Synaptotagmin 1 directs repetitive release by coupling vesicle exocytosis to the

Rab3 cycle

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Abstract

In response to Ca^{2+} influx, a synapse needs to release neurotransmitters quickly while immediately preparing for repeat firing. How this harmonization is achieved is not known. Here, we found that the Ca^{2+} sensor synaptotagmin 1 orchestrates the membrane association/disassociation cycle of Rab3, which functions in activity-dependent recruitment of synaptic vesicles. In the absence of Ca^{2+}, synaptotagmin 1 binds to Rab3 GTPase activating protein (GAP) and inhibits the GTP hydrolysis of Rab3 protein. Rab3 GAP resides on synaptic vesicles and synaptotagmin 1 is essential for the synaptic localization of Rab3 GAP. In the presence of Ca^{2+}, synaptotagmin 1 releases Rab3 GAP and promotes membrane disassociation of Rab3. Without synaptotagmin 1, the tight coupling between vesicle exocytosis and Rab3 membrane disassociation is disrupted. We uncovered the long-sought molecular apparatus linking vesicle exocytosis to Rab3 cycling and we also revealed the important function of synaptotagmin 1 in repetitive synaptic vesicle release.

Introduction

In nerve terminals, neurotransmitters are packaged into synaptic vesicles (SVs) and released by Ca^{2+}-induced exocytosis (Sudhof, 2004). Fast and precise neuronal reaction requires that SVs are clustered in front of the release site, the presynaptic active zone. SVs then dock at the active zone, where they are primed to adopt a competent “ready-for-fusion” state. An action potential induces the opening of Ca^{2+}
channels and the rising Ca\textsuperscript{2+} concentration stimulates SV-plasma membrane fusion.

The basic membrane fusion reaction is mediated by evolutionarily conserved soluble NSF attachment protein receptors (SNAREs) and related proteins like Munc13 and Munc18 (Brunger, 2005; Jahn and Fasshauer, 2012; Jahn and Scheller, 2006; Lang and Jahn, 2008; Sudhof, 2004; Weber et al., 1998). However, the Ca\textsuperscript{2+}-sensing process that starts the SNARE engine is primarily carried out by the synaptotagmin family (Chapman, 2002; Jahn and Fasshauer, 2012). Through their C2 domains, synaptotagmins bind to Ca\textsuperscript{2+}, thus triggering membrane fusion (Sudhof, 2004). After exocytosis, SVs undergo endocytosis and recycling, and are refilled with neurotransmitters for repeated rounds of release.

Rab3 protein is highly enriched in the nervous system and is specifically localized on SVs (Fischer von Mollard et al., 1994; Fischer von Mollard et al., 1991; Geppert et al., 1994; Stahl et al., 1996). Like other Rabs, Rab3 cycles on and off its target membranes according to its GTP- or GDP-bound state. On the vesicles, the active GTP-bound form of Rab3 is complexed with effector proteins like rabphilin and RIM (Rab3-interacting molecule) (Li et al., 1994; Shirataki et al., 1993; Wang et al., 1997; Wang et al., 2000), thus facilitating the recruitment/docking of SVs (Leenders et al., 2001; Nonet et al., 1997; Tsuboi and Fukuda, 2006). Ca\textsuperscript{2+}-induced exocytosis can trigger disassociation of Rab3 from SV membranes through the GTP hydrolysis process (Fischer von Mollard et al., 1994; Fischer von Mollard et al., 1991; Stahl et al., 1996), but the underlying mechanisms are not clear. The GTP-to-GDP conversion not only removes Rab3 from SVs, but also simultaneously dissociates
Rab3 from its binding effectors, which disassembles the docking complex so that both Rab3 and Rab3 effectors can be recycled for the next round of release (Wang et al., 1997; Wang et al., 2000). Given their unique features, Rab3 and synaptotagmin have been considered as the Yin and Yang of membrane fusion respectively (Geppert and Sudhof, 1998). However, the functional regulatory interaction between synaptotagmin 1 and Rab3 cycling has not been identified, nor has the mechanism by which this interaction is coupled to fast and repetitive neurotransmitter release.

