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Direct interactions between *C. elegans* RAB-3 and Rim provide a mechanism to target vesicles to the presynaptic density

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ABSTRACT

Rim is a multi-domain, active zone protein that regulates exocytosis and is implicated in vesicle priming and presynaptic plasticity. We recently demonstrated that synaptic defects associated with loss of *Caenorhabditis elegans* Rim (termed UNC-10) are accompanied by a reduction in docked vesicles adjacent to the presynaptic density. Since Rim is known to interact with the vesicle-associated GTPase Rab3A, here we asked whether UNC-10-dependent recruitment of synaptic vesicles to the presynaptic density was through an UNC-10/Rab-3 interaction. We first established that *C. elegans* Rab3 (termed RAB-3) in its GTP but not GDP-bound state interacts with UNC-10. We then demonstrated by EM analysis that *rab-3* mutant synapses exhibit the same vesicle-targeting defect as *unc-10* mutants. Furthermore, *unc-10;rab-3* double mutants phenocopy the targeting defects of the single mutants, suggesting UNC-10 and RAB-3 act in the same pathway to target vesicles at the presynaptic density. Endogenous release of *unc-10;rab-3* double mutants was similar to that of *unc-10* single mutants, but more severe than *rab-3* mutants, suggesting the common targeting defects are reflected by the milder *rab-3* release defect. Rim has recently been shown to positively regulate calcium influx through direct interactions with calcium channels. Consistent with this notion we found UNC-10 colocalized with the calcium channel, UNC-2 at *C. elegans* presynaptic densities and synaptic release in *unc-10* and *rab-3* mutants exhibit reduced calcium-sensitivity. Together these results suggest that vesicles targeted to the presynaptic density by RAB-3/UNC-10 interactions are ideally positioned for efficient calcium-dependent release.

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Rim is a component of the presynaptic density (PD), first identified as a Rab3a interacting molecule [24,26]. Subsequently, Rim has been shown to interact with several other presynaptic proteins including liprin, Munc-13 and calcium channels [11,21]. Genetic disruption of *Caenorhabditis elegans* Rim(*unc-10*) causes reduced neuromuscular transmission [12]. Similarly, mouse Rim1 knockouts exhibit release defects attributable to reduced synaptic vesicle (SV) priming and impaired long-term synaptic plasticity [2,3,14,21]. In both organisms, the actions of Rim were originally thought to occur downstream of SV docking, based on EM analysis using conventional chemical fixation [12,21]. However, re-examination of *C. elegans unc-10*(Rim) mutants using high-pressure freeze fixation [25], revealed a specific loss of docked SVs adjacent to the PD [26]. This result suggests one function of UNC-10(Rim) is to target SVs to this specialized region of the synaptic terminal [26]. Given that vertebrate Rim interacts with Rab3A [24], we postulated that UNC-10(Rim) recruits SVs to the active zone via RAB-3 interactions.

Rab3 is a monomeric G-protein that associates with SVs in its GTP-bound state and dissociates upon GTP hydrolysis [7,9,17]. Both *C. elegans rab-3* mutants and mouse Rab3A knockouts exhibit moderate synaptic defects [9,17]. To test whether these functional defects result from SV mistargeting, several studies have examined synaptic ultrastructure following Rab3 perturbation, with mixed findings. *C. elegans rab-3* mutants exhibited a redistribution of SVs to intersynaptic regions following conventional chemical fixation [17], whereas, synapses from Rab3 (A, B, C, D) knockouts appeared normal [20]. Synaptosomes from mouse Rab3A knockouts also exhibited normal SV distributions, but showed a reduction in activity-dependent SV recruitment to the active zone [13]. In contrast, neuromuscular junctions (NMJs) of Rab3A mouse mutants exhibited reduced docked SVs even under resting conditions, although vesicle distribution relative to the active zone was not examined [4]. Several studies suggest that Rab3 also regulates dense core vesicle (DCV) docking. For example, docking of DCVs in PC12 cells following Rab3A RNA interference was impaired and

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resulted in a concomitant decrease in exocytic event frequency [22]. Conversely, overexpression of Rab3A in PC12 and chromaffin cells increased the number of docked DCVs [16,23].

