RAB-6.2 and the retromer regulate glutamate receptor recycling through a retrograde pathway

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Regulated membrane trafficking of AMPA-type glutamate receptors (AMPARs) is a key mechanism underlying synaptic plasticity, yet the pathways used by AMPARs are not well understood. In this paper, we show that the AMPAR subunit GLR-1 in Caenorhabditis elegans utilizes the retrograde transport pathway to regulate AMPAR synaptic abundance. Mutants for rab-6.2, the retromer genes vps-35 and snx-1, and rme-8 failed to recycle GLR-1 receptors, resulting in GLR-1 turnover and behavioral defects indicative of diminished GLR-1 function. In contrast, expression of constitutively active RAB-6.2 drove the retrograde transport of GLR-1 from dendrites back to cell body Golgi. We also find that activated RAB-6.2 bound to and colocalized with the PDZ/phosphotyrosine binding domain protein LIN-10. RAB-6.2 recruited LIN-10. Moreover, the regulation of GLR-1 transport by RAB-6.2 required LIN-10 activity. Our results demonstrate a novel role for RAB-6.2, its effector LIN-10, and the retromer complex in maintaining synaptic strength by recycling AMPARs along the retrograde transport pathway.

Introduction

AMPA-type glutamate receptors (AMPARs) mediate much of the excitatory postsynaptic response at central nervous system synapses, and the regulated trafficking of AMPARs is a pivotal mechanism by which neurons regulate synaptic strength at excitatory synapses (Shepherd and Huganir, 2007; Henley et al., 2011). Once endocytosed into early endosomes, AMPARs can be sorted either into recycling pathways, which send them back to the plasma membrane, or into degradation pathways, which send them to the lysosome via multivesicular bodies (MVBs) and late endosomes. Recycling of previously endocytosed AMPARs from endosomal pools can occur through recycling endosomes (Gerges et al., 2004; Park et al., 2004; Hanley, 2010). Such regulated recycling is important for long-term potentiation, long-term depression, and homeostatic plasticity (Turrigiano, 2008; Kessels and Malinow, 2009; Makino and Malinow, 2009). Given the complex cell biological organization of the neuron, it is likely that additional trafficking mechanisms determine AMPAR abundance and composition at the synapse.

Recycling also occurs through retrograde transport from early endosomes back to the Golgi followed by exit from the Golgi to the plasma membrane. The retrograde pathway is particularly important for the retrieval of Golgi residents, signaling molecule chaperones, and membrane receptors, and the pathway can also be subjugated by pathogens and their toxins (Bonifacino and Rojas, 2006; Bonifacino and Hurley, 2008; Johannes and Popoff, 2008; Pan et al., 2008; Lieu and Gleeson, 2011; Pfeffer, 2011). Surprisingly, little is known about retrograde transport in neurons, and it remains unknown whether synaptic proteins or neurotransmitter receptors, such as AMPARs, use the retrograde pathway.

Retrograde transport is mediated by the retromer complex, which is comprised of sorting nexins (Vps5–SNX1/2) and the VPS26–VPS29–VPS35 subcomplex (Bonifacino and Rojas, 2006; Bonifacino and Hurley, 2008; Johannes and Popoff, 2008). The retromer is found on long tubules that extend from the early endosome.
endosome, where it shunts cargo away from the endosomal sorting complex required for transport (ESCRT) on the limiting membrane (Arighi et al., 2004; Carlton et al., 2004; Rojas et al., 2007). In the absence of retromer function, retrograde cargo is inadvertently sent down the degradation pathway by the ESCRT complex via MVBs (Arighi et al., 2004; Carlton et al., 2004). Members of the Rab6 small GTPase family of proteins also regulate retrograde transport, yet how the function of the Rab6 GTPases is integrated with that of the retromer is unclear (Echard et al., 2000; Mallard et al., 2002; Del Nery et al., 2006). A role for the retromer in AMPAR trafficking has not been described.

AMPARs also undergo regulated trafficking in the interneurons of Caenorhabditis elegans. C. elegans AMPARs are comprised of two subunits, GLR-1 and GLR-2, which function in the command interneurons where they transduce synaptic input from nose-touch mechanosensory neurons and govern overall locomotory behavior (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002; Chang and Rongo, 2005). GLR-1 and GLR-2 AMPARs also promote spontaneous reversals in the direction of locomotion (Zheng et al., 1999). Mutants that lack AMPAR function or fail to transport and maintain AMPARs at synapses have reduced nose-touch mechanosensitivity and exhibit a depressed frequency of spontaneous reversals; thus, these behaviors correlate with AMPAR synaptic abundance (Burbea et al., 2002; Shim et al., 2004; Glodowski et al., 2005). Previous genetic approaches have identified the Rab-type small GTPases RAB-5, UNC-108/RAB-2, and RAB-10 as key regulators of AMPAR trafficking in C. elegans (Glodowski et al., 2005; Chun et al., 2008; Park et al., 2009), raising the possibility that GLR-1 AMPARs are regulated by additional Rabs.

To understand how neurons regulate AMPAR recycling, we tested different candidate C. elegans Rabs for their ability to regulate GLR-1 trafficking. Here, we show that RAB-6.2, together with the retromer complex, promotes the retrograde recycling of GLR-1–containing AMPARs. We show that rab-6.2 mutants display defects in GLR-1 localization and behavior consistent with defects in retrograde transport. We show that RAB-6.2 is colocalized with LIN-10, a member of the Mint/ X11 family (Whitfield et al., 1999; Glodowski et al., 2007), regulating GLR-1 retrograde transport through this interaction. We propose that neurons use the retrograde transport pathway to regulate the synaptic abundance of neurotransmitter receptors and that the Mint family of proteins is part of the retrograde transport machinery.

