-range navigation during foraging. When GLR-1 is absent, a decrease in the number of spontaneous reversal events is observed. This aberrance is also observed in animals in which GLR-1 trafficking is impaired, suggesting that the maintenance of GLR-1 at the synapse is critical for proper function of this circuit (Glodowski et al., 2005; Zheng et al., 1999). GLR-1 synaptic populations are thought to be controlled in two main ways: recycling of GLR-1 between endosomal compartments and the plasma membrane, and trafficking to and from the Golgi. One of the factors that regulate GLR-1 trafficking is the small GTPase protein RAB-6, which can be found in

two isoforms in *C. elegans*, RAB-6.1 and RAB-6.2. While other factors mediate GLR-1 endocytosis and endosome-to-plasma membrane recycling, both RAB-6.1 and RAB-6.2 act downstream of endocytosis to regulate the trafficking of GLR-1 from endosomal compartments to the Golgi (Zhang, 2012; Zhang et al., 2012). In the following sections, I will present background on AMPA receptor trafficking mechanisms and Rab proteins in general, both in mammalian systems and in *C. elegans*, our model organism of choice.

Section I: Characteristics of AMPA-type Glutamate Receptors

AMPA Receptor Trafficking

In mammals, AMPA-type glutamate receptors are critical components of excitatory signaling in the central nervous system. Both humans and rats have four genes coding for distinct but related GluR subunits (Rosenmund et al., 1998; Wisden and Seeburg, 1993). These subunits combine to form heterotetramers at the rough endoplasmic reticulum, a process critical for proper AMPAR trafficking as only tetramers of GluR subunits can exit the ER. Subsequently, heterotetrameric AMPA receptors receive lipid modification and glycosylation at the Golgi prior to being dispatched to dendritic synapses.

The precise mechanism by which AMPA receptors are trafficked is not yet completely understood, but is generally acknowledged to be in part mediated by PDZ domain proteins, which are found in large populations in the postsynaptic density. A PDZ domain, which is composed of six beta strands and two alpha helices, recognizes and binds to specific PDZ-binding motifs that are typically found at the C-terminus of some proteins (Chang and Rongo, 2005; Kim and Sheng, 2004; Sans et al., 2001; Ye and Zhang, 2013). The trafficking of mammalian AMPA receptors is complicated by the fact that the GluR subunits bind to various different PDZ domain proteins. For example, SAP97 binds to GluR1, whereas GluR2 and GluR3 are bound by either GRIP1 or PICK1 (Fourie et al., 2013; Sans et al., 2001; Zhou et al., 2008). The association of GRIP1 with GluR2 is essential for trafficking of AMPA receptors to dendrites because GRIP1 coordinates with the kinesin motor protein KIF5 (Hirokawa et al., 2010; Mao et al., 2010;

Steiner et al., 2005). Further, individual PDZ domain proteins can contain multiple PDZ domains that can recognize a variety of binding partners, an attribute that allows PDZ domain proteins to facilitate interactions among its binding partners by physical proximity (Kim and Sheng, 2004; Ye and Zhang, 2013).

AMPA Receptors and Synaptic Plasticity

Coordination of the membrane localization of AMPA receptors by endocytosis and subsequent recycling of the receptors is the primary mechanism controlling the strength of a synapse. This regulation of synaptic strength in response to activity (i.e. synaptic plasticity) is thought to play a critical role in the ability of the brain to acquire and store information (Malinow and Malenka, 2002). Endocytosis and exocytosis of AMPA receptors facilitate changes in synaptic weight in response to changes in neural activity, a notion supported by the presence of AMPAR-containing early/sorting endosomes in the dendrite (Malinow and Malenka, 2002; Park et al., 2004; Shepherd and Huganir, 2007). Long-term potentiation – the prolonged enhancement of signaling between two neurons – results in an increase in the number of AMPA-type glutamate receptors at the synapse, and these receptors originate from nearby recycling endosomes (Park et al., 2004). Similarly, synaptic weakening (long-term depression) ultimately promotes the phosphorylation of AMPA-type glutamate receptors, resulting in their depopulation from the postsynaptic membrane (Ogasawara et al., 2008). PDZ domain proteins, discussed earlier for their role in AMPAR trafficking from the Golgi, are also required for proper endocytic recycling. PDZ domain protein SAP97, a member of the MAGUK protein family, interacts with subunit GluR1, which is inserted into the

membrane (Sans et al., 2001). Although SAP97 does interact with a number of motor proteins, knocking it out does not abolish the trafficking of GluR1, but does impair the growth of dendrites (Zhou et al., 2008). The majority of the evidence regarding the effects of AMPAR trafficking on synaptic plasticity comes from experiments using brain slices or cultured neurons. In a cell culture experiment, synaptic activity was inhibited by addition of GABA-A receptor antagonists, resulting in depopulation of AMPA receptors at the synapse (Lissin et al., 1998). When neuron cultures were treated with AMPA-receptor antagonists, the population of AMPA receptors increased at the synapses (Liao et al., 1999). Both of these treatments were undertaken over long periods of time (hours-days), demonstrating that receptor abundance can change based on neural activity. AMPA receptor depopulation was shown to occur even by acute treatment of cell cultures with glutamate, and a critical role for AMPA receptors in LTD was shown immunocytochemically by observing the decrease in AMPA-positive synapses during induction of LTD in hippocampal cell cultures (Carroll et al., 1999; Lissin et al., 1999). Cell culture experiments also confirmed that clathrin-dependent endocytosis of the receptor is the process mediating its depopulation from synaptic membranes (Carroll et al., 1999). While we can see that AMPA receptor trafficking is an important component of synaptic plasticity by contributing to LTP and LTD, this process has not yet been extensively visualized *in vivo*. By utilizing *C. elegans* as a model system, we hope to demonstrate an *in vivo* model that exhibits changes in neural activity and behavioral capacity as a result of manipulating AMPA receptor trafficking.

Section II: Components as found in *Caenorhabditus elegans*

C. elegans as a model system

While the majority of data on AMPA-type glutamate receptor trafficking has come from mammalian systems, whether from cultured neurons or brain slices, *C. elegans* is an excellent candidate for profiling the nervous system as a whole *in vivo*. Generally, *C. elegans* boasts a short generation time and is highly genetically tractable. Examination of phenotypes is simple due to the transparency of the organism, and the entire nervous system has been exhaustively mapped, down to the chemical synapses. *C. elegans* has 302 neurons that can be divided into 118 classes, 17 of which express GLR-1. Finally, GLR-1::GFP has already been demonstrated by Dr. Rongo to be both functional and highly convenient as a marker for examining potential effects on trafficking.

GLR-1 in *C. elegans*

Endocytosis of the GLR-1 receptor is accomplished in a clathrin-dependent manner by RAB-5 and the adaptin protein *unc-11* AP180 (Burbea et al., 2002; Park et al., 2009). After endocytosis, the PDZ-containing protein LIN-10 is involved in the recycling of GLR-1 from endosomes. One of the earliest players to be profiled in the GLR-1 trafficking pathway, LIN-10 was identified from a candidate gene screen for genes involved in GluR trafficking (Rongo et al., 1998). Strains bearing the *lin-10* mutation accumulate GLR-1::GFP in large endosomal structures along the ventral nerve cord,

suggesting that LIN-10 has a critical function in the proper recycling of GLR-1 from endosomes to the post-synaptic membrane (Glodowski et al., 2005; Park et al., 2009). While the carboxy terminus of GLR-1 contains a PDZ domain-interacting sequence, it is unclear whether this sequence directly interacts with the PDZ domains on LIN-10 (Glodowski et al., 2005; Rongo et al., 1998). In order to further determine how LIN-10 regulates GLR-1 recycling, several Rongo lab members (Donglei Zhang, Doreen Glodowski, and Nora Isack) performed a yeast two-hybrid screen using LIN-10 as the bait. One of the factors identified in this screen is the small GTPase protein Rab6, which exists in two isoforms: RAB-6.1 and RAB-6.2.