Here, we found that synaptotagmin 1/SNT-1 in C. elegans is crucial for the SV association of RAB-3 protein. SNT-1 promotes the GTP-bound state of RAB-3 by inhibiting RAB-3 GAP. The catalytic subunit of RAB-3 GAP (RBG-1) localizes on SVs and directly binds to SNT-1. Ca\(^{2+}\) treatment disrupts the direct association between SNT-1 and RBG-1. In addition, Ca\(^{2+}\)-binding activity of SNT-1 is essential for the dissociation of RAB-3 from SVs. Thus, our study reveals the pivotal dual role of synaptotagmin 1 in coupling SV exocytosis with the Rab3 membrane association and dissociation cycle.

**RESULTS**

**Search for components involved in RAB-3/SV association**

In *C. elegans* motor neurons, RAB-3 fused with Green Fluorescent Protein (GFP) adopts a punctate pattern of localization along the length of the ventral and dorsal cords (Mahoney et al., 2006). This punctate RAB-3 pattern is similar to that of other SV proteins, including synaptobrevin and synaptotagmin (Nonet, 1999; Nonet et al.,
1993; Zhen and Jin, 1999). A previous report also showed that most Rab3 protein is
associated with SV membranes (Fischer von Mollard et al., 1990). In the absence of
the RAB-3 GEF, AEX-3 (Iwasaki et al., 1997), GFP::RAB-3 no longer shows a
punctate pattern and becomes diffusely distributed in neuron cell bodies and axons
(Figure 1A). AEX-3 is responsible for converting RAB-3 protein from the
membrane-dissociated GDP-bound form to the membrane-associated GTP-bound
form (Figure 1C). Therefore, the punctate localization of RAB-3 in wild type likely
represents the GTP-bound, SV membrane-associated form of RAB-3, while the
diffuse GFP::RAB-3 signal may represent the dissociated GDP-RAB-3.

We speculated that mutations in components required for Rab3/SV association
may lead to a diffuse RAB-3::GFP phenotype similar to that in aex-3 animals. Hence,
we conducted a genetic screen and isolated multiple mutants in which GFP::RAB-3
lost its punctate localization pattern. Through SNP mapping, complementation testing,
and fosmid rescue, we cloned all of these mutations. Six of them (xd58, xd137, xd142,
xd143, xd148, and xd149) turned out to be new alleles of aex-3. In addition, we
obtained four rep-1 alleles (xd56, xd138, xd139, and xd140) and three hmgs-1 alleles
(xd128, xd129, and xd145). rep-1 encodes the sole Rab escort protein (Rep) (Tanaka
et al., 2008). Rep proteins bind newly-synthesized Rab proteins and facilitate the
addition of geranylgeranyl groups to Rabs (Andres et al., 1993; Seabra et al., 1992a;
Seabra et al., 1992b) (Figure 1C). HMGS-1 is orthologous to the human
hydroxymethylglutaryl-CoA synthase (HMGS) which is required for synthesis of the
geranylgeranyl moiety (Mehrabian et al., 1986; Shi and Ruvkun, 2012) (Figure 1C).
Thus, both rep-1 and hmg-1 are critical for RAB-3 membrane targeting. The diffuse GFP::RAB-3 phenotype in rep-1 and hmg-1 mutants further suggested that the diffuse signal indeed comes from membrane-dissociated RAB-3 protein.

**Synaptotagmin/SNT-1 is required for localization of RAB-3 on SVs**

In the meantime, we hypothesized that the molecules controlling the RAB-3 cycle may be associated with SV cycling. Thus, we systematically examined SV cycle-related mutants. Interestingly, we found that in snt-1(md290) animals, which lack snt-1 function, the GFP::RAB-3 puncta disappeared and the GFP signal was diffusely distributed throughout the neuronal processes, similar to aex-3, rep-1 and hmg-1 mutants (Figure 1A and 1B). The strong phenotypic similarity between snt-1 and other RAB-3/SV-association defective mutants suggests that SNT-1 plays an important role in RAB-3/SV localization.