Results

RAB-6.2 regulates GLR-1 trafficking

To screen through multiple Rabs for candidates that regulate GLR-1 trafficking, we generated transgenes that express dominant-negative GDP-locked versions of Rabs while restricting their expression to postembryonic command interneurons by using the glr-1 promoter. The trafficking of GLR-1 can be monitored using the nuds25[glr-1::gfp] transgenic strain, which expresses a rescuing GLR-1::GFP chimeric receptor that is localized to the postsynaptic face of synapses (Rongo et al., 1998; Burbea et al., 2002). Individual GLR-1::GFP-containing synapses can be observed as small (0.5–0.7 μm) puncta along the ventral cord dendrites in wild-type young adult animals (Fig. 1 B). Expression of a GDP-locked version of one of the Rabs we tested, rab-6.2, resulted in a dramatic decrease in the number and fluorescence intensity of GLR-1::GFP puncta compared with wild type (Fig. 1 C and not depicted). We obtained a mutant for the rab-6.2 gene, rab-6.2(ok2254), that contains a deletion of rab-6.2 sequences, including the promoter, both “switch” domains, and the GTPase catalytic core, resulting in a likely molecular null (Fig. 1 A). We observed a significant decrease in the number and fluorescent intensity of GLR-1 puncta along the ventral cord dendrites of rab-6.2(ok2254) mutants (Fig. 1, D–F; and Fig. S1 A), indicating that RAB-6.2 is required for proper GLR-1 localization. We also examined the localization of a presynaptic protein, SNB-1 (synaptoporelin; Nonet et al., 1998) and observed no difference in GFP-labeled SNB-1 puncta in mutants compared with wild type (Fig. 1 E and Fig. S2, A and B), indicating that the defects in GLR-1 subcellular localization are not caused by gross defects in synapse formation along GLR-1–expressing interneurons.

GLR-1 can form either homomeric channels or heteromeric channels with GLR-2 (Mellem et al., 2002; Shim et al., 2004; Chang and Rongo, 2005; Emtege et al., 2009). We introduced a transgene expressing GFP::GLR-2 (Mellem et al., 2002) into rab-6.2 mutants and found a similar number and intensity of ventral cord puncta compared with wild type (Fig. 1, E, Fig. S1 B, and Fig. S2, C and D). GLR-1 can also associate with the coreceptor STG-1 (Stargazin) and the CUB domain protein SOL-1 (Zheng et al., 1999; Wang et al., 2008). We examined STG-1::GFP and SOL-1::GFP in rab-6.2 mutants and found their pattern of localization to be similar to that of wild type (Fig. S2, G–J), CNIH-2 (cornichon homologue 2) associates with mammalian AMPARs, modulating their gating properties (Schwenk et al., 2009; Kato et al., 2010; Shi et al., 2010). We thus generated a transgene, P_glr-1::cnih-2::gfp, containing C. elegans CNIH-2 sequences fused to GFP and driven by the glr-1 promoter. We found that CNIH-2::GFP is localized to puncta along ventral cord dendrites (Fig. S1 C and Fig. S2, E and F), although at a lower number in rab-6.2 mutants compared with wild type (Fig. 1 E). These results suggest that the function of RAB-6.2 is biased toward regulating the trafficking of GLR-1 homomeric channels and some, but not all, of the cofactors known to associate with C. elegans AMPARs.

A decrease in synaptic GLR-1::GFP should result in a decrease in GLR-1 function. We found that both the spontaneous reversal rate (Fig. 1 G) and the mechanosensitivity (Fig. 1 H) of rab-6.2 mutants was significantly lower than that in wild type, indicating that a loss of endogenous GLR-1 function accompanies the drop in synaptic GLR-1 in these mutants. We also directly tested whether RAB-6.2 can influence endogenous GLR-1 channel activity. Glutamate-activated currents can be recorded from the interneuron AVA in whole-cell configuration (Mellem et al., 2002). We measured GLR-1–mediated currents elicited by the application of 1 mM glutamate (Ward et al., 2008), and we found that there was a significant reduction in current amplitude in rab-6.2 mutants versus wild type when AVA was voltage clamped at −70 mV (Fig. 1 I and Fig. S1 D). The current–voltage
relationship in neurons from wild-type animals and *rab-6.2* mutants did not differ, indicating that *rab-6.2* mutants do not have general defects in conductance (Fig. S1, E–G).

**RAB-6.2 regulates GLR-1 cell autonomously**

To determine the expression pattern of RAB-6.2, we generated transgenes containing *rab-6.2* genomic sequences (including 2 kb of upstream promoter sequences, the complete ORF, and introns) fused in frame to GFP sequences. We introduced this *rab-6.2::gfp* transgene into nematodes and found that RAB-6.2::GFP is highly expressed in body wall muscles, pharyngeal and vulval muscles, hypodermis, intestine, the gonad, coelomocytes, and neurons (Fig. 2, A–I). We also introduced this transgene into nematodes that also express a *Pglr-1::monomeric RFP* (*mRFP*) transgene (Shim et al., 2004), and we found that the relationship in neurons from wild-type animals and *rab-6.2* mutants did not differ, indicating that *rab-6.2* mutants do not have general defects in conductance (Fig. S1, E–G).
RAB-6.2::GFP is expressed in the GLR-1–expressing command interneurons (Fig. 2 J).

To confirm that the rab-6.2 gene is responsible for the mutant phenotypes and to test for the cell-autonomous function of RAB-6.2, we generated a transgene containing glr-1 promoter sequences fused to rab-6.2 cDNA sequences, with an N-terminal Venus to allow us to monitor expression and subcellular localization. We introduced this P_{glr-1::venus::rab-6.2} transgene into wild-type animals and rab-6.2 mutants, both of which express GLR-1::CFP (Chang and Rongo, 2005). We found that P_{glr-1::venus::rab-6.2} completely rescued the defects in both GLR-1::CFP puncta number and puncta fluorescence intensity (Fig. 1, E and F) as well as the spontaneous reversal and mechanosensitivity defects (Fig. 1, G and H), indicating that RAB-6.2 functions cell autonomously.

