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Regulation of Synaptic Transmission by RAB-3 and RAB-27 in *Caenorhabditis elegans*[□]

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Rab small GTPases are involved in the transport of vesicles between different membranous organelles. RAB-3 is an exocytic Rab that plays a modulatory role in synaptic transmission. Unexpectedly, mutations in the *Caenorhabditis elegans* RAB-3 exchange factor homologue, *aex-3*, cause a more severe synaptic transmission defect as well as a defecation defect not seen in *rab-3* mutants. We hypothesized that AEX-3 may regulate a second Rab that regulates these processes with RAB-3. We found that AEX-3 regulates another exocytic Rab, RAB-27. Here, we show that *C. elegans* RAB-27 is localized to synapse-rich regions pan-neuronally and is also expressed in intestinal cells. We identify *aex-6* alleles as containing mutations in *rab-27*. Interestingly, *aex-6* mutants exhibit the same defecation defect as *aex-3* mutants. *aex-6; rab-3* double mutants have behavioral and pharmacological defects similar to *aex-3* mutants. In addition, we demonstrate that RBF-1 (rabphilin) is an effector of RAB-27. Therefore, our work demonstrates that AEX-3 regulates both RAB-3 and RAB-27, that both RAB-3 and RAB-27 regulate synaptic transmission, and that RAB-27 potentially acts through its effector RBF-1 to promote soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) function.

INTRODUCTION

Neurotransmitter release is accomplished by the fusion of neurotransmitter-filled synaptic vesicles at the presynaptic nerve terminal. This process occurs through a series of highly regulated steps that include synaptic vesicle transport, docking/tethering, priming, fusion, endocytosis, recycling, and neurotransmitter refilling (Sudhof, 2004). Several genes have been assigned roles in the various steps of the synaptic vesicle cycle. Rab3 (termed RAB-3 in *Caenorhabditis elegans*), a member of the Rab family of small GTPases, regulates synaptic transmission, possibly through the docking, priming, or fusion steps (Nonet *et al.*, 1997; Weimer and Jorgensen, 2003; Schluter *et al.*, 2004).

Rabs act in a variety of cell types and regulate vesicular transport between organelles. Rabs cycle on and off membranes via a GTP-dependent mechanism (Zerial and McBride, 2001). Rab activity is regulated by two proteins, which act in an antagonistic manner. The guanine nucleotide exchange factor (GEF) exchanges GTP for GDP, and the GTPase activating protein activates the intrinsic GTPase activity of a Rab (Bernards, 2003). GDP bound Rabs are held off of the membrane by a GDP dissociation inhibitor (Wu *et al.*, 1996). The GTP/membrane-bound form of Rabs typically binds to a variety of effectors that regulate particular steps of mem-

brane transport and cell signaling (Zerial and McBride, 2001; Spang, 2004).

Rab3 was once thought to play a central role in regulating release. However, recent work has shown that a quadruple knockout of all four isoforms of Rab3 in mice only results in a 30% reduction in evoked synaptic response (Schluter *et al.*, 2004). This is consistent with work in *C. elegans* showing that mutations in the single *rab-3* gene cause only mild defects in synaptic transmission (Nonet *et al.*, 1997). Surprisingly, more dramatic phenotypes are found in Rab3 GEF knockout mice, which exhibit a marked reduction in evoked release that is not seen in the Rab3 knockout mice (Wada *et al.*, 1997; Tanaka *et al.*, 2001; Yamaguchi *et al.*, 2002). Similarly, *C. elegans aex-3* (Rab3 GEF homologue) mutants have more severe synaptic transmission defects than *rab-3* mutants and exhibit defects in the defecation motor program not seen in *rab-3* mutants (Iwasaki *et al.*, 1997). These findings suggest that the Rab3 exchange factor homologue, AEX-3, may regulate another closely related Rab besides RAB-3.

RAB-27 is a close homologue of Rab3 and is expressed in the nervous system, making it an excellent candidate Rab to be regulated by AEX-3 (Pereira-Leal and Seabra, 2001; Barral *et al.*, 2002). There are two isoforms of Rab27 in mammals, whereas there is only one isoform in *C. elegans*. Lesions in the human Rab27A gene cause Griscelli syndrome, an often fatal disease associated with albinism and immunodeficiency (Menasche *et al.*, 2000; Sanal *et al.*, 2002; Bahadoran *et al.*, 2003; Menasche *et al.*, 2003). Vertebrate Rab27 and Rab3 both bind to rabphilin, which contains a Zn²⁺ finger Rab-binding domain, and two C2 domains (Fukuda, 2003a; Fukuda *et al.*, 2004; Fukuda, 2005). However, *C. elegans* rabphilin interacts with RAB-27 but not RAB-3 via glutathione S-transferase (GST) pull-down, in vitro coimmunoprecipitation, and yeast two-hybrid assays (Fukuda, 2003a; Fukuda *et al.*

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al., 2004). Previous work demonstrated that rabphilin acts independently of RAB-3 in vertebrates and invertebrates and that rabphilin potentiates soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) function in invertebrates (Schluter *et al.*, 1999; Staunton *et al.*, 2001). Recent work by Tsuboi *et al.* demonstrates that rabphilin regulates dense core vesicle docking by binding to the SNARE protein SNAP25 (Tsuboi and Fukuda, 2005). Although Rab27 is expressed in the brain, its role in neurons and the relevance of the interaction between Rab27 and rabphilin are largely unknown (Fukuda *et al.*, 2002; Zhao *et al.*, 2002).

We set out to address some of the outstanding questions regarding synapse-specific Rab function: Why is the Rab3 GEF homologue (*aex-3*) mutant phenotype more severe than the Rab3 mutant phenotype? Does AEX-3 regulate a second Rab, potentially RAB-27? Does RBF-1 (rabphilin) function as a RAB-27 effector in *C. elegans*? Does RAB-27 play a role in synaptic transmission? We identify *rab-27* mutants in *C. elegans* and demonstrate that they have synaptic transmission defects. Our data reveal that AEX-3 regulates both RAB-3 and RAB-27. We demonstrate that RAB-3 and RAB-27 both regulate synaptic transmission. We also provide evidence that RBF-1 is an effector of RAB-27 in *C. elegans*. Together, these data suggest a model in which AEX-3 regulates RAB-3, RAB-27, and RBF-1.

MATERIALS AND METHODS

Growth and Culture of *C. elegans* and General Methods

Caenorhabditis elegans were grown at 22.5°C on solid medium as described by Wood (1988). Aldicarb plates were made by adding 2-methyl-2-[methylthio]propionaldehyde 0-[methylcarbamoyl]-oxime (aldicarb) (Chem Services, West Chester, PA) to growth medium after autoclaving. Standard methods for molecular cloning and biochemistry were used unless otherwise stated (Sambrook and Russell, 2001).

Aldicarb Assays

Twenty-five young adult animals were transferred to 1 mM aldicarb plates. Worms were scored every 30 min for total number moving. Animals were considered paralyzed if they no longer moved when prodded with a platinum wire. Each assay was performed three times.