Rab Proteins and their Roles in GLR-1 Trafficking

Rab-type small GTPase proteins are important players in the trafficking, delivery, recycling, and turnover of many membrane proteins, including AMPA-type receptors (Zerial and McBride, 2001; Zerial and Stenmark, 1993). The action of a Rab protein is controlled by its interaction with a GTPase activating protein (GAP), and a "resetting" guanine nucleotide exchange factor (GEF) that replaces GDP with GTP. Interaction with a GAP promotes hydrolysis of GTP beyond the relatively weak intrinsic GTPase activity of the Rab protein by inserting a catalytic domain containing Arg and Gln residues (Pan et al., 2006). Hydrolysis of GTP prompts the Rab protein to dissociate from the membrane, whereupon a GEF binds the Rab protein and releases the GDP, allowing a new molecule of GTP to replace it. By controlling the abundance and localization of activation and exchange factors, the Rab protein cycles "on" and "off" in both a spatial and temporal manner [Figure 1] (Gundelfinger et al., 2003). This cycling can be further

controlled by the presence of a guanosine dissociation inhibitor (GDI) that stabilizes a Rab in an "off" GDP bound state (Ullrich et al., 1993), which in turn requires a GDI-displacement factor (GDF) to remove GDI and recruit the Rab protein back to its target membrane (Sivars et al., 2003). In addition to interactions with a GAP and a GEF, Rab protein function depends upon the recruitment of additional molecules called "effectors." Effector molecules are diverse in both molecular sequence and function. Proteins involved in membrane tethering, lipid modification, membrane sorting, and motor-driven transport have all been identified as effector molecules that are recruited by various activated Rabs (Eathiraj et al., 2005; Stenmark, 2009). A number of Rab proteins have already been profiled for their role in the trafficking of GLR-1 [Figure 2]. RAB-5 is required for initial clathrin-mediated endocytosis of GLR-1, and both UNC-108/RAB-2 and RAB-10 have been shown to promote GLR-1 recycling (Chun et al., 2008; Glodowski et al., 2007; Park et al., 2009). In a yeast two-hybrid screen utilizing the PDZ domain of LIN-10 as bait, RAB-6 was identified as a candidate for involvement in this pathway. RAB-6 is present in the *C. elegans* genome in two isoforms: RAB-6.1 and RAB-6.2. Both molecules can interact with LIN-10 via yeast two-hybrid. RAB-6.2 has thus far been more thoroughly characterized, so I will discuss it first.

RAB-6.2: RAB-6.2 was characterized and demonstrated to have a role in the regulation of GLR-1 trafficking, alongside the retromer complex (Zhang et al., 2012). LIN-10, which had been already identified as a regulator of GLR-1 recycling, was further characterized as an effector of RAB-6.2. In constitutively active *rab-6.2(GTP)* mutants, the GLR-1::GFP protein is driven to the cell body, resulting in a decrease in the number of punctate structures visible in the ventral nerve cord and an accumulation of the

receptor in Golgi-localized puncta in the cell body (Zhang et al., 2012). Consistent with a true effector, both of these phenotypes are suppressed in a *lin-10* mutant background. Further, RAB-6.2[GTP] drives LIN-10::GFP into puncta along the ventral nerve cord, suggesting that the localization of LIN-10 is partially regulated by RAB-6.2 (Zhang et al., 2012). In addition to providing an informative piece of the GLR-1 trafficking puzzle, the identification of LIN-10 as an effector for RAB-6.2 provides an informative example for the identification of future Rab effector molecules in this system. RAB-6.2 has also been shown to be active in the intestine, where it acts to regulate retrograde trafficking of the Wntless ortholog protein MIG-14, also in a retromer-dependent fashion (Yang et al., 2008; Zhang et al., 2012). Unlike the situation for GLR-1 trafficking, LIN-10 is not required as a RAB-6.2 effector for regulating MIG-14 transport.

RAB-6.1: Work in our lab has shown that RAB-6.1 and RAB-6.2 have overlapping functions and appear to work in concert in some as yet uncharacterized manner to coordinate retrograde transport in non-neuronal tissues (Zhang, 2012). As with RAB-6.2, expressing constitutively active RAB-6.1[GTP] results in trafficking of GLR-1::GFP to the neuron soma, with decreased puncta distribution in the ventral nerve cord [Figure 3.2]. In neurons, *rab-6.1* mutants fail to recycle GLR-1 in a manner similar to that observed in *rab-6.2* mutants, and the effect of removing each RAB is cumulative, with double-mutants showing a stronger trafficking defect than either single mutant alone. In the intestine, RAB-6.1 trafficking of MIG-14/Wntless appears to have a different intracellular route than trafficking of that molecule by RAB-6.2. In *rab-6.2* knockout mutants, MIG-14::GFP accumulates in early and late endosomes that are spatially separated from the Golgi apparatus. By contrast, in *rab-6.1* knockout mutants,

MIG-14 is trafficked to early and late endosomes located proximally to the Golgi (Zhang, 2012).

The Retromer Complex

RAB-6.2 is not the only component of retrograde trafficking. A multi-protein complex called the retromer is involved in the recycling of receptors from endosomes to the trans-Golgi network. In mammalian systems, the retromer complex is composed of a core particle, VPS35; the associated factors VPS26, VPS29, VPS5, and VPS17; and the sortings nexins SNX-1, SNX-2, SNX-3, and SNX-27 (Cullen and Korswagen, 2011). These components are expressed in a number of tissues, including the brain, where they have been implicated in Alzheimer's disease. This appears to be the result of improper processing of amyloid precursor protein (APP), which is thought to be a consequence of trafficking of APP via alternate pathways (Jiang et al., 2014). Initially, VPS35 and VPS26, two important components of the retromer, show reduced expression in patients with Alzheimer's disease (Small et al., 2005). Additionally, expression of the neuronal retromer receptor sorLA is downgraded in patients with late-onset Alzheimer's (Scherzer et al., 2004). The VPS26 subunit of the retromer ordinarily recognizes and binds to sorLA, resulting in sorting and processing of APP (Fjorback et al., 2012). The prevailing hypothesis at present suggests that individuals with impaired retromer function have some flaw in this interaction, resulting in improper trafficking of APP and overprocessing of APP to beta amyloid (Aβ). This idea carries implications for broader retromer-mediated trafficking studies in that defects in components of the retromer complex might create phenotypic variations in the trafficking of an observed cargo molecule that are

unrelated to more specific trafficking pathways, a notion supported by the RAB-6.2 trafficking scheme, which is to some extent also performed by the retromer (Zhang et al., 2012).

The *C. elegans* retromer is a complex of a cargo-selective trimer (Vps26, Vps29, Vps35) and a heterodimer of membrane-binding proteins (SNX-1 and SNX-2) that act in concert to coordinate the sorting of cargo from endosomes to the trans-golgi network (Cullen, 2008; Cullen and Korswagen, 2011; Wassmer et al., 2009). Our lab identified a role for the retromer complex in the recycling of GLR-1 by examining GLR-1::GFP in mutant backgrounds for one of several retromer components, observing a decrease in the number and intensity of GLR-1 puncta in the ventral nerve cord for each case (Zhang et al., 2012). The J-domain-containing protein RME-8 is a critical component of both clathrin-dependent endocytosis machinery and downstream trafficking of those cargoes through interactions with the retromer subunit SNX-1 (Shi et al., 2009; Zhang et al., 2001). In experiments designed to determine whether RAB-6.2 functions in the same pathway as the retromer, RME-8 and RAB-6.2 (along with LIN-10) were shown to colocalize in *C. elegans* neurons, but also that RAB-6.2 is capable of trafficking GLR-1 to the Golgi in the absence of functional RME-8 (Zhang et al., 2012). This suggests potentially distinct but complementary roles for RAB-6.2-mediated retrograde trafficking and retrograde trafficking mediated by the retromer complex [Figure 4].

Section III: Research Questions and Approach

Though we know that the trafficking of GLR-1 is regulated by RAB-6.2, a number of questions remain to be answered. How does RAB-6.2 perform this function? What other factors are involved in the process? Does RAB-6.2 have a more general retrograde trafficking function in *C. elegans*? The experiments outlined in this proposal to identify factors that modulate the function of RAB-6.2 aim to address those questions. The role of effectors in the Rab-mediated endosomal trafficking schema has been well-documented, but the roles of some specific effectors less so. We also know that RAB-6.1 is involved in trafficking events in both the neurons and intestinal cells, but we do not yet know precisely how. Does RAB-6.1 complement the role of RAB-6.2? Do they work in parallel pathways? Does RAB-6.1 require the retromer? Together with prior results from our lab, we detail our experiments to refine our knowledge about RAB-6.1 as a component of trafficking machinery.

Results

To identify candidate effector proteins for RAB-6.2, we performed a yeast two-hybrid screen using *rab-6.2 (GTP)* as bait. We screened the *C. elegans* ORFeome library and repeated the screen five times, for a total of approximately 60,000 entry clones tested. We subsequently analyzed candidates from the screen using a candidate-gene approach to identify which, if any, were true effectors of RAB-6.2.

There are a number of yeast two-hybrid systems commercially available. We used the DupLEX-A screen kit, which uses LexA as the binding protein, acid blob domain protein B42 as the activator, and LacZ as the reporter gene. Briefly, our yeast two-hybrid

screens utilized a bait protein (in our case, *rab-6.2 [GTP]*) fused to the DNA-binding domain of a transcription factor (LexA). A library of genes was created in which each gene was expressed as a fusion protein attached to the activator domain, in this case B42. Transcription of the reporter gene resulted in an easy identification of colonies in which interaction occurred, and those colonies were picked and sequenced using primers in the kit. Sequence data was analyzed using BLAST, resulting in a candidate gene for further study. This process was repeated until we were satisfied that we had saturated the screen. A useful marker for this was the already-known effector LIN-10; we were able to pull LIN-10 out of the screen multiple times, which indicated we had saturated the extent of the screen and had the added benefit of verifying the yeast two-hybrid method as a valid way to identify candidate effector proteins.