**RAB-6.2 regulates GLR-1 trafficking at a step after endocytosis**

One explanation for the decrease in GLR-1::GFP puncta observed in rab-6.2 mutants is that RAB-6.2 facilitates the retrograde transport of previously endocytosed GLR-1 receptors and that, in the absence of RAB-6.2 activity, these receptors are trafficked for degradation. We tested this model by determining whether endocytosis is required for the receptor turnover that we observe in rab-6.2 mutants, as mutations that block GLR-1 endocytosis should suppress rab-6.2. The clathrin adaptin AP180 orthologue UNC-11 is a key mediator of GLR-1 endocytosis, and loss-of-function mutations in unc-11 suppress the turnover and/or internal accumulation of receptors observed in membrane-recycling mutants (Nonet et al., 1999; Burbea et al., 2002). We analyzed GLR-1::GFP localization in rab-6.2; unc-11 double mutants and found that mutations in unc-11 prevent the decrease in GLR-1::GFP puncta number and fluorescence intensity observed in rab-6.2 mutants (Fig. 3, A, B, and E; and Fig. S1 A). GLR-1 endocytosis is also facilitated by the direct ubiquitination of four key lysine residues on the C-terminal tail sequences of the receptor itself (Burbea et al., 2002). The nuIs108[P_{glr-1::glr-1(4kr)::gfp}] transgene expresses a GLR-1::GFP in which the ubiquitinated lysines are mutated to arginines,
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...endosome and depress receptor endocytosis and turnover. Unlike for wild-type GLR-1::GFP, GLR-1(4KR)::GFP puncta number and fluorescence intensity is not altered in rab-6.2 mutants compared with wild type (Fig. 3, C–E; and Fig. S1 A). Moreover, expression of GLR-1(4KR)::GFP, but not wild-type GLR-1::GFP, restored mechanosensitivity to rab-6.2 mutants (Fig. 3 F). Our results indicate that clathrin-mediated endocytosis and receptor ubiquitination are required for the turnover of the GLR-1 receptor and loss of GLR-1 synaptic function observed in rab-6.2 mutants, suggesting that RAB-6.2 regulates GLR-1 trafficking at a step after endocytosis and receptor ubiquitination.

RAB-6.2 regulates the exit of GLR-1 from endosomes and GLR-1 turnover

The increase in GLR-1::GFP turnover observed in rab-6.2 mutants suggested that GLR-1 might be improperly sorted from early endosomes to MVBs, late endosomes, and eventually lysosomes via ESCRT-mediated transport. We tested this possibility by blocking ESCRT-mediated transport in rab-6.2 mutants and observing whether GLR-1 would accumulate in endosomes. The VPS-4 AAA ATPase facilitates the movement of endocytosed cargo from early endosomes to MVBs, and expression of a dominant-negative VPS-4 mutant protein can significantly reduce trafficking from early endosomes to MVBs and the late endosome (Babst et al., 2002; Yeo et al., 2003). Neuronal expression of dominant-negative VPS-4 from the transgene nls145[Pglr-1::vps-4(dn)] blocks the movement of GLR-1 receptors from early endosomes to MVBs and decreases their ubiquitin-mediated turnover (Chun et al., 2008). We found that expression of dominant-negative VPS-4 in rab-6.2 mutants restored GLR-1::GFP puncta number and fluorescence intensity to wild-type levels (Fig. 4, A–C; and Fig. S1 A), indicating that the ESCRT pathway is required for GLR-1 turnover when retrograde transport is blocked. Consistent with this model, we detected lower levels of GLR-1 in rab-6.2 mutants by Western blotting in whole-nematode lysates (Fig. 4 F).

If the pathways for retrograde recycling and transport to MVBs are both blocked, GLR-1 should eventually accumulate in early endosomes and thus decrease synaptic GLR-1 function. We therefore measured the spontaneous reversal rate and mechanosensitivity of GLR-1 in rab-6.2 mutants compared with GLR-1 mutants that also express dominant-negative VPS-4. Although dominant-negative VPS-4 can prevent GLR-1 turnover, it cannot restore either the decreased frequency in reversal rates (Fig. 4 D) or the decreased mechanosensitivity (Fig. 4 E) observed in rab-6.2 mutants, suggesting that the GLR-1 receptors in rab-6.2 nls145[Pglr-1::vps-4(dn)] animals are not accumulating at synaptic sites.

To determine the specific site of GLR-1 accumulation in these animals, we used the endosomal markers SYN-13...
animals, Venus::RAB-5 is localized to a small number of puncta along the ventral cord dendrites, and a small number of GLR-1::CFP puncta colocalized with these Venus::RAB-5 puncta (Fig. 4 K). In rab-6.2 mutants, Venus::RAB-5 becomes distributed throughout the dendrites, consistent with an enlargement of early endosomes caused by arrested retrograde transport (Fig. 4 L). The remaining GLR-1::CFP in rab-6.2 mutants is colocalized with Venus::RAB-5, and this colocalization is also observed when MVB trafficking is blocked by dominant-negative VPS-4 (Fig. 4, L and M). Collectively, these results indicate that RAB-6.2 regulates the exit of endocytosed GLR-1 receptors out of early endosomes in neurons.