Strains Used

Isolation of *aex-3(js815)*. The *aex-3(js815)* mutant allele was isolated from a PCR screen using a knockout deletion library as described in Liu *et al.* (1999). Briefly, two rounds of nested PCR were used to screen an ethyl methane sulfonate (EMS) mutagenized library. Round 1 consisted of two outer primers and two inner primer primers: outer, 5'-TGTTAATGGCATTITTCAGACG-3' and 5'-TCAACCCAGAGGTGAACCTTTT-3'; and poison, 5'-ATGATCGGCT-TGAGAAGAAGA-3' and 5' GGGCTCAAGTGAATTTTTGTG-3'. Round 2 consisted of a pair of inner or nested primers: 5'-ACAATTTGTGTCCAAGT-CAACG-3' and 5'-ATCTTTCGACAGACGCTCAACT-3'. The *js815* allele has a 555-bp deletion (aa 159-264) that causes a frame shift and a premature stop. PCR using primers 5'-TGTTAATGGCATTITTCAGACG-3', 5'-TCAACCCA-GAGGTGAACCTTTT-3', and 5'-ATGATCGGCTTGAGAAGAAGA-3' was used to genotype *js815*.

Cloning of *aex-6* Mutations. Mutations in the *rab-27* gene were identified by sequencing various *aex-6* alleles (see Supplemental Figure S1 for identified mutations).

Other Strains Used. Single mutants *rab-3(js49)* and *rbf-1(js232)* (Nonet *et al.*, 1997; Staunton *et al.*, 2001). Double mutants were made by standard crossing procedures *jsIs682*; *aex-3(js815)*, *aex-3(js815)*; *jsEx740*, *aex-6(sa24)*; *jsEx740*, *aex-6(sa24)*; *rab-3(js49)*, *jsIs423*; *aex-3(js815)*, *aex-6(sa24)*; *jsIs423*, *rab-3(js49)*; *rbf-1(js232)*, and *aex-6(sa24)*; *rbf-1(js232)*.

Transgenic Animals. Transgenic animals were constructed as described in Mello *et al.* (1991). Briefly, plasmids (see below) purified with QIAGEN (Valencia, CA) columns were coinjected with pJM23 (*lin-15* marker) into *lin-15(n765ts)* mutant animals. Progeny were grown at 22.5°C, and transgenic animals were selected based on the absence of the *lin-15* phenotype. *jsEx640* (NM1023-GFP::RAB-3) was integrated into chromosome III as described

previously (Hope, 1999) and is referred to as *jsIs682* (GFP::RAB-3). The *jsIs682* (GFP::RAB-3) transgene rescues the aldicarb resistance of *rab-3(js49)* and is likely a functional GFP fusion (our unpublished data). *jsIs423* (RBF-1::GFP) was a spontaneous integration of *jsEx86* (Staunton *et al.*, 2001). *jsEx623* (NM1030-*rab-27* promoter driving GFP) and *jsEx740* (NM1112-GFP::RAB-27) were also used (see Supplemental Figure S1 for *rab-27* clones used). The *jsEx740* (GFP::RAB-27) transgene rescues the aldicarb resistance and defecation (AEX) defects found in *aex-6(sa24)* and is likely a functional GFP fusion (our unpublished data).

Plasmid Construction

NM1030-*rab-27* Promoter Driving Enhanced GFP (eGFP). A 3.3-kb *rab-27* promoter was amplified in a PCR using oligonucleotides 5'-AAACATCACTGC-AGCGATTGCACAACCTCAAGGCTCTC-3' and 5'-AATTCATGGGATAGTCG-TAGTACCCACTTCC-3' digested with PstI and NcoI and inserted into PstI-NcoI NM1019.

NM1112-*rab-27* Promoter Driving eGFP Fused to the *rab-27* Coding Region. A 1.8-kb *rab-27* genomic region was amplified in a PCR using oligonucleotides 5'-CCCTGTACATGGGTGACTACGACTATCTCATC-3' and 5'-AATTCGGC-CGATAATCAGCAATTTGCACAATAGGAAGAAGCAGCCGATGGGTC-3', digested with BsrGI and EagI, and inserted into BsrGI-EagI NM1030.

pMK2: A Small Genomic *rab-3* Clone. pMK2 consists of the 4.5-kb BglII *rab-3* genomic fragment of cosmid F11G1 inserted in BamHI-digested pBluescript II in the orientation such that the PstI site of the vector is proximal to the *rab-3* promoter in the insert.

NM1019-*rab-3* Promoter Driving eGFP. A 1.3-kb *rab-3* promoter fragment was amplified from pMK2 using oligonucleotides 5'-CCCTCACTAAAGGGA-ACAAAAG-3' and 5'-CGCCTTGAGGTAAACCGACCGGTGCCATCTGAA-AA-3', digested with PstI and AgeI, and inserted into AgeI/PstI NM990. This vector consists of eGFP and the 3' untranslated region of the *unc-10* gene inserted in the yeast URA3 shuttle vector pRS426 (Christianson *et al.*, 1992). The sequence of NM990 is available at thalamus.wustl.edu/netlab/.

NM1023-*rab-3* Promoter Driving eGFP and the *rab-3* Coding Region. The coding region of *rab-3* was amplified from cDNA with oligonucleotides 5'-TCGGCTGCAGTTAGCAATTGCATTGCTGTTG and 5'-GGTTTGATACAT-GCGCGCTGCGGACAACCTC, digested with BsrGI and EagI, and inserted into BsrGI/EagI-digested NM1019.

NM1122-RAB-27 Protein Expression Vector. *rab-27* was amplified from cDNA using oligonucleotide 5'-CATGGCCATGGGTGACTACGACTATCTC-3' and 5'-TCGGGTCGACGCATAGGAAGAAGCGGCCG-3', digested with NcoI and SalI, and inserted into NcoI-SalI pHO2d (Fasshauer *et al.*, 1997). The final construct contained the complete *rab-27* coding sequences minus the last four amino acids fused to a linker sequence [ASTSLNSG] and ending in a His₆-tag.

Production of RAB-27 Antisera

NM1122 was transformed into BL21-Codon Plus-RIL cells (Stratagene, La Jolla, CA). The His₆-tagged fusion protein was expressed using a 1 mM isopropyl β-D-thiogalactoside induction at room temperature for 18 h. The fusion protein was purified on Ni-NTA agarose (QIAGEN) in RB buffer (20 mM HEPES, pH 7.4, 200 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1% β-mercaptoethanol, 5% glycerol, and 5-500 mM gradient of imidazole), and dialyzed into phosphate-buffered saline. Antiserum 209 and 217 were raised in rabbits (Covance, Denver, PA).

Immunohistochemistry

Immunohistochemistry was performed using Bouin's fixative for whole-mount staining as described previously (Nonet *et al.*, 1997). Mouse anti-RAB-3 antibodies (m937.3) were used as described previously (Nonet *et al.*, 1997). Rabbit anti-RAB-27 antibodies (RB209) were used at a 1:10,000 dilution. Primary antibodies were visualized with goat anti-mouse IgG Alexa 488 and goat anti-rabbit IgG Alexa 568 (Invitrogen, Carlsbad, CA) at a 1:1000 dilution.