Together these observations suggest that Rim and Rab3 both regulate synaptic function and vesicle docking, although definitive evidence that they act together to target synaptic vesicles to the PD has yet to be obtained. To test whether Rim and Rab3 act in the same targeting pathway we examined interactions between *C. elegans* UNC-10(Rim) and RAB-3.

C. elegans RAB-1, -3, -5, -8, -11, -27, and -37 were amplified from first strand cDNA, and cloned into the yeast LexA binding domain vector pBHA (see supplemental methods for details). GTP-bound, GDP-bound and $\Delta C \times C$ geranyl-geranylation motif lesions were introduced by site-directed mutagenesis (see supplemental methods for details). The N-terminal domain of Rim was introduced into the GAL4 activation domain vector pACT II. UNC-10 clones containing a.a. 1–143, 1–187, and 1–331, all showed activity. Tom Sudhof provided pLexNRAB3A(T36N), pLexNRAB3A(Q81L), and pREYRim-100 [24]. Plasmids were co-transformed into the yeast strain L40 (MATa, ade2, trp1Δ1, leu2-2,113, his3Δ200, LYS2::lexAop$_{(x4)}$-HIS3, URA3::lexAop$_{(x8)}$-LacZ) using a LiAC/ssDNA/PEG method. Interactions were visualized using an X-gal filter lift assay and quantified using ONPG assays (see supplemental methods for details).

Wild-type (N2 Bristol), *unc-10*(*md1117*), *rab-3*(*js49*) and *unc-10*(*md117*);*rab-3*(*js49*) animals were prepared for EM by high-pressure freeze fixation as described previously [26].

40–50 nm thick serial sections spanning complete NMJs, defined as contiguous synaptic profiles with a PD were analyzed. For each synaptic profile, the number of SVs and distance from SV membranes perpendicular to the plasma membrane, and circumferential distance along the membrane to the center of the closest PD, were measured using ImageJ with the scorer blinded to genotype identity.

Animals were prepared for immunoEM analysis as previously described [26]. UNC-2 goat primary antibody (Santa Cruz Biotechnologies) was diluted 1:10 and anti-goat-15 nm gold bead conjugated antibodies were diluted 1:80. The distance from each bead to the plasma membrane, and to the center of the closest PD was measured.

Electrophysiological methods were as previously described [10].

Vertebrate Rim is known to bind selectively to activated Rab3 through a zinc finger containing N-terminal domain [6,24]. To determine whether the zinc finger domain of UNC-10(Rim) interacts with *C. elegans* RAB-3, we performed yeast two hybrid assays using the zinc finger region of both *C. elegans* and vertebrate Rim. In the two hybrid assay, *C. elegans* and vertebrate Rim both interacted with the activated *C. elegans* GTP-bound RAB-3(Q81L) mutant, but not the GDP-bound RAB-3(T36N) mutant (Fig. 1A). This interaction was independent of the C-terminal $C \times C$ geranyl-geranylation motif that mediates membrane-association of RAB-3 (Fig. 1A). Furthermore, the *C. elegans* Rim-RAB-3 interaction was selective for RAB-3 as Rim failed to interact with wild-type or GTP-bound mutant forms of *C. elegans* RAB-1, -5, -8, -11, -27 and -37 (Fig. 1B). Vertebrate and *C. elegans* Rim showed very similar RAB-3 specificity (data not shown), suggesting the Rim/RAB-3 interaction has been highly conserved during evolution. To confirm the interaction under more native conditions we prepared Triton-X100 solubilized membrane extracts from WT *C. elegans* (see supplemental methods for details). However, neither UNC-10(Rim) nor the active zone-associated protein ELKS-1 was solubilized in Triton-X100 complicating the immunoprecipitation protocol. Instead, we spiked a Triton-X100 solubilized membrane extract with a Rim zinc finger fusion protein at 0.5 ng/ul and performed immunoprecipitations from the extract. Antibodies directed against synaptobrevin (SNB-1) efficiently immunoprecipitated SNB-1, but did not pull down either Rim or RAB-3 indicating that the membranes were efficiently solubilized (Fig. 1C). By contrast, immunoprecipitation of Rim pulled down RAB-3, but not SNB-1 (Fig. 1C). Pre-immune sera failed to immunoprecipitate any of the three proteins (Fig. 1C).