We observed mRFP::SYN-13 and GLR-1::GFP colocalization in neuron cell bodies as previously described (Chun et al., 2008; Park et al., 2009). We coexpressed mRFP::SYN-13 with GLR-1::GFP in both rab-6.2 and rab-6.2 nls145[Pgbp::vps-4(dn)] animals. Although we did not see a significant change in the amount of GLR-1::GFP colocalized with mRFP::SYN-13 puncta in rab-6.2 mutants (~30%; Fig. 4, G, H, and J), we found that the mRFP::SYN-13-labeled structures are increased in size, with nearly 70% of GLR-1::GFP colocalized, in rab-6.2 mutants when MVB trafficking is blocked by dominant-negative VPS-4 (Fig. 4, I and J). We also coexpressed GLR-1::CFP with Venus::RAB-5 in both rab-6.2 and rab-6.2 nls145[Pgbp::vps-4(dn)] animals. In wild-type animals, Venus::RAB-5 is localized to a small number of puncta along the ventral cord dendrites, and a small number of GLR-1::CFP puncta colocalized with these Venus::RAB-5 puncta (Fig. 4 K). In rab-6.2 mutants, Venus::RAB-5 becomes distributed throughout the dendrites, consistent with an enlargement of early endosomes caused by arrested retrograde transport (Fig. 4 L). The remaining GLR-1::CFP in rab-6.2 mutants is colocalized with Venus::RAB-5, and this colocalization is also observed when MVB trafficking is blocked by dominant-negative VPS-4 (Fig. 4, L and M). Collectively, these results indicate that RAB-6.2 regulates the exit of endocytosed GLR-1 receptors out of early endosomes in neurons.
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RAB-6.2 associates with GLR-1 and Golgi
To examine RAB-6.2 subcellular localization, we generated transgenes containing the glr-1 promoter sequences fused to sequences encoding GFP, Cerulean, or Venus fused in frame to the N-terminal sequences of RAB-6.2. We found that RAB-6.2 is localized to punctate structures in the neuron cell body and along the ventral cord dendrites. We introduced the $P_{glr-1}:venus::rab-6.2$ transgene into nematodes that express the Golgi resident protein mannosidase (MANS):YFP (Rolls et al., 2002; Glodowski et al., 2005) and observed nearly complete colocalization between Cerulean::RAB-6.2 and MANS::YFP, indicating that RAB-6.2 is localized on or near Golgi structures (Fig. 5 A). We introduced the $P_{glr-1}:venus::rab-6.2$ transgene into nematodes expressing GLR-1::CFP, and we found that GLR-1::CFP and Venus::RAB-6.2 are colocalized at or near individual puncta in both the cell body (Fig. 5 B) and the ventral cord dendrites (Fig. 5 C). Our results indicate that a minority of RAB-6.2 and GLR-1 puncta are colocalized, consistent for a cargo molecule and a specific Rab GTPase regulator of its trafficking.

To test whether a guanine nucleotide-bound state regulates RAB-6.2 subcellular localization, we introduced either GDP-locking (RAB-6.2(GDP), mutation T24N) or GTP-locking (RAB-6.2(GTP), mutation Q69L) mutations into the GFP-tagged transgene. Whereas wild-type GFP::RAB-6.2 is found in puncta along dendrites (Fig. 5 D), GFP::RAB-6.2(GDP) was diffusely distributed in the dendrites and cell body cytosol (Fig. 5 E and not depicted). In contrast, we found GFP::RAB-6.2(GTP) in a punctate pattern, with higher levels of punctate fluorescence intensity than those observed for the wild-type GFP::RAB-6.2 protein (Fig. 5 F). Thus, activation of RAB-6.2 by GTP binding results in its punctate localization.

RAB-6.2 can drive GLR-1 retrograde transport back to soma Golgi
To test whether the activation of RAB-6.2 has an instructive role in directing GLR-1 retrograde transport, we generated $P_{glr-1}:rab-6.2(gtp)$, a transgene that expresses GTPase-defective, constitutively active RAB-6.2 via the glr-1 promoter. Expression of RAB-6.2(GTP) resulted in the accumulation of few GLR-1::GFP ventral cord puncta and, instead, resulted in the accumulation in several large puncta in the neuron cell bodies (Fig. 6, A–D, I, and J). We examined GLR-1::GFP in animals that express RAB-6.2(GTP) and are also blocked for endocytosis. We found that both the decrease in GLR-1 ventral cord puncta and the accumulation of GLR-1 in large cell body puncta caused by RAB-6.2(GTP) are prevented in unc-11 mutants (Fig. 6, E–J). Similarly, GLR(4KR)::GFP puncta levels remain steady in the dendrites of animals expressing RAB-6.2(GTP) (Fig. 6 J; and Fig. S1 A). Moreover, coexpression of dominant-negative VPS-4 does not suppress the decrease in dendritic GLR-1 or the accumulation of GLR-1 in the cell body in animals expressing RAB-6.2(GTP) (Fig. S1 A and not depicted). Thus, RAB-6.2(GTP) promotes the retrograde redistribution rather than the turnover of previously endocytosed GLR-1 receptors.

If RAB-6.2(GTP) is driving GLR-1 out of synapses, we would expect a reduction in GLR-1 function. We therefore measured the spontaneous reversal rate and mechanosensitivity of animals expressing RAB-6.2(GTP) and found that both behaviors were reduced (Fig. 6 K and not depicted). To determine the site of GLR-1 accumulation in the cell body, we expressed RAB-6.2(GTP) in $ols25[glr-1::glr-1::cfp]$ transgenic animals that also express MANS::YFP, which resides in the Golgi (Rolls et al., 2002; Shim et al., 2004). In wild-type cell bodies, little GLR-1::CFP is colocalized with MANS::YFP (Fig. 6 L).