Imaging and *C. elegans* Neuroanatomy

All images (with the exception of Figure 3C) were taken with the anterior end on the left, the posterior on the right, dorsal side on the top, and ventral on the bottom. These images were taken of the anterior (head) neurons that surround a large muscular organ termed the pharynx. These neurons form a synaptic-rich nerve ring, which is essentially void of neuronal cell bodies. Synaptic proteins occur as a fairly sharp ring in this region of the nervous system. The neuronal cell bodies that form synapses within the nerve ring are found both anterior and posterior to the nerve ring. There are ~50 neurons proximal to the anterior side and 100 neurons proximal to the posterior side. In mutants that disrupt synaptic localization, the signal seems diffuse, and the outline of a number of cell bodies will become increasingly apparent. For a guide to *C. elegans* anatomy, we suggest www.wormatlas.org.

Western Analysis

Western analysis was performed as described in Weimer *et al.* (2003). Briefly, mixed staged animals were grown to near starvation and diluted in 5× the volume of packed worms with a sucrose:HEPES solution (0.36 M sucrose, 12 mM HEPES, and protease inhibitors). Worms were sonicated four times for 10 s on ice. Lysates were spun down at $16,000 \times g$ for 15 min. Each lysate contained about 3 mg/ml protein, and 15 μ g was loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose (Osmonics, Minnetonka, MN). Primary anti-RAB-27 (rabbit 209) was used at a 1:5000 dilution. Secondary goat anti-rabbit IgG H & L horseradish peroxidase (HRP) (Zymed Laboratories, South San Francisco, CA) was used at a 1:10,000 dilution. Blots were detected using an ECL kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Measurement of GTP-bound Rab

Assay was carried out essentially as described in Coppola *et al.* (2002). COS-7 cells were transfected with 0.5 μ g of plasmids encoding FLAG-tagged *C. elegans* RAB-3 (FLAG-Rab3) or RAB-27 (FLAG-Rab27). The aforementioned cells were cotransfected with 3.5 μ g of plasmid encoding either an empty expression vector (pEF-BOS), T7-tagged AEX-3 (T7-AEX-3), or a C-terminal deletion mutant (Δ a1083-1409) of AEX-3 (T7-AEX-3- Δ C) (Oishi *et al.*, 1998; Coppola *et al.*, 2002). The cotransfected cells were incubated for 2 d at 37°C, harvested, and homogenized in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1 mM PMSF, 100 μ M pepstatin A, and 100 μ M leupeptin). The homogenates were treated with 1% Triton X-100 for 1 h and centrifuged at $17,400 \times g$ for 10 min. Expression of T7-AEX-3 or T7-AEX-3- Δ C was determined by Western analysis with anti-T7 tag antibodies (Figure 3A, IB: T7-AEX-3). The relative abundance of FLAG-Rab3 or FLAG-Rab27 in each lysate was determined by immunoblotting with HRP-conjugated anti-FLAG tag antibody (1/5000 dilution) (Figure 3A, IB: FLAG-Rab Input). The amount of FLAG-Rab3 or FLAG-Rab27 input in each experimental condition was equalized before pulling-down, so that approximately the same amount of FLAG-Rab was used in each experiment (Figure 3A, IB: FLAG-Rab Input). The equalized lysate (FLAG-Rab3 or FLAG-Rab27) was incubated for 1 h at 4°C with a T7-/GST-tagged Rab binding domain (RBD) of mammalian Rim2 or rabphilin (T7-GST-Rim2-RBD or T7-GST-Rph-RBD, respectively; (Fukuda, 2004; Fukuda *et al.*, 2004), which had been coupled to glutathione-Sepharose 4B (GE Healthcare). After washing the beads with lysis buffer three times, the amount of FLAG-Rab that was pulled-down was analyzed by 12.5% SDS-PAGE followed by immunoblotting with the HRP-conjugated anti-FLAG tag antibody (1/5000 dilution) (Figure 3A, IB: FLAG-Rab pull-down). HRP-conjugated anti-T7 tag antibody (1/5000 dilution) was used for detection of T7-GST-Rim2-RBD and T7-GST-Rph-RBD (Figure 3A, IB: T7-GST-RBD).

Electrophysiology

The technique for recording evoked postsynaptic currents (ePSCs) at the *C. elegans* neuromuscular junction has been described previously (Wang *et al.*, 2001), which was based on a technique originally developed by others (Richmond *et al.*, 1999). Postsynaptic currents were amplified with a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA) and acquired with the Clampex software (Molecular Devices). Data were sampled at a rate of 10 kHz after filtering at 2 kHz. The recording pipette solution contained 120 mM KCl, 20 mM KOH, 5 mM Tris, 0.25 mM CaCl₂, 4 mM MgCl₂, 36 mM sucrose, 5 mM EGTA, and 4 mM Na₂ATP (pH adjusted to 7.2 with HCl). The external solution included 140 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 5 mM MgCl₂, 11 mM dextrose, and 5 mM HEPES (pH adjusted to 7.2 with NaOH).

The frequency and mean amplitude of miniature postsynaptic currents (mPSCs) of each experiment were determined using MiniAnalysis (Synaptosoft, Decatur, GA) with the amplitude detection threshold set at 10 pA. The analysis was assisted by visual inspection to include undetected smaller events (≥ 5 pA) and to exclude falsely detected events resulting from baseline fluctuations. Amplitudes of ePSCs were measured with Clampfit (Molecular Devices). The averaged amplitude of the two largest ePSCs from each experiment was used for statistical analysis. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. A $p < 0.05$ is considered statistically significant.

RESULTS

RAB-27 Is a Synaptic Protein

The closest *C. elegans* homologue of the vertebrate Rab27 family members is the gene Y87G2A.4, which we have named *rab-27* in accordance with standard *C. elegans* nomenclature (Horvitz *et al.*, 1979; Pereira-Leal and Seabra, 2001). To determine which cells express RAB-27, we constructed transgenic animals that express GFP under the *rab-27* promoter (see Supplemental Figure S1 and *Materials and Methods* for *rab-27* constructs and protein sequence alignment).