We generated a number of candidate genes (Table 1), which were initially profiled for likelihood based on known function and expression patterns (for example, proteins with known GAP domains like TBC-1).

Name	Description
TBC-1	GTPase activating protein (GAP)
W09C5.7	SAC1 family phosphoinositide phosphatase (Golgi-associated)
COGC-1	subunit of Conserved Oligomeric Golgi Complex
COGC-3	subunit of Conserved Oligomeric Golgi Complex
COGC-6	subunit of lobe B of Conserved Oligomeric Golgi Complex
VHA-13	subunit A of cytoplasmic domain of ATPase
VHA-3	subunit c of membrane-bound domain of ATPase (same as vha-2)
VHA-2	subunit c of membrane-bound domain of ATPase
APL-1	orthologous to human amyloid precursor protein (cargo molecule)
VPS-52	ortholog of Vps52p Golgi associated retrograde protein
VPS-53	part of GARP complex with vps-52 and -54
VPS-54	part of GARP complex with vps-52 and -53
ELKS-1	C. elegans homolog of vertebrate ELKS (Glu, Leu, Lys, Ser - rich proteins)

Table 1: Yeast two-hybrid candidate genes and their functions.

With a number of candidate genes in hand, we next wanted to examine how each candidate influenced the trafficking of GLR-1. Using LIN-10 as a precedent, we hypothesized that knocking out genuine effector candidates would result in defects in the trafficking of GLR-1, which we visualized by examining GLR-1::GFP. Additionally, we tested whether double mutant strains containing both the *rab-6.2 (GTP)* transgene and candidate knockouts suppressed the *rab-6.2 (GTP)* phenotype. Finally, we hypothesized that in addition to being colocalized at or near sites of RAB-6.2 accumulation within the cells, the localization of a true effector should be regulated by RAB-6.2.

To test our first hypothesis, we crossed a knockout strain of our candidate gene into a strain expressing the GLR-1::GFP transgene, and then examined the GLR-1:GFP localization for any aberrancies. Based on GLR-1::GFP localization phenotypes of known RAB-6.2 trafficking pathway components, any phenotype resulting in changes in fluorescence intensity or a reduction in the number of punctate structures in the ventral nerve cord was considered interesting as it might suggest a role for the gene in the RAB-6.2 mediated trafficking pathway.

Profiling the localization of a candidate is informative for two main reasons. Primarily, if RAB-6.2 (GTP) and a candidate colocalize, it supports the idea that they are participating in a direct interaction. Secondly, an alteration in the localization pattern of a candidate gene between the candidate in a wild-type background and the same candidate in the *rab-6.2(GTP)* background suggests that the localization of the candidate is regulated by RAB-6.2. This was demonstrated by Donglei Zhang in his experiments that identified LIN-10 as an effector.

Lastly, suppression of the RAB-6.2(GTP) gain of function phenotype provides evidence that a candidate gene is not only an effector for RAB-6.2 in the trafficking of a given cargo, but also that it is a required effector for this activity.

Ultimately, the yeast two-hybrid screen and subsequent analysis yielded two interesting candidates. The first, W09C5.7 (hereafter SAC-2) in the above table, is analogous to hSac2 phosphatase in humans and mice. SAC2 phosphatases are inositol 5-phosphatases that act on phosphatidylinositol 4,5-bisphosphate and phosphatidyl inositol 3,4,5-triphosphate, two phospholipid molecules that have been implicated in endosomal trafficking events. The second candidate, VHA-2/3, are genes coding for identical subunits of a vacuolar ATPase that participates in the acidification of endosomes. In the next section, I describe both of these candidates in more detail.

Section IV: Candidates of Interest from the Yeast Two-Hybrid Assay

Candidate 1: SAC-2

Initially, *sac-2* knockout mutants were examined in transgenic animals expressing GLR-1::GFP and in transgenic animals expressing *rab-6.2(GTP)* and GLR-1::GFP. This was done to examine the effect of removal of SAC-2 on the trafficking of GLR-1, both alone and in the presence of constitutively active RAB-6.2. No strong effect on GLR-1::GFP trafficking phenotypes were observed. Subsequent analysis by spontaneous reversal assay, a more sensitive measurement that tests for impairment in GLR-1-mediated behavioral function, showed a statistically significant decrease in the number of spontaneous reversal events in *sac-2(ok2743)* animals compared with wild type (N2) animals. This result may suggest that SAC-2 does not exclusively act in GLR-1 trafficking, but has a more general role in sensorimotor activity. SAC-2::GFP localizes to structures in the nerve ring cell body and appears diffusely distributed in the ventral nerve cord, although a small number of punctate structures can be discerned. In a *rab-6.2(ok2254)* knockout background, SAC-2::GFP did not appear in the ventral nerve cord, whereas the size and intensity of localized SAC-2::GFP in the cell bodies increased dramatically. We also observed that in a *rab-6.2(GTP)* background, SAC-2::GFP remained diffuse in the ventral nerve cord similarly to SAC-2::GFP in an otherwise wild-type background [Figure 5]. However, the intensity of the fluorescent signal in the cell bodies appears to be decreased relative to the otherwise wild-type strain. This difference is observable even under a low-power UV dissection microscope. In the wild-type

background, SAC-2::GFP fluorescence is readily observable under the low-power objective, whereas in the *rab-6(GTP)* background, magnification with a higher power 10X objective was required to visualize the signal.

Taken together, these results suggest that SAC-2 localization is regulated by the presence of activated RAB-6.2, which seemingly acts to keep SAC-2 localized away from the Golgi via a mechanism we do not yet understand. The phenotypes we have observed are quite different from those we initially predicted. We expected true effector candidates to be driven to the cell body in the presence of *rab-6.2(GTP),* but we observed the opposite effect. This may suggest that SAC-2 is an effector of some other Rab protein and that the localization of SAC-2 by RAB-6.2 helps regulate the function of that other Rab. Alternatively, RAB-6.2 might have a previously uncharacterized anterograde trafficking function with respect to SAC-2.

Candidate 2: VHA-2/3

VHA-2 and VHA-3 are genes coding for identical subunits of a vacuolar ATPase that participates in ATP hydrolysis-coupled proton transport to acidify membrane compartments (Oka et al., 1998). VHA-2 and VHA-3 are functionally identical and vary by expression in tissues and at specific times during development (Oka et al., 2001). Candidate gene analysis showed that in a *rab-6.2(GTP)* background, knocking out VHA-3 resulted in a partial rescue of the GLR-1::GFP trafficking phenotype, to an extent comparable to *rab-6.2(GTP) lin-10* double mutant strains. However, no change in GLR-1::GFP localization was observed in double knockout *rab-6.2(ok2254), vha-3* strains, nor

in *vha-3* single mutants. In order to support the idea that VHA-2/3 is interacting with RAB-6.2, we generated a GFP-tagged construct of VHA-2. VHA-2 cDNA was to build our GFP-tagged construct as no vha-3 cDNA was available. In an otherwise wild-type background, the GFP signal was quite weak, suggesting either an enhanced turnover of the overexpressed protein or that the GFP tag was internal to acidified compartments, compromising the fluorescence. In either case, the distribution observed was restricted to the cell bodies of GLR-1-expressing neurons, with no visible signal in the ventral cord dendrites. This phenotype was identical when the VHA-2::GFP transgene was expressed in either a *rab-6.2* knockout or a *rab-6.2* GTP-locked background. Ultimately, this candidate was deemed unattractive for further study. Although it is not unreasonable to consider a compartmental acidification component as an effector for a factor involved in endosomal trafficking, the dimming of the GLR-1::GFP signal in the neuron cell bodies in a *rab-6.2[GTP]* background might also be interpreted as the result of broader impairment to endosomal trafficking resulting from loss of the VHA-3, rather than a specific influence on GLR-1 trafficking via RAB-6.2 action.

Section V: Investigating the role of RAB-6.1

Our approach to RAB-6.1 centered around five experiments aimed at understanding different possible interactions with RAB-6.2 and the retromer.

Firstly, we tested whether RAB-6.1 or RAB-6.2 influence the subcellular localization of each other. If the two Rabs function in separate pathways, then the localization of one RAB should not be altered in the mutant background of the other. To address this question, we generated strains containing either GFP-tagged RAB-6.1 or RAB-6.2 in the knockout mutant background for the other Rab. We found that the abundance of punctate structures containing RAB-6.2::GFP does not change when that transgene is expressed in a *rab-6.1(tm2124)* null mutant background [Figure 6]. Additionally, the intensity of the GFP signal in the nerve ring-region neuron cell bodies does not change in the absence of functional RAB-6.1. Our results indicate that while the localization of RAB-6.2 does not appear to be dependent upon the presence of active RAB-6.1, RAB-6.2 may play a role in the localization of RAB-6.1.