Figure 5. RAB-6.2 associates with Golgi and GLR-1. (A and A′) Cerulean::RAB-6.2 [A] and MANS::YFP [A′] in PVC cell bodies. [A″] Merged image showing Cerulean::RAB-6.2 colocalization to puncta with MANS::YFP (arrowheads). [B−C″] GLR-1::CFP [B and C] and Venus::RAB-6.2 [B′ and C′] in PVC cell bodies [B−B″] and dendrites [C−C″]. [B′′ and C′′] Merged images show colocalization at specific points (arrowheads). [D−F] Subcellular localization of the indicated GFP::RAB-6.2 variant: wild type [D], GDP-locked mutant [E], and GTP-locked mutant [F]. Bars, 5 µm.
containing the LIN-10 PTB domain in bacteria and then tested the ability of these proteins when bound to glutathione agarose beads to pull down in vitro translated HA-tagged RAB-6.2 protein. We also tested the ability of GST::LIN-10(PTB) to interact with mutant RAB-6.2 protein locked in either its GTP-bound or GDP-bound state. GST alone could not pull down HA::RAB-6.2 protein (Fig. 7 A). However, GST::LIN-10(PTB) could specifically pull down HA::RAB-6.2(GTP) but not HA::RAB-6.2(GDP) (Fig. 7 A). We also expressed either GST alone, GST::RAB-6.2(GTP), or GST::RAB-6.2(GDP) in bacteria and then tested the ability of these proteins to pull down an in vitro translated HA-tagged LIN-10 PTB domain. We found that GST::RAB-6(GTP), but not GST alone, GST::RAB-6(GDP), specifically pulled down HA::LIN-10(PTB) (Fig. 7 B). Thus, activated RAB-6.2 can physically bind to LIN-10.

We next introduced the Pglr-1::venus::rab-6.2 transgene into animals that express a LIN-10::CFP chimeric protein to test whether LIN-10::CFP drives GLR-1::CFP into MANS::YFP-decorated Golgi (Fig. 6 M). In contrast, expression of RAB-6.2(GTP) drives GLR-1::GFP into MANS::YFP-decorated Golgi (Fig. 6 M).

**Activated RAB-6.2 interacts with LIN-10**

One possible effector target of RAB-6.2 could be LIN-10, a PDZ/phosphotyrosine binding (PTB) scaffolding molecule that regulates GLR-1 recycling (Glodowski et al., 2007; Park et al., 2009). The PTB domain of Mint3, a mammalian LIN-10 homologue, can directly bind to mammalian Rab6 (Teber et al., 2005; Thyrock et al., 2010). We therefore tested whether LIN-10 PTB domain bait and various C. elegans candidate Rab prey could interact by yeast two-hybrid assay. We tested 19 different Rab preys and found an interaction for RAB-6.1 and RAB-6.2, members of the Rab6 subfamily (Fig. S3).

To confirm the physical interaction between the LIN-10 PTB domain and RAB-6.2, we expressed either GST or GST::LIN-10(PBTB), which contains GST plus 207 amino acids containing the LIN-10 PTB domain in bacteria and then tested the ability of these proteins when bound to glutathione agarose beads to pull down in vitro translated HA-tagged RAB-6.2 protein. We also tested the ability of GST::LIN-10(PTB) to interact with mutant RAB-6.2 protein locked in either its GTP-bound or GDP-bound state. GST alone could not pull down HA::RAB-6.2 protein (Fig. 7 A). However, GST::LIN-10(PTB) could specifically pull down HA::RAB-6.2(GTP) but not HA::RAB-6.2(GDP) (Fig. 7 A). We also expressed either GST alone, GST::RAB-6.2(GDP), or GST::RAB-6.2(GTP) in bacteria and then tested the ability of these proteins to pull down an in vitro translated HA-tagged LIN-10 PTB domain. We found that GST::RAB-6(GTP), but not GST alone, GST::RAB-6(GDP), specifically pulled down HA::LIN-10(PTB) (Fig. 7 B). Thus, activated RAB-6.2 can physically bind to LIN-10.

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We next introduced the Pglr-1::venus::rab-6.2 transgene into animals that express a LIN-10::CFP chimeric protein to test whether LIN-10::CFP drives GLR-1::CFP into MANS::YFP-decorated Golgi (Fig. 6 M). In contrast, expression of RAB-6.2(GTP) drives GLR-1::GFP into MANS::YFP-decorated Golgi (Fig. 6 M).
determine where in neurons this physical interaction occurs. LIN-10 is localized to Golgi structures in neuron cell bodies and punctate structures along ventral cord dendrites (Glodowski et al., 2005). We found that Venus::RAB-6.2 is colocalized with LIN-10::CFP in neuron cell bodies (Fig. 7 C). Along dendrites, Venus::RAB-6.2 is colocalized adjacent to sites of LIN-10 localization (Fig. 7 D), indicating that these two proteins are found at related subcellular compartments within neurons.

Both LIN-10 and RAB-6.2 are broadly expressed in multiple tissues, including the intestine (Fig. 2; Whitfield et al., 1999). *C. elegans* intestinal cells are polarized epithelial cells that are well suited for subcellular trafficking experiments (Grant and Sato, 2006). To examine LIN-10 and RAB-6.2 in epithelial cells, we generated transgenes containing the *vha-6* intestine-specific promoter sequences driving sequences encoding GFP, tagRFP, or mCherry fused in frame to the complete RAB-6.2, MANS, or LIN-10 reading frame sequences. Both LIN-10::GFP and tagRFP::RAB-6.2 proteins were localized to punctate structures in the intestinal cytosol (Fig. S4 A). Similarly, both GFP::RAB-6.2 and MANS::mCherry were completely colocalized (Fig. S4 B). Finally, we found that LIN-10::GFP and MANS::mCherry were localized adjacent to one another in the intestine (Fig. S4 C). Collectively, our results suggest that RAB-6.2 is localized tightly to MANS-containing Golgi structures and that LIN-10 is localized to adjacent Golgi-associated structures.