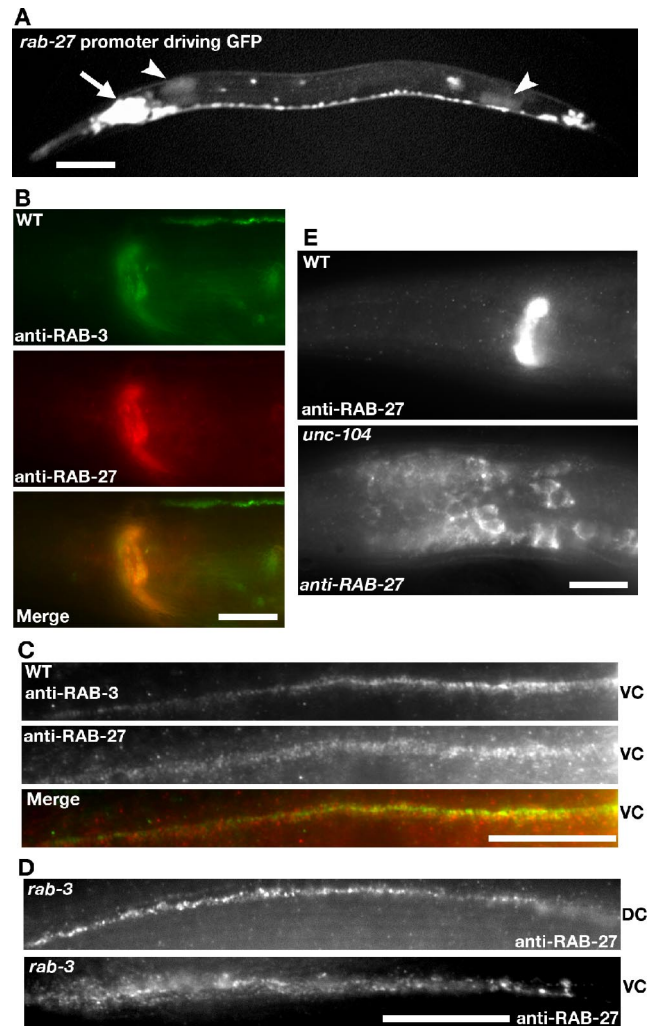


Figure 1. Synaptic Localization of RAB-27. (A) *rab-27* is expressed in the nervous system. Animals carrying a transgene expressing GFP under the *rab-27* promoter. Expression is seen in the neuronal ganglion (arrow) and intestinal cells (arrow head). Bar, 20 μ m. (B) Colocalization of RAB-3 and RAB-27 in synapse-rich regions. Wild-type animals costained with RAB-3 (green) and RAB-27 (red) antisera. Immunoreactivity is most apparent at synapse-rich regions of the nerve ring. Bar, 10 μ m. (C) Colocalization of RAB-3 and RAB-27 at the synapse. Wild-type animals costained with RAB-3 (green) and RAB-27 (red) antisera. RAB-3 and RAB-27 seem to colocalize within the synapse-rich ventral cord (VC), although their colocalization is not complete. Bar, 10 μ m. (D) RAB-27 synaptic localization is independent of RAB-3. *rab-3(js49)* mutant animals were stained for RAB-27. Immunoreactivity remained abundant in the synapse-rich regions of the dorsal cord (DC) and VC. Bar, 10 μ m. (E) RAB-27 localization is UNC-104 dependent. Wild-type and *unc-104(e1265)* mutant animals stained with RAB-27 antisera. RAB-27 staining is found in cell bodies anterior and posterior to the synaptic-rich nerve ring in *unc-104* mutant animals. Bar, 10 μ m.

This *rab-27* reporter is detected pan-neuronally and in the intestine (Figure 1A). *C. elegans rab-27* expression is consistent with that of mammalian Rab27B, which is also expressed in both brain and intestine as well as other secretory cells (Barral *et al.*, 2002; Zhao *et al.*, 2002). To determine the subcellular localization of RAB-27, we raised antibodies against the full-length recombinant RAB-27 protein. Immunostaining of wild-type and *rab-3(js49)* mutant animals

shows that the RAB-27 protein is enriched in synapse-rich regions of the nervous system, such as the nerve ring, dorsal cord, and ventral cord (Figure 1, B–D). RAB-27 largely colocalizes with synaptic vesicle-associated RAB-3, although this colocalization is not complete (Figure 1, B and C). RAB-27 immunoreactivity in *rab-27* mutants is absent suggesting that the RAB-27 antibody is specific (see below). RAB-27 immunostaining is normal in *rab-3* mutants, suggesting that RAB-27 localization is independent of RAB-3 function (Figure 1D). Although the GFP reporter shows RAB-27 expression in the intestine, no immunoreactivity was detected in the intestine, possibly due to diffuse localization, low expression, or the absence of endogenous expression in the intestine.

In *C. elegans*, synaptic vesicle- and dense core vesicle-associated proteins share a unique dependence on the kinesin UNC-104 for anterograde transport (Hall and Hedgecock, 1991; Jacob and Kaplan, 2003; Zahn *et al.*, 2004). Consistent with RAB-27 being present on either synaptic and/or dense-core vesicles, we found that RAB-27 is retained in cell bodies in *unc-104(e1265)* mutants (Figure 1E). RAB-3 is similarly mislocalized in *unc-104(e1265)* mutants (Nonet *et al.*, 1997). Thus, *C. elegans* RAB-27 displays characteristics of a synaptic vesicle and/or dense-core vesicle-associated protein, although we do not exclude the possibility that RAB-27 is associated with another membrane organelle.

aex-6 Encodes RAB-27

To address the role of RAB-27 in the nervous system, we set out to identify mutations in the *rab-27* gene. We reasoned that if RAB-27 is indeed regulated by AEX-3 (see below), then *rab-27* mutants might have similar phenotypes as *aex-3* mutants, and they might have been isolated in previous genetic screens. *aex-6* emerged as a candidate to encode RAB-27 through the aforementioned reasoning. *aex-6* was originally isolated as a mutant defective in the anterior body wall muscle contraction (aBoc) and expulsion steps of the defecation motor program in *C. elegans* (Thomas, 1990). In addition, *aex-6* mutants are egg-laying defective, lethargic, and form dauers at high temperatures (Thomas, 1990; Ailion and Thomas, 2003). *aex-3* mutants share many of these phenotypes. We performed a series of analyses to determine whether *aex-6* encodes RAB-27. Several lines of evidence support this prediction. First, *aex-6* mutants map to an interval on chromosome I that encompasses the *rab-27* locus (Thomas, 1990). Second, sequencing of three *aex-6* alleles reveal point mutations in the *rab-27* coding region, including two alleles containing early stop codons (Supplemental Figure S1). Third, RAB-27 immunoreactivity is absent in *aex-6* alleles both by immunohistochemistry and by Western blot analyses (Figure 2, A and B). Fourth, a transgene containing GFP-tagged RAB-27 rescues both the aldicarb and AEX phenotypes (our unpublished observation; see Supplemental Figure S1 for transgene constructs). Thus, *aex-6* encodes RAB-27. We will continue to refer to mutations in the *rab-27* gene as *aex-6*.