In the other case, our data suggests that RAB-6.2 may play a role in the localization of RAB-6.1. Although the density of RAB-6.1::GFP-containing punctate structures in the ventral nerve cord did not appear to be significantly affected when expressed in a *rab-6.2(ok2254)* null mutant background, a subtle reduction in the intensity of the fluorescent signal was observed in the nerve ring-region neuron cell bodies [Figure 7]. Because the trafficking of RAB-6.1 does not appear to be abolished in these mutants, it is unlikely that RAB-6.2 is the sole determinant of RAB-6.1 localization. We consider it likely that while active RAB-6.2 may influence the

localization of RAB-6.1 indirectly, the localization of either RAB-6 does not depend upon the presence or activity of the other.

In our second experiment, we tested whether RAB-6.1 and RAB-6.2 act in the same or in parallel pathways – particularly whether one requires the other – with respect to GLR-1 trafficking. To accomplish this, we tested the effect of expressing constitutively active (GTP-bound conformation locked) forms of each RAB-6 in a null mutant background for the other. These strains were then analyzed for ventral nerve cord puncta density and size, and for fluorescence intensity of PVQ neuron cell bodies.

In strains expressing either RAB-6.1[GTP] or the RAB-6.2[GTP] transgene, GLR-1::GFP is trafficked from the ventral nerve cord to Golgi structures in neuron cell bodies [Figure 3C-D; Figures 8 and 9]. We found that RAB-6.2[GTP] can still drive GLR-1::GFP out of the ventral cord and back to the cell bodies even in the absence of RAB-6.1 function [Figure 8]. Similarly, RAB-6.1[GTP] can still drive GLR-1::GFP out of the ventral cord and back to the cell bodies even in the absence of RAB-6.2 function. However, the presence of RAB-6.1[GTP] in a *rab-6.2(ok2254)* null mutant background appears to result in disorganized localization of the GLR-1::GFP localization to as yet unidentified punctate structures in the neuronal cell body [Figure 9E].

Section VI: Discussion and Future Direction

Potential RAB-6.2 effector SAC-2

RAB-6.2 appears to regulate SAC-2 subcellular localization; however, GLR-1 trafficking appears grossly normal in *sac-2* mutants, raising the possibility that SAC-2 might regulate other retrograde cargo. For example, another potential candidate is the Wntless analog MIG-14, the trafficking of which is regulated by RAB-6.2 and the retromer in the intestine. If SAC-2 is a true effector for RAB-6.2-mediated trafficking of MIG-14, we would expect a similar phenotype to be observed in *sac-2* knockout mutant strains when visualizing MIG-14::GFP. Confirmation of the notion that MIG-14::GFP degradation was occurring could be done by mutating the turnover factor CUP-5, which directs traffic to the lysosome.

Determining the identity of cellular bodies that are decorated with SAC-2::GFP is another important piece of the puzzle. This would be accomplished via double-labeling experiments with known compartment-associated molecules, such as mannosidase for the Golgi, RAB-5 for early endosomes, and RAB-7 for late endosomes. Based on the function of most inositol 5-phosphatases and the cellular structures on which their target PtdIns molecules are found, we would expect SAC-2 co-localization with endosomal compartments and almost certainly with the Golgi.

Although we identified SAC-2 as a candidate based on a presumably physical interaction in our yeast two-hybrid assay, the limitations of that technology requires that the interaction be confirmed biochemically. To do this, we would employ a glutathione S-transferase pull-down assay to verify the physical interaction of SAC-2 with rab-6.2

(GTP). A SAC-2::GST fusion protein would need to be cloned and expressed in *E. coli*. This fusion protein would then be attached to GSH-coated beads and exposed to lysate from C. elegans animals expressing rab-6.2(GTP). SDS-PAGE analysis would be used to evaluate the results.

Identifying and Evaluating New Candidate Effectors for RAB-6.2

In the yeast two-hybrid screen, we identified a number of candidate genes based on the physical interaction between active RAB-6.2 and potential effector proteins. By performing a clonal ethyl methane sulfonate (EMS) mutagenesis screen to generate mutant lines, candidate genes could be identified based on satisfaction of a functional requirement. This would be scored via epifluorescence microscopy for the ability of a given mutation to suppress the *rab-6.2(GTP)* gain-of-function phenotype. We have already prepared a strain carrying the *rab-6.2[GTP]* transgene along with GLR-1::GFP in the neurons and intestinal MIG-14::GFP to allow simultaneous evaluation of trafficking defects in those two tissues. This will facilitate identification and prioritization of mutants for further study.

Commonly, mutagenesis screens performed to saturation yield two main classes of mutants: genes with multiple mutant alleles in the candidate pool, and genes with only single alleles. As mentioned previously, isolating a gene with multiple alleles in the candidate pool increases the likelihood of obtaining a null mutant, which is the most genetically useful and informative and will be high on the list of candidates to be cloned. Mono-allelic genes are potentially more challenging to work with, as it is possible to obtain dominant gain-of-function mutants with roles in RAB-6.2-mediated trafficking that might be difficult to elucidate. One exception here (although improbable) could be a

GEF protein for RAB-6.2 in the case where a mutation alters the binding affinity of the GEF such that it is capable of binding the GTP-locked form of RAB-6.2 in a way that impairs the ability of the Rab to traffic GLR-1. This could result in apparent suppression of the *rab-6.2(GTP)* phenotype.

Thus, the highest priority for further study would be multi-allelic genes that exhibit direct suppression of the *rab-6.2(GTP)* phenotype AND have GLR-1 and MIG-14 trafficking phenotypes when crossed away from *rab-6.2(GTP).*

Interesting mutants obtained from this screen would be sequenced using whole genome sequencing and subjected to candidate gene analysis, including construction of fluorescently tagged versions for injection and evaluation. Chiefly, we would want to know if a candidate gene's localization is consistent with that of RAB-6.2 and its cargo, and if RAB-6.2 can drive localization of the candidate in a retrograde fashion.

RAB-6.1

Our findings with RAB-6.1 largely support previous work indicating that RAB-6.1 and RAB-6.2 play overlapping roles, but act independently of one another to traffic cargo to the cell body. One potential confounding factor is the observation that RAB-6.1[GTP] appears to traffic GLR-1::GFP to multiple, seemingly disorganized bodies in the soma when expressed in a *rab-6.2 (ok2254)* null mutant background. This finding complicates the picture of RAB-6.1/RAB-6.2 mediated retrograde trafficking; previously, we considered a pathway in which RAB-6.1 acts on cargo molecules at a point after its arrival in the neuron cell body (as conducted by RAB-6.2). Now, we must consider the possibility that RAB-6.2 acts to determine the somatic destination of cargo, at least in the

case of GLR-1. To investigate this possibility, double-labeling experiments will need to be conducted to determine the identity of the subcellular compartments containing GLR-1 in this background. As with the experiment I described above to look at SAC-2 localization, we would use markers mannosidase for the Golgi, RAB-5 for early endosomes, and RAB-7 for late endosomes.

Additional experiments need to be conducted to better understand the role of the retromer complex in the RAB-6.1 and RAB-6.2 retrograde trafficking pathways. Previous work in our lab using retromer components RME-8 and VPS-35 has shown that RAB-6.2 is able to traffic GLR-1 to the cell body in the absence of retromer function (Zhang, 2012; Zhang et al., 2012), but the retromer and RAB-6.1 is less well understood. In the intestine, preliminary results from RNAi experiments for each RAB-6 isoform indicate that absence of RAB-6.1 or RAB-6.2 affects trafficking of both MIG-14 and retromer component RME-8, suggesting a potential role for RAB-6 in retromer-mediated trafficking in the intestine [Figure 11]. However, these strains are not healthy when grown on RNAi plates to knock down rab-6.2, suggesting a possible interaction between RME-8 overexpression and loss of RAB-6.2.

Can constitutively active RAB-6.1 also overcome the requirement of the retromer? Strains for this analysis have been generated, but analysis has been precluded by the slow growth and strong egg-laying defect observed in *rab-6.1[GTP], vps-35(hu68)* double mutants. Overcoming these challenges may require generation of a balanced strain to facilitate generation of suitable numbers of animals for imaging and analysis.