**LIN-10 is an effector of RAB-6.2**

To test whether LIN-10 is a RAB-6.2 effector, we first examined whether RAB-6.2 acted to recruit LIN-10 by introducing the *odls22[P_glc-1::lin-10::GFP]* transgene into *rab-6.2* mutants. Although LIN-10::GFP is punctate in wild type (Fig. 8 A), it is diffusely distributed in the cytosol of *rab-6.2* mutants (Fig. 8 B). We next introduced transgenes expressing either RAB-6.2(GDP) or RAB-6.2(GTP) into *odls22* animals. Like in *rab-6.2* mutants, LIN-10::GFP is not localized to puncta when RAB-6.2(GDP) is expressed (Fig. 8 C). In contrast, expression of RAB-6.2(GTP) drove LIN-10::GFP into a more punctate localization pattern (Fig. 8 D), suggesting that the activation of RAB-6.2 regulates LIN-10 subcellular localization. Using fluorescence density thresholding (Umemura et al., 2005), we were able to approximate the relative levels of LIN-10::GFP present in puncta versus the unlocalized baseline along the ventral cord (Fig. 8 E). We observed no difference.
effector. As GLR-1 receptors accumulate in elongated endosomes in lin-10 mutants, we speculate that LIN-10 also plays a separate, RAB-6.2–independent role in transporting GLR-1 from early endosomes to the degradation pathway.

Another mediator of retrograde transport is the retromer complex. Thus, we examined GLR-1::GFP localization in the retromer mutants snx-1(tm847) and vps-35(hu68) as well as mutants for the SNX-1–associated rme-8(b1023). We found a significant decrease in the number and fluorescent intensity of GLR-1::GFP puncta in all three of these mutants (Fig. 9, A–D, and G; and Fig. S1 A) similar to the phenotype observed in rab-6.2 mutants, suggesting that the retromer also promotes GLR-1 recycling. Mutations that impaired GLR-1 endocytosis or ubiquitination blocked the effects of these retromer mutants (Fig. 9 G, Fig. S1, and not depicted), indicating that the retromer regulates GLR-1 trafficking at a step after endocytosis. Finally, turnover

in GFP::RAB-6.2 puncta in wild type (Fig. 8 F) compared with lin-10 mutants (Fig. 8 G), indicating that whereas RAB-6.2 regulates LIN-10 subcellular localization, the reverse is not true.

If LIN-10 is a RAB-6.2 effector, the effect of RAB-6.2 on GLR-1 retrograde transport should require LIN-10 activity. Although RAB-6.2(GTP) can drive GLR-1::CFP to colocalize with MANS::YFP in wild type (Fig. 6 M), it cannot in lin-10 mutants (Fig. 8 H). We also examined GLR-1::GFP ventral cord localization in transgenic animals expressing RAB-6.2(GTP), which are also mutated for lin-10. In lin-10 mutants, GLR-1 accumulates in elongated postendocytic endosomes that are readily distinguished based on their size and morphology (Glodowski et al., 2007; Park et al., 2009). We found that if LIN-10 activity is absent, GLR-1::GFP accumulates in elongated endosomes along ventral cord dendrites regardless of the presence of RAB-6.2(GTP) (Fig. 8, I–K). Thus, RAB-6.2 promotes the retrograde transport of GLR-1 receptors back to Golgi at least in part through its interaction with LIN-10 as an effector. As GLR-1 receptors accumulate in elongated endosomes in lin-10 mutants, we speculate that LIN-10 also plays a separate, RAB-6.2–independent role in transporting GLR-1 from early endosomes to the degradation pathway.

The retromer regulates GLR-1 recycling

Another mediator of retrograde transport is the retromer complex. Thus, we examined GLR-1::GFP localization in the retromer mutants sns-1(tm847) and vps-35(hu68) as well as mutants for the SNX-1–associated rme-8(b1023). We found a significant decrease in the number and fluorescent intensity of GLR-1::GFP puncta in all three of these mutants (Fig. 9, A–D, and G; and Fig. S1 A) similar to the phenotype observed in rab-6.2 mutants, suggesting that the retromer also promotes GLR-1 recycling. Mutations that impaired GLR-1 endocytosis or ubiquitination blocked the effects of these retromer mutants (Fig. 9 G, Fig. S1, and not depicted), indicating that the retromer regulates GLR-1 trafficking at a step after endocytosis. Finally, turnover
Figure 9. The retromer regulates GLR-1 retrograde transport. (A–F) GLR-1::GFP in wild type (A), snx-1(tm847) (B), vps-35(hu68) (C), rme-8(b1023) (D), rab-6.2(GTP)–expressing vps-35(hu68) (E), and rab-6.2(GTP)–expressing rme-8(b1023) (F) animals. (G) Mean fluorescent intensity of GLR-1 puncta (plus sign indicating wild type). (H and I) The mean integrated fluorescent intensity for individual PVC cell bodies (H) and nose-touch mechanosensitivity (I). (J) Mean nose-touch mechanosensory response is plotted for each trial during a train of nose-touch trials over a 5-min period. Genotypes are indicated by color. Rescue indicates rab-6.2(ok2254) mutants that express wild-type Venus::RAB-6.2 from the glr-1 promoter. Bars, 5 µm. ANOVA with Dunnett’s multiple comparison to wild type (***, P < 0.001). Error bars are SEM. AU, arbitrary unit.
of GLR-1 in these retromer mutants is blocked when dominant-negative VPS-4 is expressed, suggesting that, in the absence of retromer function, GLR-1 is shunted by the ESCRT complex to MVBs, late endosomes, and lysosomes for proteolysis (Fig. 9 G and Fig. S1 A).