*snt-1* encodes the synaptotagmin 1 homologue in *C. elegans* (Nonet et al., 1993). Neuronal synaptotagmins function as Ca$^{2+}$ sensors for synaptic exocytosis, but their role in Rab3 localization has not been revealed. To determine whether loss of snt-1 function indeed leads to the diffuse RAB-3 phenotype, we examined other snt-1 alleles. We found that the n2665, md220, md125 and md172 alleles of snt-1 all display a diffuse GFP::RAB-3 phenotype similar to md290 (Figure 1-figure supplement 1, panel A). Both the snt-1 and rab-3 genes are broadly expressed in the nervous system (Nonet et al., 1993; Nonet et al., 1997). To determine whether snt-1 influences RAB-3 in all neurons, we examined RAB-3 localization with a pan-neuronal marker
Prab-3::GFP::RAB-3. In wild-type animals, GFP::RAB-3 displays a punctate pattern, while in snt-1 mutants, GFP::RAB-3 is completely diffuse in neuronal processes, including the nerve ring, ventral cord, and dorsal cord regions (Figure 1-figure supplement 1, panel B and C). These data indicate that the effect of snt-1 on RAB-3 localization is widely preserved in the nervous system. In addition, the diffuse GFP::RAB-3 phenotype was fully rescued when wild-type snt-1 was introduced into mutant animals (Figure 1-figure supplement 1, panel D), suggesting that SNT-1 is indeed essential for localization of RAB-3 on SVs.

snt-1 affects RAB-3 localization through an SV-independent mechanism

The diffuse RAB-3 phenotype in snt-1 may be caused by failure of SV clustering at the synaptic terminal. Therefore, we examined the localization of another synaptic vesicle protein SNB-1. SNB-1 is the *C. elegans* synaptobrevin homologue (Nonet, 1999). In worm DD and VD motor neurons, SNB-1 is distributed evenly in punctate structures along neuronal processes, similar to RAB-3 (Figure 2A) (Zhen and Jin, 1999). In snt-1 animals, some of the SNB-1 puncta are enlarged, but the punctate distribution of SNB-1 is not altered (Figure 2A and 2B). This observation is consistent with recent findings (Yu et al., 2013), suggesting that SV clustering is probably not affected by snt-1.

unc-104 encodes the cytosolic kinesin responsible for synaptic vesicle trafficking from cell bodies to nerve terminals (Hall and Hedgecock, 1991). In the absence of UNC-104 kinesin, few SVs are transported to synaptic termini, while neuron cell
bodies have a surfeit of SVs. To further address whether the diffuse RAB-3 phenotype is indeed caused by the dissociation of RAB-3 from SVs in snt-1 mutants, we performed a serial mutant analysis utilizing both GFP::RAB-3 and SNB-1::GFP markers. Because the snt-1;unc-104 double mutant animals are arrested during larval development, the synaptic phenotypes were examined in newly-hatched L1 animals (larval stage 1). In L1 animals, among DD, VD, and AS motor neurons, only DDs are born (Sulston, 1976; Sulston and Horvitz, 1977), and they form pre-synapses along the ventral cord. Thus, in wild-type L1 animals labeled by $\text{Punc-25}::\text{SNB-1}::\text{GFP}$, the GFP signal could only be detected along the ventral cord (Figure 2C and 2C”) but not the dorsal cord (Figure 2C”). In snt-1 mutants, the SNB-1::GFP distribution is indistinguishable from wild type (Figure 2D, 2D’, and 2D”). In unc-104 single mutants, we found that SNB-1::GFP accumulated in cell bodies (Figure 2E and 2E”, white arrow) and little GFP signal could be detected outside of cell bodies or on the dorsal cord (Figure 2E”), which is consistent with the role of UNC-104 in SV transport. In snt-1;unc-104 double mutants, the SNB-1::GFP signal accumulated in cell bodies (Figure 2F and 2F”, white arrow) like in unc-104 single mutants, suggesting that further removal of SNT-1 in unc-104 mutant animals does not alter the dependence of SVs on UNC-104 for intracellular trafficking. Interestingly, the effect of snt-1 or snt-1 unc-104 mutations on RAB-3 and SNB-1 is quite different. As shown in Figure 2H and 2J, GFP::RAB-3 is still diffuse in cell bodies and axons in both snt-1 and snt-1 unc-104 animals and GFP signal could be detected even in the non-synaptic dorsal cord region (Figure 2H’ and 2J’). In contrast, in unc-104 animals, the RAB-3
puncta are retained within cell bodies, just like SNB-1 is in *unc-104* mutants (Figure 2I and 2I’). These data strongly support the notion that the diffuse phenotype of RAB-3 is not caused by the dispersion of SV clusters, but rather by the specific dissociation of RAB-3 from SV membranes.