Section VII: Figures

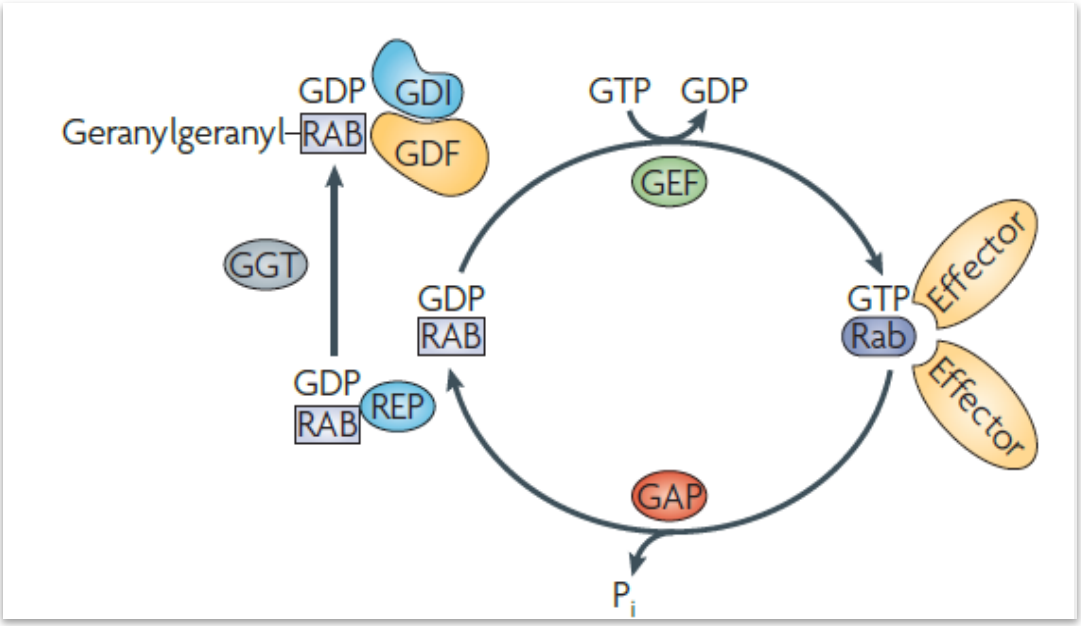

Figure 1: The rab switch and its circuitry (Stenmark 2009)

In this figure from Stenmark's 2009 review of Rab protein function, the oscillation of a Rab protein between "off" (GDP-bound) and "on" (GTP-bound) states is shown. Starting on the left, the process of Rab protein geranyl-geranylation is shown, catalyzed by a geranylgeranyl- transferase. The prenylated Rab is then down bound to a Guanosine Dissociation Inhibitor (GDI), which prevents unregulated displacement of GDP. Interaction of the GDI with a GDI Displacement Factor (GDF) allows GEF-catalyzed exchange of GDP for GTP and thus "activation" of the Rab protein, which then interacts with effector molecules to perform its function. Association with a guanosine activator protein (GAP) results in hydrolysis of GTP and subsequent binding of GDP.

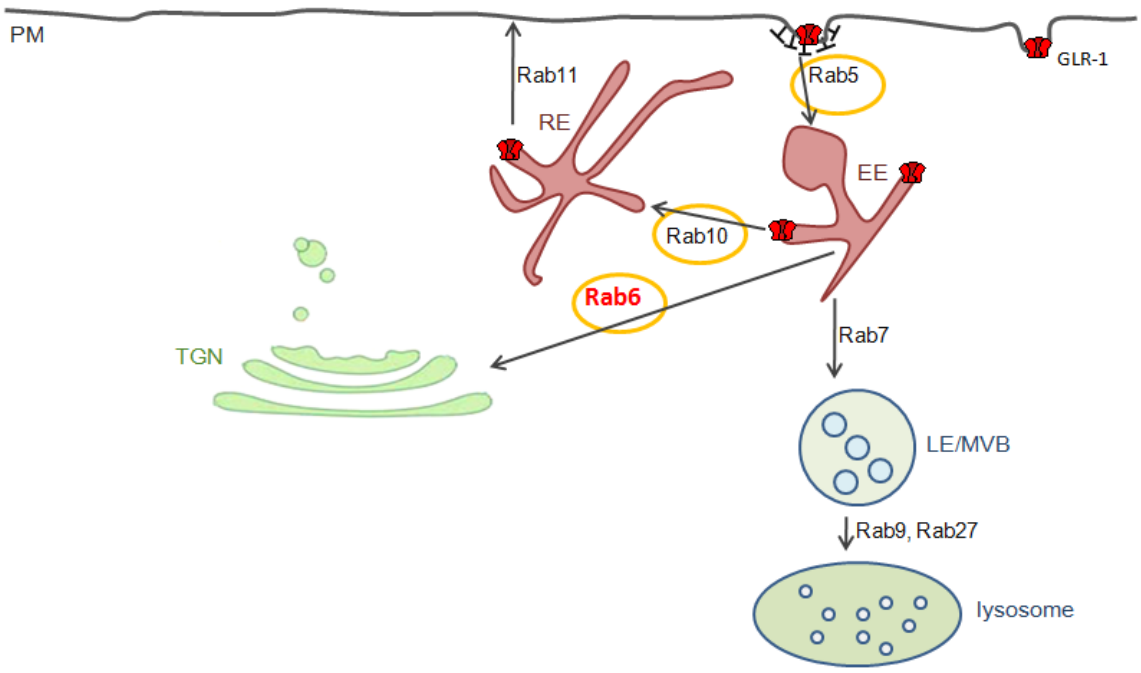

Figure 2: Schematic of relevant Rab proteins for GLR-1 trafficking in *C. elegans.*

[Figure originally produced by D. Zhang, 2011]

In this figure, the general functions of various Rab proteins within GLR-1 expressing C. elegans neurons are shown. Glutamate receptor GLR-1 is shown in red. Beginning from the top, Rab5 participates in clathrin-mediated endocytosis, from which GLR-1 is translocated into the early endosome. From this point endocytosed GLR-1 has several possible fates. It can be transported to a recycling endosome in a process regulated by Rab10 (from which point it is recycled to the plasma membrane in a process regulated by Rab11). It can be send down a degradative pathway in a process mediated by Rabs 7, 9, and 27. Finally, it can be trafficked into the cell body in a process regulated by Rab6, with a destination of the Golgi apparatus.

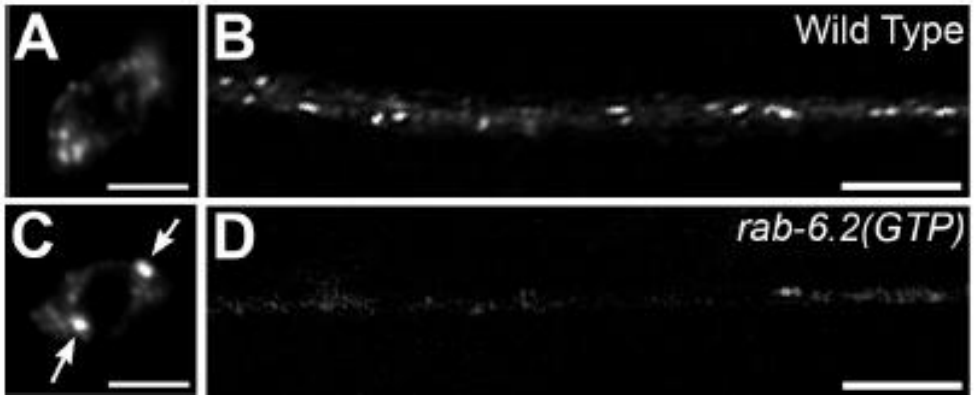

Figure 3: Rab-6.2 Gain of Function

(D. Zhang et al., 2012): GLR-1::GFP in wild-type and constitutively active rab-6.2 (GTP) background. Points A and C show neuron cell bodies in the nerve ring region of the animal, while B and D show the ventral nerve cord. (Zhang 2012). Note the punctate nature of GLR-1::GFP is clear in the wild-type animal, but greatly reduced in the constitutively active *rab-6.2 (GTP)* animal.

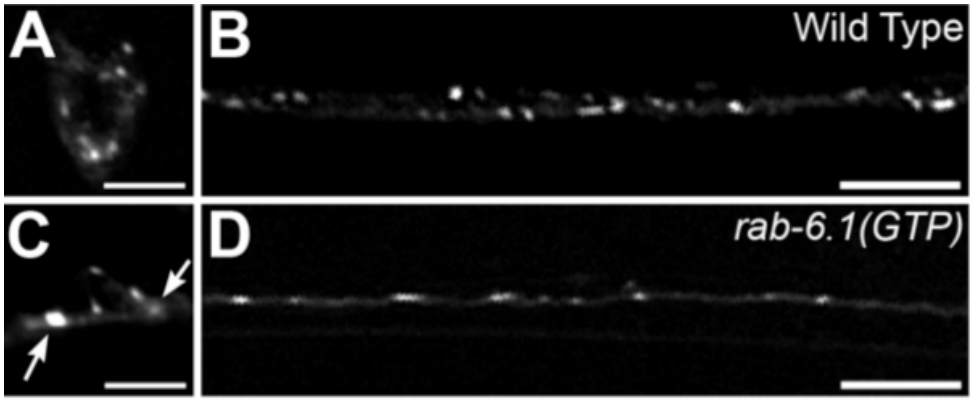

Figure 4: Rab-6.1 Gain of Function

(D. Zhang 2012): GLR-1::GFP in wild-type and constitutively active rab-6.1[GTP] background. Points A and C show neuron cell bodies in the nerve ring region of the animal, while B and D show the ventral nerve cord. Note the punctate nature of GLR-1::GFP in the ventral nerve cord, which is reduced in the rab-6.1[GTP] animal.