AEX-3 Regulates Both RAB-3 and RAB-27

The similarity between the *rab-27* and RAB3 GEF *aex-3* mutant phenotypes suggested that both RAB-3 and RAB-27 might be regulated by this guanine nucleotide exchange factor. Association of the Rab protein with the vesicle membrane often requires the activity of the GEF protein (Pfeffer, 2005). In particular, RAB-3 association with synaptic vesicles requires AEX-3 (Iwasaki *et al.*, 1997). Thus, we tested whether RAB-27 is also mislocalized in *aex-3* mutants. We found that, like RAB-3, RAB-27 is more abundant in cell

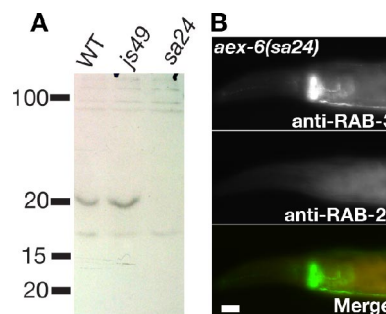


Figure 2. *aex-6(sa24)* is a null allele. (A) Western blot confirming loss of RAB-27 in *aex-6(sa24)*. Approximately 15 μ g of total worm lysate from wild-type, *rab-3(js49)*, and *aex-6(sa24)* were separated by SDS-PAGE and blotted to nitrocellulose. Band of approximately 20 kDa disappears in *aex-6(sa24)* mutants. $n = 3$. (B) *aex-6(sa24)* mutant animals lack RAB-27. *aex-6(sa24)* mutant animals were costained for RAB-3 and RAB-27. Although RAB-3 was properly localized to the synapse-rich nerve-ring, RAB-27 was not detected. Bars, 10 μ m.

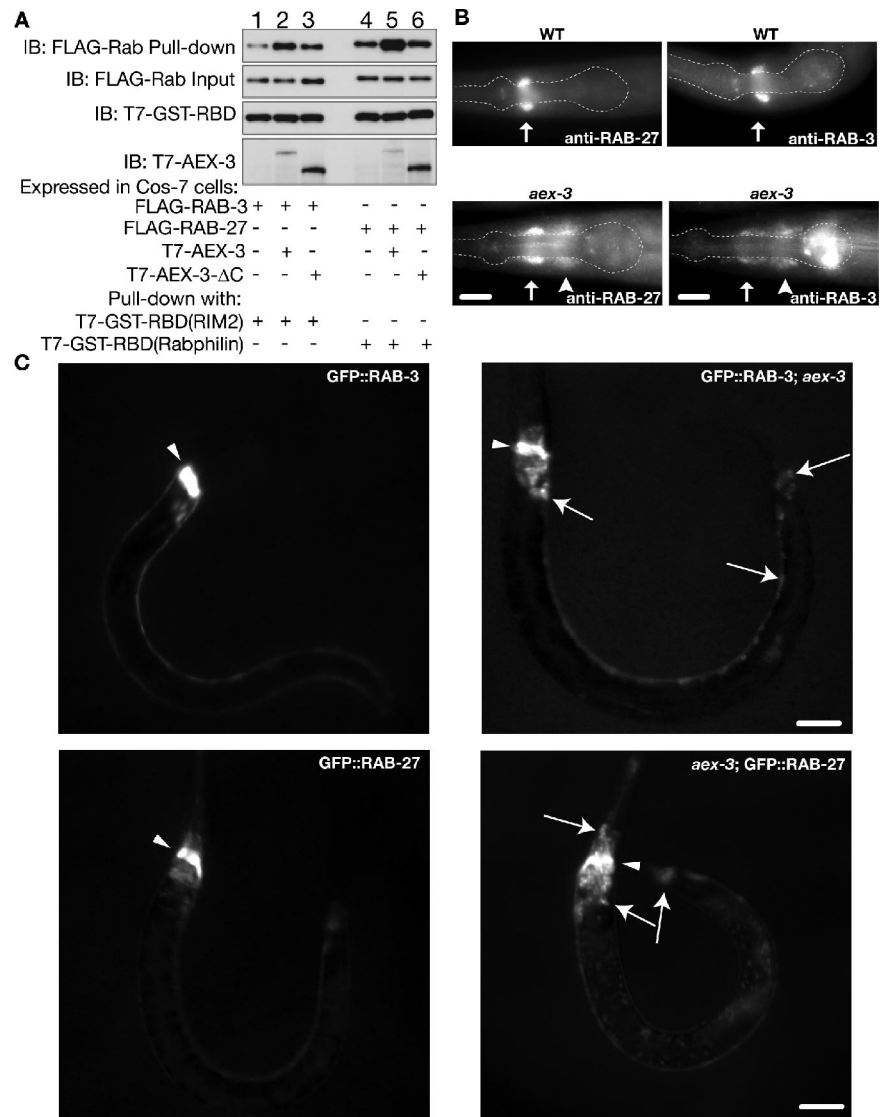
bodies in *aex-3(js815)* mutants than in wild-type animals (Figure 3B). A transgene carrying an N-terminal GFP fusion to RAB-27 (GFP::RAB-27) was similarly mislocalized in *aex-3(js815)* (Figure 3C). The mislocalization of RAB-27 was less drastic than that of a GFP::RAB-3 transgene (Figure 3, B and C). It is worth noting that each GFP fusion rescued the corresponding Rab loss-of-function mutation (see *Materials and Methods*). These results are consistent with AEX-3 regulating both RAB-3 and RAB-27.

To obtain biochemical evidence that AEX-3 regulates both RAB-3 and RAB-27, we assayed levels of GTP-bound Rabs in the presence or absence of AEX-3 (Coppola *et al.*, 2002). GTP-bound RAB-3 specifically binds to Rim2, and GTP-bound RAB-27 specifically binds to rabphilin (Supplemental Figure S2, A and B; Fukuda, 2003a). GDP-bound forms of these Rab proteins do not bind these targets. We used GST-tagged Rab-binding domains of Rim2 and rabphilin in pull-down assays from COS-7 cells. Coexpression of AEX-3 with RAB-3 increased binding of RAB-3 to Rim (Figure 3A). Similarly, coexpression of AEX-3 with RAB-27 increased binding of RAB-27 to rabphilin (Figure 3A). It is the nucleotide exchange function of AEX-3 that is likely responsible for this activity, because an AEX-3 mutant (T7-AEX-3- Δ C) that lacks GEF activity does not stimulate binding (Figure 3A; Coppola *et al.*, 2002). By contrast, AEX-3 did not stimulate RAB-5 binding to its effector Rabenosyn-5 (Supplemental Figure S2C; Nielsen *et al.*, 2000). These results strongly suggest that AEX-3 regulates the activity of both RAB-3 and RAB-27.