**SNT-1 promotes the GTP-bound form of RAB-3**

How does mutation of *snt-1* affect the SV membrane association of RAB-3? Previous studies showed that the localization of RAB-3 on SV membranes is tightly associated with its GTP-bound state (Zerial and McBride, 2001). Therefore, we tested whether the loss of RAB-3 from SVs in *snt-1* mutants is caused by reduction of GTP-bound RAB-3. The active GTP-Rab3 binds to the RBD domain of its effector RIM, while the inactive GDP-Rab3 does not. Previous reports demonstrated that the RBD domain of mammalian RIM2 could bind to the worm GTP-RAB-3 (Mahoney et al., 2006; Wang et al., 1997). Thus, we performed pull-down assays to examine the GTP-RAB-3 level *in vivo*. In wild-type worm lysates, the active GTP-bound form of RAB-3 protein was efficiently pulled down by GST-RBD (Figure 3A). In contrast, the amount of GTP-RAB-3 pulled down by RIM2 RBD was significantly reduced in *snt-1* lysates (Figure 3A and 3B). In the absence of RAB-3 GEF, the GDP-bound RAB-3 cannot be converted to the GTP-bound RAB-3. Indeed, in *aex-3* animals, the amount of RAB-3 that can be pulled down by GST-RBD is also greatly decreased (Figure 3A and 3B). Thus *snt-1*, similar to *aex-3*, affects the level of GTP-bound RAB-3 *in vivo*. GTP-RAB-3 is associated with SV membranes, while GDP-RAB-3 is diffuse in
the cytosol. Thus, we performed cell fractionation experiments to further examine the
GTP or GDP status of RAB-3. In wild type, RAB-3 is highly enriched in membrane
fractions (Figure 3C), which is consistent with the SV localization of GTP-RAB-3. In
contrast, the RAB-3 protein distribution is shifted to the soluble fraction when snt-1 is
removed, suggesting a cytosolic localization of RAB-3 in snt-1 mutants (Figure 3C).
These data suggest that snt-1 indeed promotes the GTP-bound form of RAB-3.