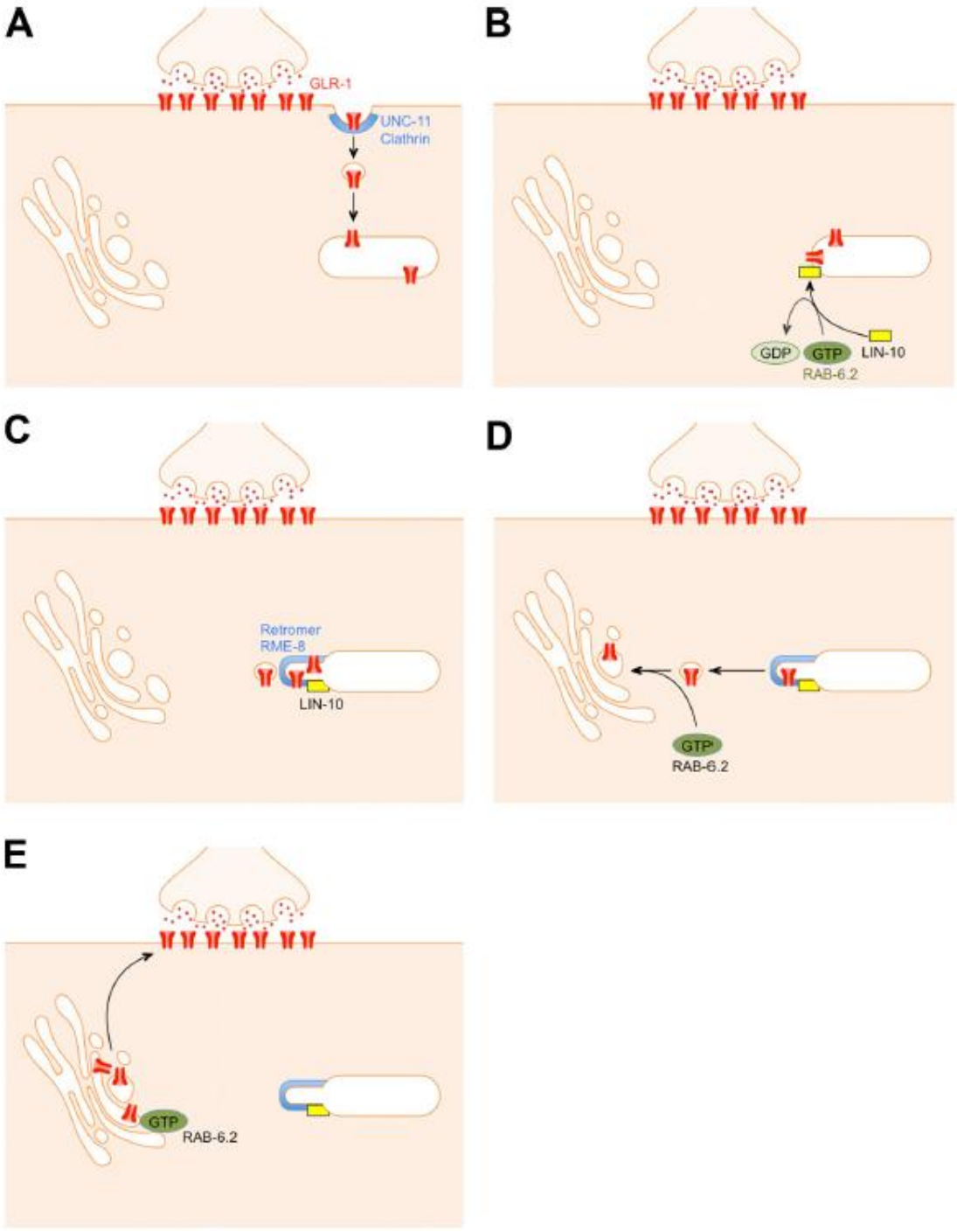

Figure 5: A model for Rab-6.2 retrograde trafficking

A. Unc-11/AP180 regulated clathrin-dependent endocytosis of GLR-1 and transport to early endosome.

B. Activated RAB-6.2 transports effector LIN-10 to early endosome.

C. LIN-10 sequesters AMPAR into endosomal tubules with retromer and RME-8; tubules mature into transport vesicles.

D. Activated RAB-6.2 regulates transport of budding cargo vesicles from early endosome to *trans*-Golgi

E. At Golgi, processing of GLR-1 (association with co-receptors, etc) and re-sorting back to target membranes.

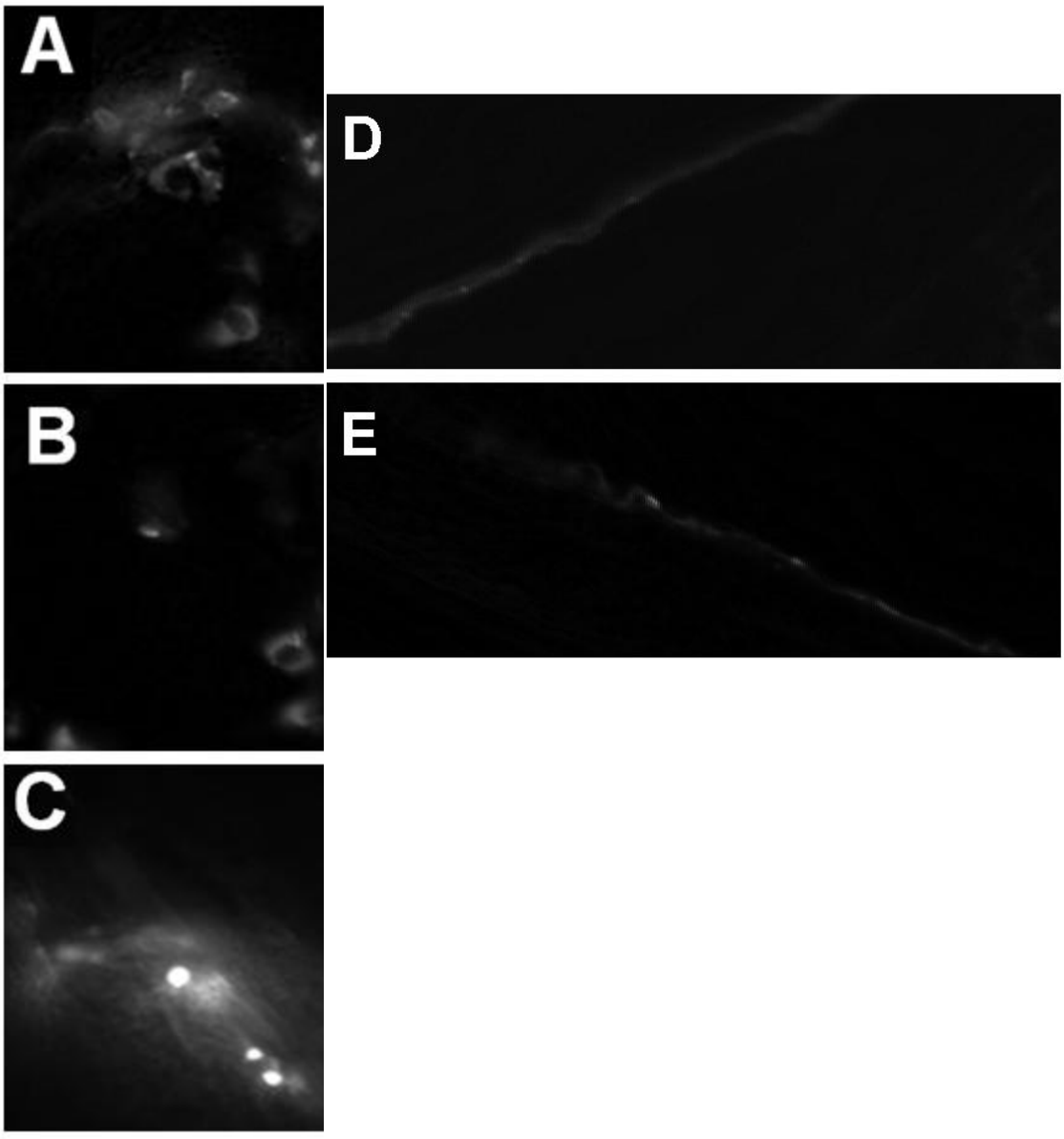

Figure 6: SAC-2::GFP

A. SAC-2::GFP alone. This image highlights the localization of SAC-2::GFP to the cell body of neurons in the nerve ring region.

B. Transgenic animal expressing SAC-2::GFP with *rab-6.2(GTP)*. Similar cell body distribution as wild-type.

C. Transgenic animal expressing SAC-2::GFP in a *rab-6.2* knockout background showing increased localization to structures in the cell body. This animal showed no visible GFP signal in the ventral nerve cord.