To examine whether RAB-6.2 and the retromer regulate GLR-1 via the same pathway, we performed an epistasis analysis between rab-6.2 and the retromer mutations. Double mutants for rab-6.2 and either rme-8 or vps-35 did not show a dramatically stronger effect on GLR-1 cluster number, suggesting either that these genes function in the same genetic pathway or that the retrograde pathway is completely blocked in the absence of either RAB-6.2 or the retromer alone, occluding a stronger phenotype in the double mutant (Fig. 9 G and Fig. S1 A).

We also generated double mutants that contain a GTP-locked P_{GTP}:rab-6.2(gtp) transgene, which can drive GLR-1::GFP retrograde transport from dendrites back to the cell body, and either a vps-35 (Fig. 9 E) or rme-8 (Fig. 9 F) mutation. We found that RAB-6.2(GTP) can drive GLR-1::GFP retrograde transport back to the cell body regardless of the presence of VPS-35 or RME-8 activity (Fig. 9 H), suggesting that RAB-6.2 and the retromer complex can independently mediate GLR-1 retrograde transport under certain circumstances.

If RAB-6.2 and the retromer both promote GLR-1 retrograde recycling, we would expect RAB-6.2 and retromer components to be localized either together or nearby. The J domain protein RME-8 associates with the retromer complex, where it helps regulate clathrin dynamics at early endosomes (Shi et al., 2009). Thus, we coexpressed Cerulean::RAB-6.2 with Venus::RME-8 in the command interneurons, and we observed that the two proteins were colocalized (Fig. 10 C and D), often with Venus::RME-8...
When GLR-1 is overexpressed, AMPARs are sequestered into endosomal tubules that in turn give rise to retrograde cargo vesicles that are sent to MVBs for eventual degradation. In contrast, AMPARs can either be sent to MVBs for eventual degradation, or they can be recycled back to the synapse. We propose that RAB-6.2 participates in both pathways, such as the retrograde pathway, are used for subunit-specific AMPAR regulation.

The rapid habituation observed in the glr-1 mutants is fully rescued by the expression of a rescuing wild-type Venus::RAB-6.2 from the glr-1 promoter, suggesting that RAB-6.2 and retrograde transport of AMPARs not only maintain glutamatergic efficacy but also regulate the habituation kinetics of the touch circuit in the postsynaptic interneurons.

**Discussion**

Here, we have shown that RAB-6.2 and LIN-10 recycle AMPARs along a retrograde transport pathway in neurons so as to maintain synaptic strength (Fig. S5). GLR-1 AMPARs undergo activity-dependent endocytosis in a process that requires UNC-11/AP180 (Fig. S5 A; Burbea et al., 2002; Grunwald et al., 2004; Glodowski et al., 2007). Once endocytosed, AMPARs can either be sent to MVBs for eventual degradation, or they can be recycled back to the synapse. We propose that RAB-6.2 performs two functions to promote the recycling fate for these receptors. First, RAB-6.2, in its GTP-bound form, interacts with LIN-10, delivering LIN-10 to early endosomes (Fig. S5 B). We suggest that LIN-10, along with the retromer complex and RME-8, sequesters AMPARs into endosomal tubules that in turn give rise to retrograde cargo vesicles for the receptors (Fig. S5 C). Second, RAB-6.2, in its GTP-bound form, regulates the trafficking of these cargo vesicles to Golgi, including dendritic output of Golgi as well as cell body Golgi (Fig. S5 D). Interestingly, RAB-6.2–decorated Golgi and LIN-10/RME-8–decorated endosomes are adjacent to one another in neurons and epithelia cells, perhaps indicating that Golgi-proximal endosomes favor this pathway. Once at Golgi, AMPARs can affiliate with new coreceptors and be sorted back to synaptic membranes (Fig. S5 E). Our results suggest that GLR-2–containing AMPARs do not rely on this pathway, indicating that different trafficking pathways, such as the retrograde pathway, are used for subunit-specific AMPAR regulation.
GLR-1–containing channels but not GLR-2–containing channels; thus, GLR-1 homomic channels and GLR-1/2 heteromeric channels might use different pathways for their recycling. Interestingly, these subunits have different affinities for scaffolding molecules, show different trafficking patterns in response to previous mechanosensory experience, and yield different habituation kinetics within the touch circuit (Emtage et al., 2009). Thus, RAB-6.2–mediated trafficking might play an important role in this or other types of behavioral plasticity in the touch circuit by dictating the specific GLR-1/GLR-2 subunit composition at the synapse. Indeed, animals displayed faster habituation to touch in mutants defective for the retrograde transport of GLR-1–containing AMPARs. Future tests of this idea will require a more thorough means for discriminating the subunit combinations.

The role of retrograde transport in neurons is poorly understood with one important exception: the amyloid precursor protein (APP). Changes in retromer gene expression are correlated with Alzheimer’s disease (AD), and depletion of the retromer increases the production of Aβ, one of the key pathological causes of AD (Small, 2008). Neuronal SorLA (sorting protein-related receptor) associates with APP and helps regulate APP retrograde transport to the Golgi; SorLA is also reduced in AD brains (Scherzer et al., 2004; Andersen et al., 2005; Offe et al., 2006; Nielsen et al., 2007). Retrograde transport also appears to help recycle the β-secretases BACE1 and BACE2, which influence Aβ production (He et al., 2005; Wahle et al., 2005). Thus, APP retrograde transport might keep Aβ production low in healthy neurons by keeping APP out of the endosome, the site where it becomes processed to produce Aβ. Consistent with this idea, the Mints, which are orthologues of LIN-10, regulate APP and presenilin trafficking, as well as APP processing into Aβ, although the exact mechanism remains controversial (Sastre et al., 1998; Mueller et al., 2000; Hill et al., 2003; Lee et al., 2003; Xie et al., 2005; Sano et al., 2006; Ho et al., 2008; Saito et al., 2008). Our results indicate that Mint proteins are effectors for Rab6 GTases in neurons and could help explain how an evolutionarily conserved choice point in trafficking regulated by Mint-Rab6 has been co-opted for a pathological function in human disease.