RAB-3 and RAB-27 Control Synaptic Transmission

Because both RAB-3 and RAB-27 are expressed in the nervous system and both are regulated by AEX-3, we hypothesized that these Rabs regulate synaptic release. To test this hypothesis, we used a well-established pharmacological assay to analyze the changes in cholinergic signaling (Miller *et al.*, 1996). In this assay, animals are exposed to the cholinesterase inhibitor aldicarb, and a time course of paralysis is used to quantify defects in synaptic function. As previously demonstrated, *rab-3* mutants show a moderate resistance to aldicarb (Nonet *et al.*, 1997), whereas *aex-3* mutants exhibit stronger resistance compared with wild-type animals (Figure 4A). *aex-6(sa24)* mutants are slightly more aldicarb resistant than wild-type animals, indicating only a slight disruption of cholinergic signaling. Consistent with our hypothesis that RAB-3 and RAB-27 act to regulate synaptic transmis-

Figure 3. AEX-3 regulates RAB-3 and RAB-27. (A) AEX-3 increases the amounts of GTP-bound RAB-3 and RAB-27 in COS-7 cells. Lysates were prepared from COS-7 cells co-transfected with either FLAG-Rab3 alone (lane 1), FLAG-Rab3, and T7-tagged AEX-3 (T7-AEX-3; lane 2), FLAG-Rab3 and T7-tagged AEX-3 deletion mutant (T7-AEX-3- Δ C; lane 3), FLAG-Rab27 alone (lane 4), FLAG-Rab27 and T7-AEX-3 (lane 5), or FLAG-Rab27 and T7-AEX-3- Δ C (lane 6). After normalizing the amount of input FLAG-Rab between each co-transfected condition (IB: FLAG-Rab Input), the cell lysates were incubated with equal amounts of T7-/GST-tagged Rab binding domain (T7-GST-RBD) of mammalian Rim2 or rabphilin (IB: T7-GST-RBD). Because the RBD binds specifically to the GTP-bound form of each Rab, the amount of Rab pulled-down by a GST-tagged RBD is a readout for the amount of GTP-bound Rab in the lysate (IB: FLAG-Rab pull-down). The amounts of RAB-3 or RAB-27 pulled down are dramatically increased when they are coexpressed with full-length T7-AEX-3 (lanes 2 and 5), but not with coexpression of an empty vector (lanes 1 and 4) or a mutant form of AEX-3 (T7-AEX-3- Δ C, lanes 3 and 6). The expression of T7-AEX-3 or T7-AEX-3- Δ C was analyzed by immunoblotting with anti-T7 tag antibody (IB: T7-AEX-3). Ratio of band intensities of FLAG-Rab pull-down compared with FLAG-Rab input: lane 1 (0.55), lane 2 (1.86), lane 3 (0.88), lane 4 (1.52), lane 5 (3.08), and lane 6 (1.83). (B) *aex-3* mutant animals mislocalize RAB-3 and RAB-27. Wild type (top) and *aex-3(js815)* (bottom) stained with RAB-27 (left) and RAB-3 (right) antisera. Both RAB-3 and RAB-27 are mislocalized from synapse-rich regions (arrows) in *aex-3* mutants to cell bodies (arrowheads). The pharynx is outlined as an anatomical guide. Bars, 10 μ m. (C) *aex-3* mutant animals mislocalize GFP-tagged RAB-3 and RAB-27. Top left, visualization of GFP from a GFP-tagged RAB-3 transgenic animal (*jsIs682*) shows primarily nerve ring staining. Top right, GFP-tagged RAB-3 (*jsIs682*) is mislocalized from the nerve ring to neuronal cell bodies in *aex-3(js815)* mutant animals. Arrows mark representative neuronal cell bodies. Arrowheads mark the dorsal side of the nerve ring. Bottom left, visualization of GFP from a GFP-tagged RAB-27 transgenic animal (*jsEx740*) shows primarily nerve ring staining. Bottom right, GFP-tagged RAB-27 (*jsEx740*) is mislocalized from the nerve ring to neuronal cell bodies in *aex-3(js815)* mutant animals. Arrows mark representative neuronal cell bodies. Bars, 20 μ m.



sion, and that AEX-3 regulates both proteins, *aex-6*; *rab-3* double mutants exhibit stronger resistance to aldicarb than either single mutant and are comparable in resistance to *aex-3* mutants.

To confirm our findings from the aldicarb assay, we analyzed miniature and evoked postsynaptic currents at the *C. elegans* neuromuscular junction. Consistent with the aldicarb data, *rab-3(js49)* mutants exhibit a reduction of evoked release, or ePSCs, to ~55% of wild-type (Figure 4, B and C). Remarkably, the evoked release defects in *aex-3(js815)* and *aex-6(sa24)*; *rab-3(js49)* (~43% and ~25% of wild type, respectively) mirror the results of the aldicarb assays (Figure 4, B and C). We also find that *rab-3(js49)* mutants exhibit a robust ~35% decrease in miniature postsynaptic current (mPSC) frequency compared with wild-type animals (Figure 4, B and D). Interestingly, *aex-3(js815)* and *aex-6(sa24)*; *rab-3(js49)* double mutants exhibit an even greater decrease in mPSC frequency (~53 and ~34% of wild-type, respectively). In contrast, mPSC amplitude was not affected significantly

in any of the mutants we tested, except for a modest <14% reduction in the *aex-6(sa24)*; *rab-3(js49)* double mutant, suggesting there is little disruption of postsynaptic function (Figure 4, B and E). These data strongly suggest that pre-synaptic RAB-3 and RAB-27 both regulate synaptic transmission.

RAB-27 Regulates RBF-1

Because *rab-3* and *aex-6* mutant animals have distinct (e.g., defecation defect in *aex-6* mutants) and overlapping (e.g., synaptic transmission defect) phenotypes and both Rabs are regulated by the same Rab3 GEF homologue (AEX-3), we hypothesized that RAB-3 and RAB-27 have distinct effectors. In vertebrates, rabphilin was originally identified as a Rab3 effector (Shirataki *et al.*, 1993; Li *et al.*, 1994; Ostermeier and Brunger, 1999); however, this does not seem to be the case in *C. elegans* (Staunton *et al.*, 2001). Similarly, studies in mice have shown that rabphilin is not required for Rab3 function (Schluter *et al.*, 1999). Later work demonstrated that rabphi-