D. Ventral nerve cord of animal pictured in (A). Punctate structures are few and dim.

E. Ventral nerve cord of animal pictured in (B). Punctate structures are few and dim.

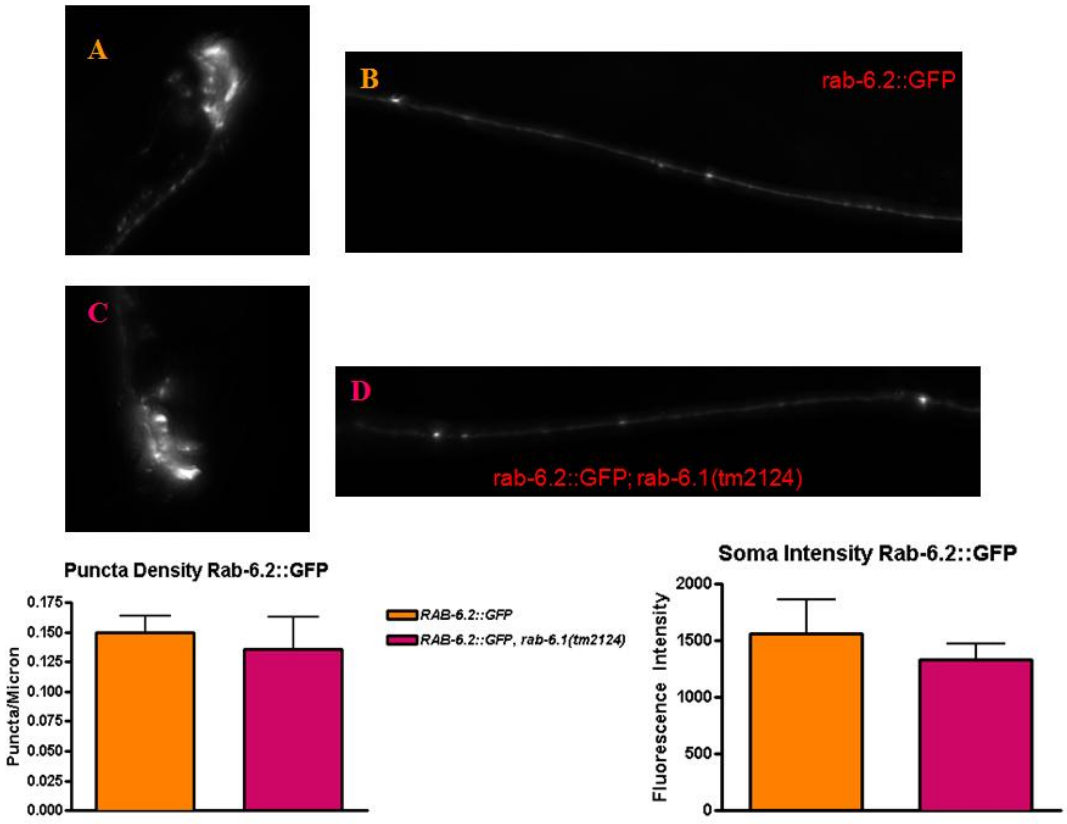

Figure 7: RAB-6.2::GFP is not driven by RAB-6.1

(A,C) RAB-6.2::GFP in nerve ring-region cell bodies. (B,D) RAB-6.2::GFP in the ventral nerve cord. (E) Comparison of density of the population of RAB-6.2::GFP-containing punctate structures in the ventral nerve cord in the indicated strains. The distribution of these punctate structures (and thus RAB-6.2::GFP) does not appear to differ appreciably in a *rab-6.1* null background ($p = 0.6488$). (F) Intensity of fluorescent signal from RAB-6.2::GFP in nerve ring region neuron cell bodies. Here too, data does not suggest that RAB-6.2::GFP localization changes based on the presence of rab-6.1 ($p=0.5496$).

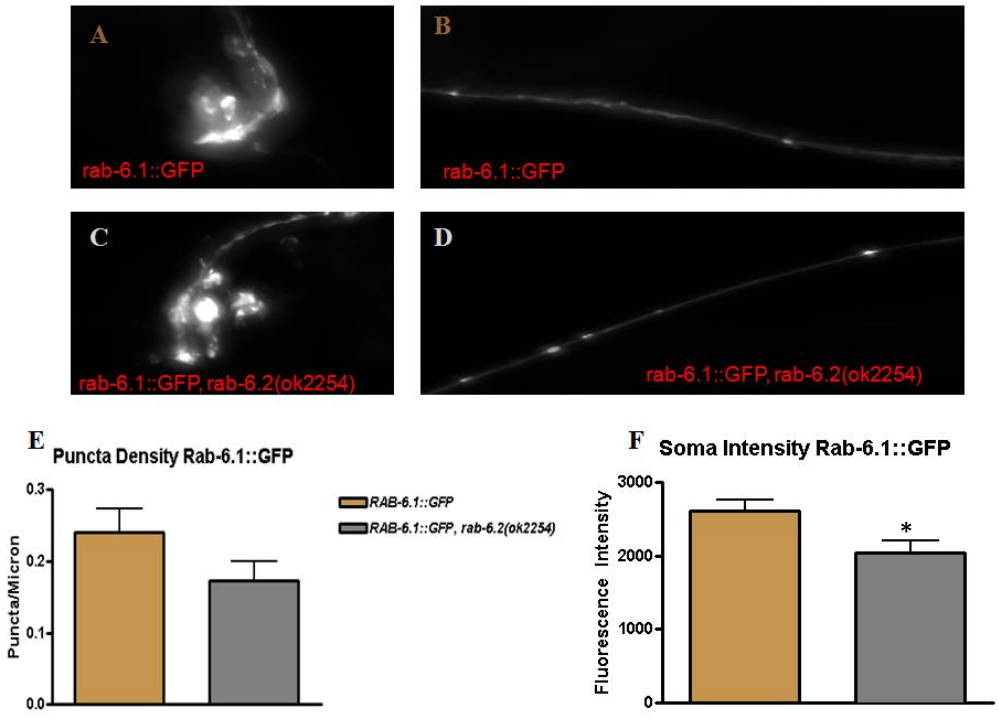

Figure 8: Rab-6.1::GFP is not driven by RAB-6.2

(A,C) RAB-6.1::GFP in nerve ring-region cell bodies. (B,D) RAB-6.1::GFP in the ventral nerve cord. (E) Comparison of density of the population of RAB-6.1::GFP-containing punctate structures in the ventral nerve cord in the indicated strains. Although the presence of RAB-6.1::GFP –containing punctate structures appears to decrease in a rab-6.2 null background, the difference shown is not statistically significant ($p=0.1604$). (F) Intensity of fluorescent signal from RAB-6.1::GFP in nerve ring region neuron cell bodies. Data indicates that in the absence of functional rab-6.2, intensity of this signal decreases in those cell bodies ($p = 0.02$).

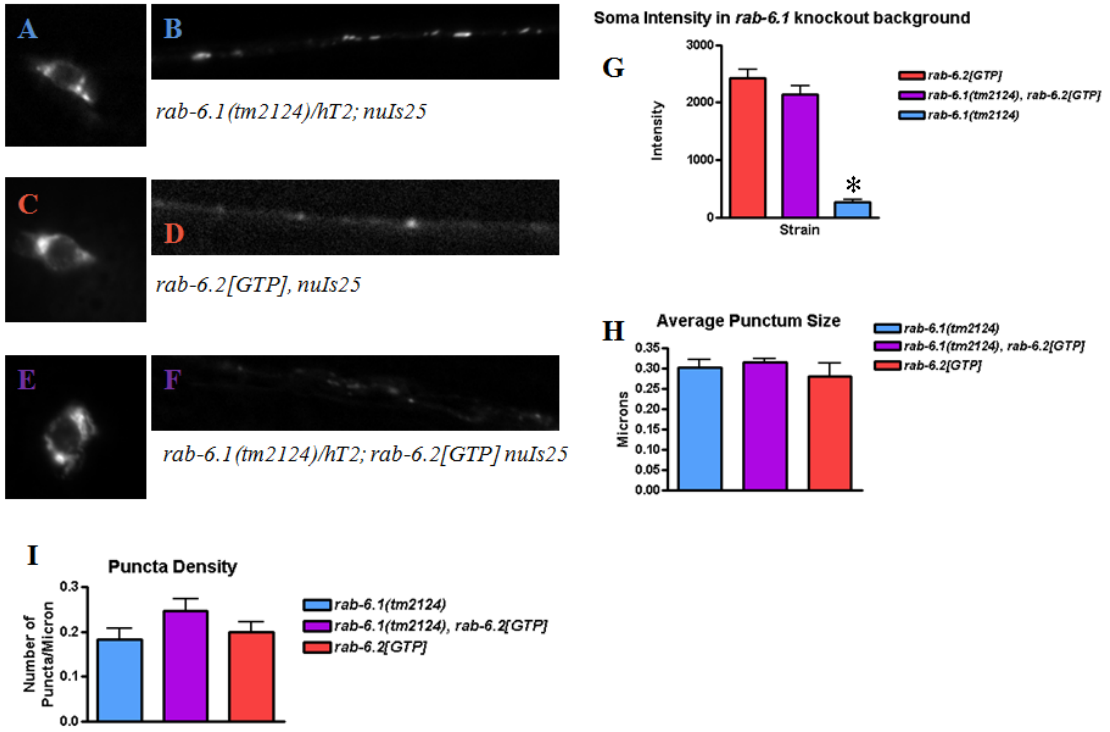

Figure 9: RAB-6.2 can drive GLR-1::GFP in the absence of RAB-6.1

Images and quantification of GLR-1::GFP in animals with the indicated genotypes.