Materials and methods

Strains

Animals were grown at 20°C on standard nematode growth media (NGM) plates seeded with OP50 Escherichia coli. Some strains were provided by the Caenorhabditis Genetics Center. Strains were backcrossed to our laboratory N2 strain to minimize other genetic variation. The following strains were provided by the following institutions: (within an empirically established tolerance factor) were counted as co-localized. Object number was calculated by counting the mean number of clusters per 100 μm of dendrite length.

The quantification of PVC cell body fluorescence was performed using ImageX (v4.0.11) software using the fluorescent color voxels colocalization function. We applied an empirically derived threshold to all images for both the GLR-1::GFP channel and the mRFP::SYN-13 channel to eliminate background cover slips fluorescence and other noise (typically, <5% of pixels for each channel). The fluorescent intensity values for both the GLR-1::GFP and mRFP::SYN-13 channels were then scatter plotted for each pixel. Pixels with similar intensity values for both channels (within an empirically established tolerance factor) were counted as co-localized. To acquire the fraction of GLR-1::GFP colocalized with mRFP::SYN-13, the number of colocalized pixels was normalized to the number of GLR-1::GFP pixels under threshold. To maximize our resolving power while observing the relatively small C. elegans neuron cell bodies, we restricted our analysis to a single focal plane taken through the middle of each cell body.
Behavioral assays

The reversal frequency of individual animals was assayed as previously described but with some modifications [Zheng et al., 1999]. Single young adult hermaphrodites were placed on NGM plates in the absence of food. The animals were allowed to adjust to the plates for 5 min, and the number of spontaneous reversals for each animal was counted over a 5-min period. 20 animals were tested for each genotype, and the reported scores reflect the mean number of reversals per minute. No-touch mechanosensations was assayed by placing young adult hermaphrodites on NGM plates with food. Individual young adult animals were allowed to collide with a human hair 10 consecutive times within a 5-min period. Activation of the reversal behavior was scored immediately after each contact with the hair stimulus, and the score was summed over the 10 trials. 30 or more animals were tested for each genotype, and the reported scores reflect the mean number of responses.

Patch clamp whole-cell recording

In vivo whole-cell recordings were performed at room temperature with an amplifer (EPC-10; HEKA) and Patchmaster software (HEKA) using a protocol described in previous studies (Wang et al., 2008; Ward et al., 2008). In brief, the head of glued worms was dissected, and the AVA neurons were exposed for patch clamp recordings in the bath solution. Recording pipettes were pulled from borosilicate glass. The pipette solution contained 115 mM K-glucionate, 25 mM KCl, 50 mM Hepes, 0.1 mM CaCl₂, 1 mM BAPTA, 5 mM MgATP, and 0.5 mM NaGTP [315 mM Os, pH adjusted to 7.35, with KOH]. The bath solution contained 150 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, 15 mM Hepes, 10 mM glucose (325 mM Os, pH adjusted to 7.35, with KOH). Voltages were clamped at ~70 mV. Current data were sampled at 22 kHz. 1 mM glutamate was applied for 500 ms by pressure ejection.

GST pull-downs

Complete coding sequences for the LIN-10 PTB domain, RAB-6.2, and mutant forms of RAB-6.2 were introduced into the GST expression vector pGEX2T-GW, and either GST alone or GST-tagged proteins were expressed in E. coli strain BL21 and purified using glutathione–Sepharose 4B beads (GE Healthcare) as described previously (Pant et al., 2009). Bacterial lysates were prepared in 2x yeast tryptone medium and were induced at an OD₆₀₀ of 0.5 with 0.1 mM IPTG and grown overnight at 15°C. Lysates were exposed for patch clamp recordings in the bath solution. Current data were sampled at 22 kHz. 1 mM glutamate was applied for 500 ms by pressure ejection.

GLR-1::GFP Western blotting

Lysates were prepared from adult worms using a stainless steel dounce homogenizer [DuraGrind; Wheaton] and buffer A (50 mM Hepes, pH 7.7, 50 mM potassium acetate, 2 mM magnesium, 1 mM EDTA, and 250 mM sucrose), a protease inhibitor cocktail [Roche], and 10 mM N-ethylmaleimide. Membranes were isolated from clarified lysates by ultracentrifugation and then suspended in buffer A plus β-mercaptoethanol, SDS, and DTT. Proteins were separated from membrane lysates by SDS-PAGE, and GLR-1::GFP or actin were simultaneously detected by Western blotting using a combination of anti-GFP antibodies [GeneTex, Inc.] and antactin antibodies [MP Biomedicals]. Quantitation was performed using ImageJ, averaging normalized GLR-1::GFP/actin ratios over four independent experiments.

Yeast two-hybrid interactions

Yeast two-hybrid experiments were performed by placing the indicated bait and prey cDNA sequences into the pEG202 bait vector and pG4.5 prey vector. The resulting plasmids were cotransformed, along with the reporter plasmid psh118-34, into yeast strain EGY48, and transformed yeast were recovered on –Leu–His–Trp–Ura dropout plates. Resulting colonies were diluted in series on –Leu–His–Trp–Ura dropout plates to test for interactions based on growth.

Online supplemental material

Fig. S1 shows supplemental quantification data. Fig. S2 shows the localization of other synaptic proteins in rab-6.2 mutants. Fig. S3 demonstrates that the LIN-10 PTB domain interacts with RAB-6.2 in a yeast two-hybrid assay. Fig. S4 shows that RAB-6.2 is localized to Golgi in intestinal epithelial cells. Fig. S5 shows a model for RAB-6.2 regulation of AMPAR trafficking. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201104141/D1C.

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References


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