Since UNC-10(Rim) is an integral active zone protein [26] that interacts with RAB-3::GTP, predicted to be SV-associated, we hypothesized that UNC-10(Rim) recruitment of SVs to the PD requires RAB-3. To test this model we used high-pressure freeze fixation to resolve the ultrastructural phenotype of *rab-3* mutants. *rab-3*(*js49*) mutant synapses contained normal numbers of SVs (23.78 ± 0.45 SVs per profile, $n = 79$ for WT vs. 23.08 ± 0.81 SVs per profile, $n = 39$ profiles for *rab-3* (ns $p > 0.4$)) (Fig. 2A and B). However, there was a specific reduction in SVs contacting the plasma membrane near the presynaptic density, relative to the wild-type

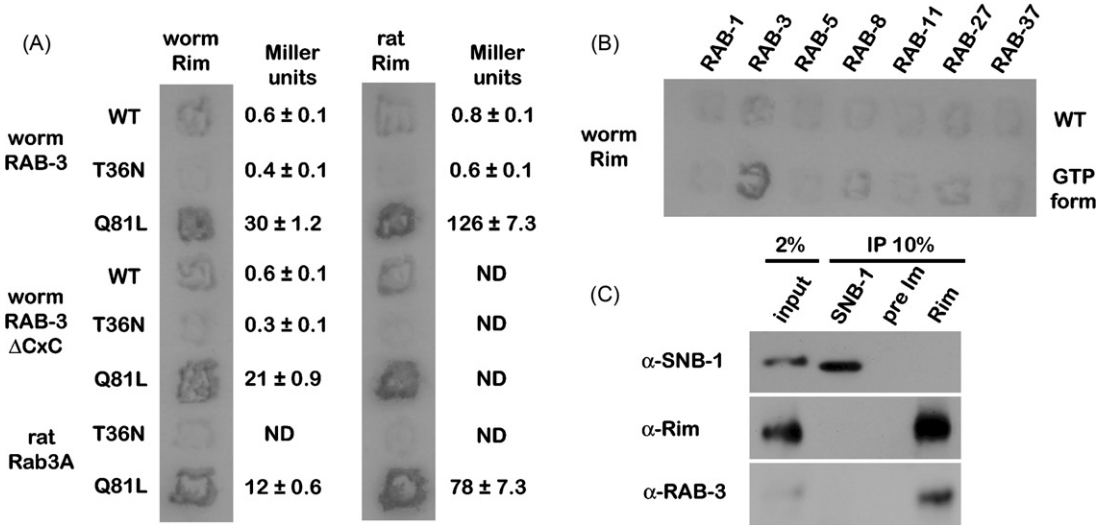

Fig. 1. UNC-10 interacts with RAB-3^{GTP}. (A) The Rim–RAB-3 interaction is conserved across species boundaries. X-gal filter lift two hybrid assays reveal interactions between worm UNC-10 Rim$_{1-187}$ and rat Rim $_{1-345}$ and both rat and worm GTP-bound mimic mutant RAB-3(Q81L), but not the GDP-bound mimic RAB-3(T36N). (B) Interactions between worm UNC-10 Rim$_{1-187}$ were highly selective for RAB-3 compared to other RABs. (C) Rim interacts with RAB-3 in worm extracts. Whole worm solubilized extracts spiked with purified His6Rim zinc finger fusion protein were immunopurified using anti-vSNARE SNB-1, anti-Rim, and pre-immune sera, separated by SDS-PAGE and analyzed by Western blot using antibodies against SNB-1, Rim and RAB-3. 2% of the input and 10% of each immunoisolate were loaded on the gels.

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