(A, C, and E) GLR-1::GFP in the soma of the PVQ neurons.

(B, D, and F) GLR-1::GFP in the ventral nerve cord.

(G) Intensity of GFP signal in PVQ neuron cell bodies ($p<0.0001$).

(H) Average size of an individual punctum in the ventral nerve cord ($p=0.5091$).

(I) Density of GLR-1::GFP punctate structures in the ventral nerve cord shown in number of puncta per micron of cord distance observed ($p=0.1828$).

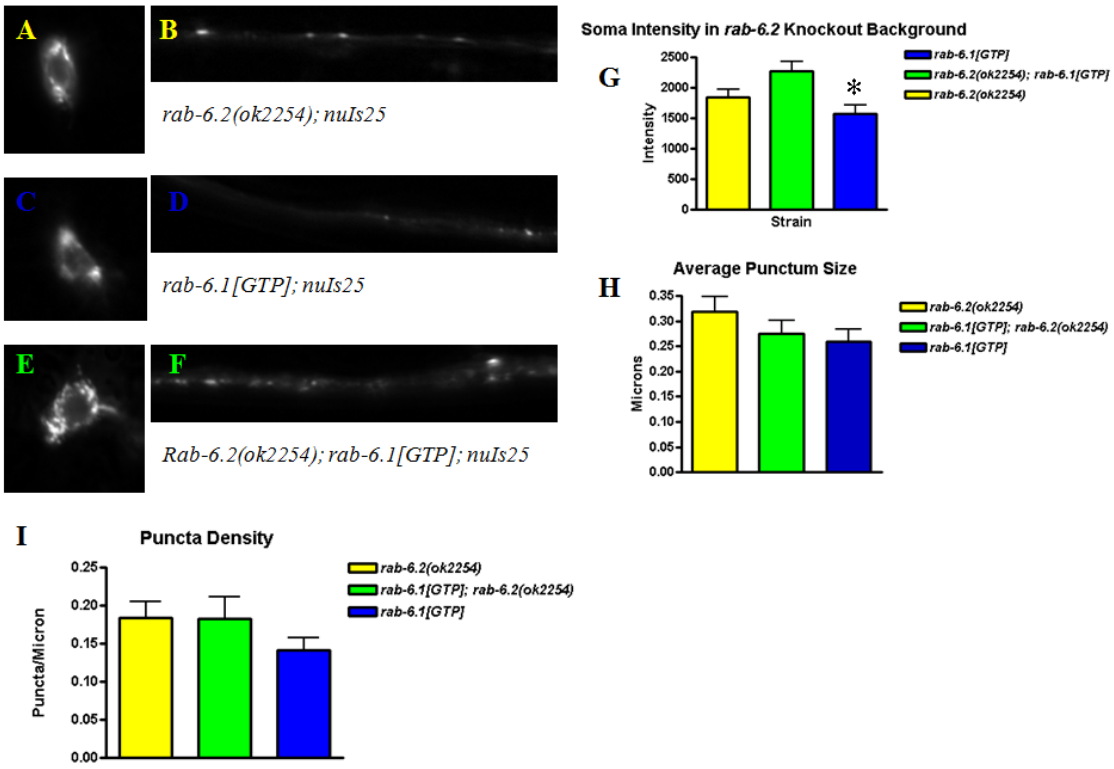

Figure 10: Rab-6.1 can drive GLR-1 in the absence of RAB-6.2

Images and quantification of GLR-1::GFP in animals with the indicated genotypes.

(A, C, and E) GLR-1::GFP in the soma of the PVQ neurons.

(B, D, and F) GLR-1::GFP in the ventral nerve cord.

(G) Intensity of GFP signal in PVQ neuron cell bodies (p=0.0068).

(H) Average size of an individual punctum in the ventral nerve cord (p=0.2715).

(I) Density of GLR-1::GFP punctate structures in the ventral nerve cord shown in number of puncta per micron of cord distance observed (p=0.3393).

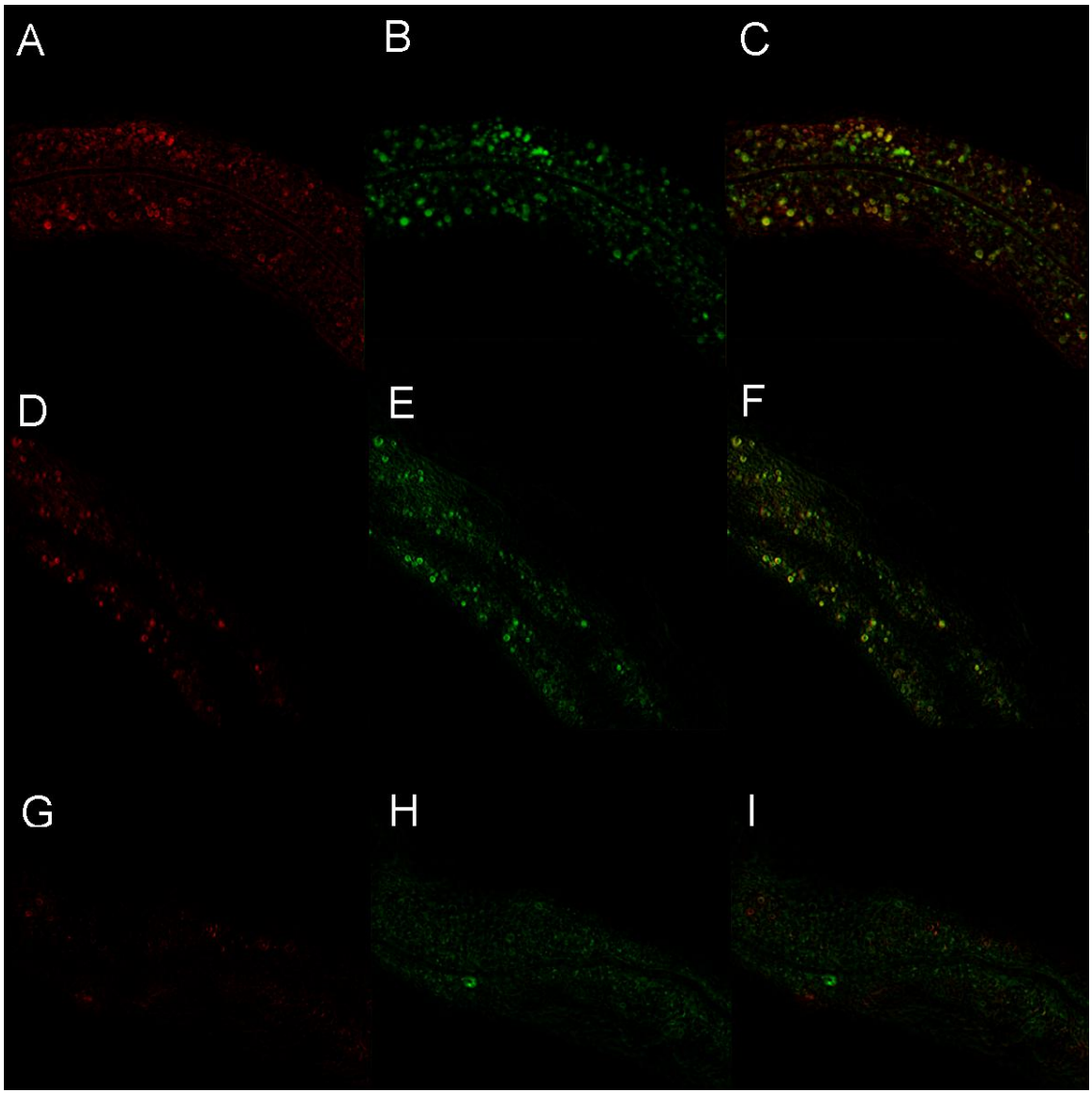

Figure 11: RME-8::mCherry and MIG-14::GFP in the intestine

This figure shows the effect of removing either rab-6 isoform on intestinal localization of retromer component RME-8 and rab-6 cargo MIG-14. Images A, B, and C show RME-8::mCherry, MIG-14::GFP, and the merged image treated with empty RNAi vector L4440. Images D, E, and F show the same markers in animals grown two generations on RNAi for rab-6.1. Images G, H, and I show the same markers in animals grown two generations on RNAi for rab-6.2.

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