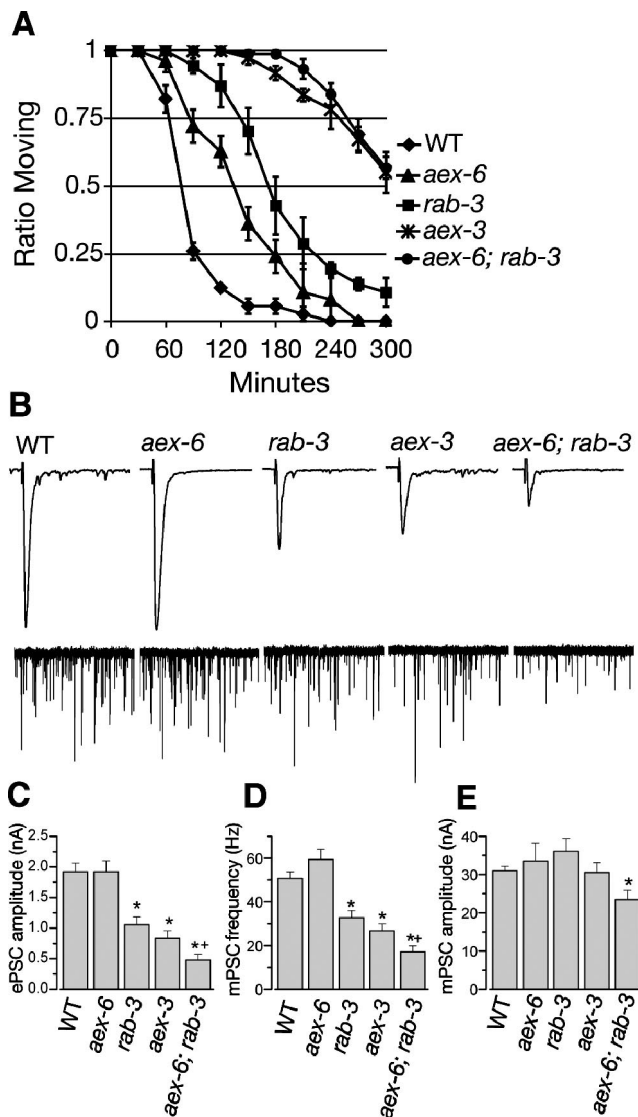


Figure 4. Synaptic transmission defects in *aex-3* and *aex-6*. (A) Aldicarb resistance of *aex-3* and *aex-6*. Wild-type (diamond), *aex-6(sa24)* (triangle), *rab-3(js49)* (square), *aex-3(js815)* (star), and *aex-6(sa24); rab-3(js49)* (circle) were exposed to 1 mM aldicarb and scored for percentage of the animals still moving every 30 min. Error bars represent SEM. (B) Less evoked release in *aex-6; rab-3* double mutant, and *aex-3* mutant animals than in *rab-3* or *aex-6* mutant animals. Representative traces of evoked release (top) and miniature postsynaptic currents, mPSC (bottom) of wild-type, *aex-6(sa24)*, *rab-3(js49)*, *aex-3(js815)*, and *aex-6(sa24); rab-3(js49)* double mutant animals. (C) The mean evoked release, or ePSC, was determined for each mutant. Although there is no significant change in evoked release between wild-type (WT) and *aex-6(sa24)* mutant animals, there is a significant reduction between WT and *rab-3(js49)*, *aex-3(js815)*, and *aex-6(sa24); rab-3(js49)* mutant animals (* $p < 0.05$). Consistent with RAB-3 and RAB-27 both regulating synaptic transmission, there is a significant decrease in evoked release between the *aex-6(sa24); rab-3(js49)* double mutants and either single mutant (* $p < 0.05$). WT ($n = 40$), *aex-6(sa24)* ($n = 16$), *rab-3(js49)* ($n = 28$), *aex-3(js815)* ($n = 30$), and *aex-6(sa24); rab-3(js49)* ($n = 20$). (D) The decrease in the frequency of miniature postsynaptic currents, or mPSCs, mirrored the decrease in evoked release (C). *rab-3(js49)*, *aex-3(js815)*, and *aex-6(sa24); rab-3(js49)* mutant animals all exhibit a significant decrease in the frequency of mPSCs from wild-type (* $p < 0.05$). There is a significant decrease in the frequency of mPSCs between *aex-6(sa24); rab-3(js49)* double mutants and either single mutant (* $p < 0.05$). WT ($n = 32$), *aex-6(sa24)* ($n = 9$), *rab-3(js49)* ($n = 16$,

lin interacts with both vertebrate and invertebrate Rab27 (Fukuda, 2003a; Fukuda *et al.*, 2004). To determine whether RBF-1 is an effector of RAB-27 in vivo, we looked at RBF-1 localization in *aex-3* and *aex-6* mutant animals. We found that GFP-tagged RBF-1 (RBF-1::GFP) is mislocalized from synapse-rich regions in *aex-3(js815)* and *aex-6(sa24)* mutant animals, but RBF-1::GFP is normally localized in *rab-3(js49)* animals (Figure 5A; Staunton *et al.*, 2001). We also noted that RBF-1::GFP fluorescence is weaker in *aex-6* mutant animals than in wild type. The intensity of RBF-1::GFP decreases rapidly as the *aex-6* mutant animals develop beyond the young adult stage (our unpublished data). These results suggest that RBF-1 is an effector of RAB-27. To provide functional evidence that RBF-1 is an effector of RAB-27, we tested whether *rbf-1* mutants enhance the aldicarb resistance found in *rab-3* mutants. We found that *rab-3; rbf-1* mutant animals exhibit an aldicarb resistance similar to *aex-6; rab-3* mutant animals (Figure 5B). In contrast, *aex-6; rbf-1* mutant animals do not exhibit an enhancement in aldicarb-resistance with respect to *aex-6* single mutants (Figure 5C). These results strongly suggest that the synaptic transmission defect of *aex-6* mutants is due to the loss of RBF-1 function. Our data show that RAB-3 and RAB-27 function together to regulate synaptic transmission; however, RAB-27 function diverges from RAB-3 through its effector protein RBF-1.

DISCUSSION

Here, we identify AEX-3 as a regulator of RAB-27 and characterize the role of RAB-27 in the nervous system. In peripheral tissues, Rab27 has been studied due to its role in Griscelli syndrome. The immunodeficiency of Griscelli syndrome patients is due to a defect in lytic granule exocytosis from cytotoxic T-cells and may be mediated by effectors such as Munc13-4 and granuphilin (Feldmann *et al.*, 2003; Goishi *et al.*, 2004; Shirakawa *et al.*, 2004; Torii *et al.*, 2004; Yamamoto *et al.*, 2004; Neeft *et al.*, 2005). In addition, Rab27 plays a role in exocytosis of dense core granules from both endocrine and exocrine cells (Chen *et al.*, 2003; Fukuda, 2003b; Izumi *et al.*, 2003; Chen *et al.*, 2004; Goishi *et al.*, 2004; Imai *et al.*, 2004; Matsumoto *et al.*, 2004). Until now, the role of Rab27 in the nervous system was unknown. Previous work on the role of Rabs in synaptic transmission focused primarily on Rab3 (Sudhof, 2004). In this study, we identified mutations in the *rab-27* gene and showed that these mutants exhibit mild synaptic and behavioral defects. We also showed that the exchange factor homologue AEX-3 regulates both RAB-3 and RAB-27 activity. This is noteworthy because AEX-3 is the first exchange factor homologue found to regulate Rab27 in invertebrates or mammals. Also, few exchange factors have been shown to regulate multiple Rabs (Jones *et al.*, 2000). Our findings also suggest that other mechanisms, in addition to exchange factors, specifically control different Rab functions. RAB-3 and RAB-27 seem to be coregulated at the synapse, but functionally diverged through their distinct effectors.

Many exocytic proteins are conserved from yeast to vertebrates. Several of these proteins seem to play critical, if not essential, roles in exocytosis. These include the SNARE proteins and certain SNARE regulators (Bennett and Scheller,

aex-3(js815) ($n = 16$), and *aex-6(sa24); rab-3(js49)* ($n = 10$). (E) There is no significant change in the amplitude of mPSCs between any of the mutants and wild type, with the exception of the *aex-6(sa24); rab-3(js49)* double mutant animals (* $p < 0.05$). n values are the same as in D.

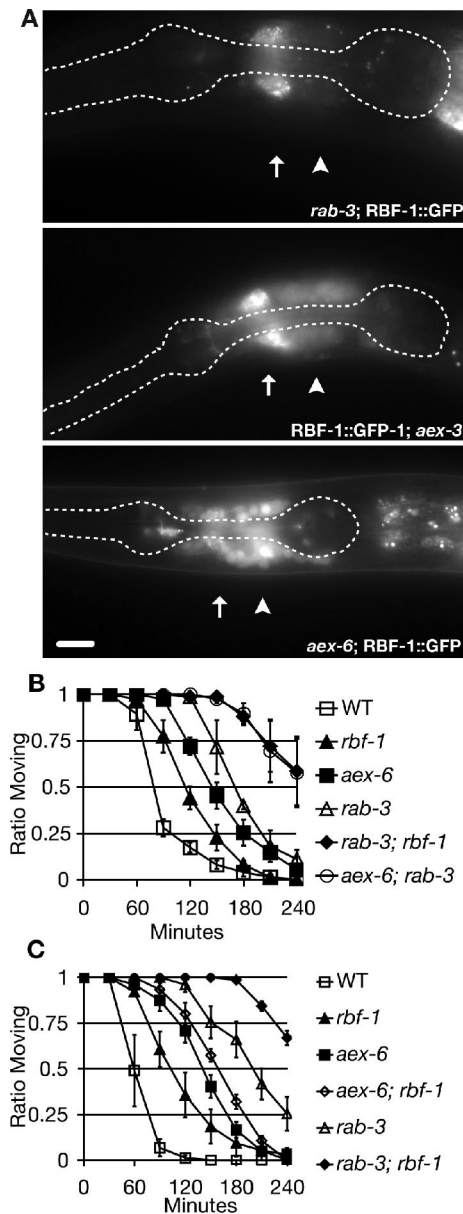


Figure 5. RBF-1 is an effector of RAB-27. (A) RBF-1::GFP localization is dependent on AEX-3 and AEX-6. Visualization of GFP from an integrated RBF-1::GFP transgenic animal (*jsIs423*) shows primarily nerve ring staining. RBF-1::GFP is mislocalized to neuronal cell bodies in *aex-3(js815)* mutant animals, and the mislocalization is more severe in *aex-6(sa24)* mutant animals. The pharynx is outlined as an anatomical guide. Synaptic-rich regions are marked with an arrow, and neuronal cell bodies are indicated by an arrowhead. Bars, 10 μ m. (B) *rbf-1* enhances *rab-3*. Wild type (open box), *rbf-1(js232)* (closed triangle), *aex-6(sa24)* (closed box), *rab-3(js49)* (open triangle), *rab-3(js49); rbf-1(js232)* (closed diamond), and *aex-6(sa24); rab-3(js49)* (open circle) were exposed to 1 mM aldicarb and scored for percentage of the animals still moving every 30 min. Error bars represent SEM. (C) *rbf-1* does not enhance *rab-27*. Wild type (open box), *rbf-1(js232)* (closed triangle), *aex-6(sa24)* (closed box), *aex-6(sa24); rbf-1(js232)* (open diamond), *rab-3(js49)* (open triangle), and *rab-3(js49); rbf-1(js232)* (closed diamond) were exposed to 1 mM aldicarb and scored for percentage of the animals still moving every 30 min. Error bars represent SEM.

1993; Sollner, 2003; Weimer and Jorgensen, 2003; Sudhof, 2004). The yeast exocytic Rab Sec4 is required for exocytosis

(Salminen and Novick, 1987). Surprisingly, knockout of Rab3, an exocytic Rab, in mice and *rab-3* mutant *C. elegans* exhibit only a mild decrease in synaptic transmission (Nonet *et al.*, 1997; Schluter *et al.*, 2004). One outstanding question is whether exocytic Rabs are required for exocytosis in metazoans. Our results demonstrate a role for two distinct Rab proteins (RAB-3 and RAB-27) in synaptic transmission. Although these results do not determine whether exocytic Rabs are required for synaptic transmission (or exocytosis in metazoans), they do demonstrate the ability of distinct Rabs, such as RAB-3 and RAB-27, to play similar or redundant roles in the same tissue. Our findings imply that there may even be a third or fourth Rab, similar to Rab3 or Rab27, regulating synaptic transmission.

We document that RAB-27 is found primarily at the synapse-rich regions of the *C. elegans* nervous system, notably the nerve ring and both ventral and dorsal nerve cords. RAB-27 localization only partially overlaps with that of RAB-3, suggesting that RAB-27 is likely on a distinct set of exocytic vesicles from RAB-3. One possibility is that RAB-27 is found mostly on dense-core vesicles, and RAB-3 is mostly on synaptic vesicles. Alternatively, RAB-3 and RAB-27 may be on different subpopulations of synaptic-, dense-core, or even a novel set of exocytic vesicles. Our data are consistent with RAB-3 and RAB-27 both being localized to exocytic vesicles of the nervous system.

We show that RBF-1 is an effector of RAB-27 in *C. elegans*. RBF-1 is related to the mammalian protein granuphilin, which is thought to form a complex with Rab27 and the SNARE protein syntaxin (Torii *et al.*, 2004). Recently, the C2B domain of rabphilin was shown to interact with SNAP25 and may regulate docking (Tsuboi and Fukuda, 2005). In addition, data from our laboratory demonstrates that RBF-1 potentiates SNARE function through the SNAP25 homologue RIC-4 (Staunton *et al.*, 2001). Together, these findings suggest a mechanism in which the vesicle protein RAB-27 links/docks vesicles to release sites via an interaction with rabphilin, which then binds to a SNARE protein. Our model is consistent with work demonstrating that mutations in *C. elegans rab-3*, a close homologue of *rab-27*, have fewer synaptic vesicles at synapses and that Rab3a knockout mice lack activity-dependent recruitment of synaptic vesicles to the active zone (Nonet *et al.*, 1997; Leenders *et al.*, 2001). This model is also strengthened by our data showing a decrease in mPSC frequency in *rab-3* mutants and a further reduction in mPSC frequency in *aex-3* and *aex-6; rab-3* double mutants, which could be indicative of a decrease in the readily releasable pool. It is worth noting that other studies of Rab3A knockout mice have not observed alterations in either the readily releasable pool or in the numbers of docked or total synaptic vesicles at synapses (Schluter *et al.*, 2004) and have argued that Rab3a acts at the fusion step rather than in earlier docking steps. Whether these differences represent synapse-type specific or organism specific differences will require a more detailed understanding of the mechanisms of Rab modulation of synaptic release.

Our results illustrating that Rab27, a gene linked to Griscelli syndrome, regulates synaptic transmission are consistent with recent findings from patients with Chediak-Higashi syndrome, a disorder related to Griscelli syndrome (Tardieu *et al.*, 2005). The authors found that after successful bone-marrow transplantation to treat the immunological defects, patients later develop severe neurological disorders in the absence of any structural damage that could have resulted from the disease. These neurological disorders could be the result of impaired neuronal dense-core or synaptic vesicle release. We find that RAB-27 clearly plays a role in

synaptic transmission at least partially through its effector RBF-1. We propose that the vertebrate homologue of AEX-3 may play a role in the molecular mechanisms of melanosome movement, secretory vesicle release, and Griscelli syndrome via RAB-27.

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