**SNT-1 promotes the GTP-RAB-3 level by inhibiting GTP hydrolysis**

How does loss of function of snt-1 lead to the reduction of GTP RAB-3? One
possibility is that SNT-1 may regulate the RAB-3 GTP-GDP cycle by promoting GEF
activity. We performed the following experiments to test this possibility. Firstly,
AEX-3 is the GEF molecule for both RAB-3 and RAB-27 (Mahoney et al., 2006). If
snt-1 indeed affects AEX-3 activity, we would expect that the localization of RAB-27
on SVs will be affected by the absence of SNT-1. We made a GFP::RAB-27 reporter
and expressed it in motor neurons in worms. The GFP::RAB-27 protein is enriched in
synaptic regions and displays a punctate expression pattern similar to RAB-3 (Figure
3D). In *aex-3* mutants, GFP::RAB-27 becomes diffuse, consistent with the role of
AEX-3 as a GEF for RAB-27 (Figure 3D). In contrast, GFP::RAB-27 still displays a
punctate distribution indistinguishable from wild type in snt-1 mutants (Figure 3D),
suggesting that the GEF activity of AEX-3, at least for RAB-27, is not altered by
mutation of snt-1. Second, if SNT-1 promotes AEX-3 GEF activity, we would expect
that increasing the *aex-3* expression level may rescue the diffuse RAB-3 phenotype in
snt-1 mutants. However, no such rescue was observed (Figure 3E). Lastly, we
examined the expression level of AEX-3 and found that it was indistinguishable in
wild-type and snt-1 animals (Figure 3F). Together, these results suggest that it is
unlikely that snt-1 regulates the GTP-RAB-3 level by promoting RAB-3 GEF activity.
Alternatively, the decreased GTP-RAB-3 level may be caused by increased
RAB-3 GTPase activity in snt-1 mutants. Rab3 GTPase activity is greatly facilitated
by Rab3-specific GTPase-activating protein (GAP). Rab3 GAP is composed of the
catalytic subunit Rab3GAP1 and the noncatalytic subunit Rab3GAP2. rbg-1 and
rbg-2 encode Rab3GAP1 and Rab3GAP2 respectively in worms (Figure 4-figure
supplement 1, panel A and B) (Fukui et al., 1997; Nagano et al., 1998). In the absence
of Rab3 GAP, the RAB-3 synaptic enrichment is enhanced, which is consistent with
the role of Rab3 GAP in assisting GTP hydrolysis (Figure 4-figure supplement 1,
panel C and D). If RAB-3 GTP hydrolysis activity is indeed increased in snt-1
mutants, we would expect that loss of GAP function will suppress the snt-1 mutant
phenotype. Indeed, in rbg-1;snt-1 double mutants, we found that the diffuse
GFP::RAB-3 phenotype of snt-1 single mutants is significantly suppressed (Figure 4A
and 4B). Furthermore, mutation of the rbg-2 gene also suppressed the diffuse RAB-3
phenotype in snt-1 mutants (Figure 4-figure supplement 1, panel E). In contrast, the
diffuse GFP::RAB-3 signal caused by aex-3 mutation could not be suppressed by
rbg-1 (Figure 4A and 4B). We next performed RIM2- RBD pull-down assays to test
whether the GTP-RAB-3 level was restored in rbg-1;snt-1 mutants. In contrast to the
greatly reduced GTP-RAB-3 level in snt-1 lysates, the amount of GTP-bound RAB-3
is significantly increased in \textit{rbg-1;snt-1} samples (Figure 4C). Taken together, these results suggest that \textit{snt-1} indeed regulate the RAB-3/SV association specifically by inhibiting RAB-3 GTP hydrolysis.

\textbf{Rab3GAP1/RBG-1 localizes on SVs}

As the catalytic subunit of Rab3 GAP, RBG-1 can interact with RAB-3 (Figure 4-figure supplement 1, panel F). To understand how SNT-1 inhibits RAB-3 GTP hydrolysis, we examined the sub-cellular localization of RBG-1. We created a functional mCherry::RBG-1 construct and injected it into \textit{rbg-1} mutant animals. In wild-type animals, the mCherry::RBG-1 signal displayed a punctate expression pattern along the ventral and dorsal cords. Double staining further showed that the mCherry::RBG-1 puncta co-localized with the SV marker SNB-1::GFP (Figure 5C). Next we tested whether the punctate localization of RBG-1 relies on the UNC-104-based intracellular transport system like other SV associated proteins. We found that in \textit{unc-104} mutants, the mCherry::RBG-1 puncta no longer appeared in the putative synaptic region; instead they were retained in the cell bodies (Figure 5B). Together, the data above suggest that RBG-1 is localized on SVs.

\textbf{SNT-1 is required for the SV localization of RBG-1}

SNT-1 resides on SVs and its function in RAB-3 localization is executed by RAB-3 GAP. Could the SV localization of RBG-1 be regulated by \textit{snt-1}? We examined RBG-1 distribution in \textit{snt-1} animals and found that the RBG-1/SV co-localization is
lost and mCherry::RBG-1 fluorescence becomes diffuse (Figure 5D). In contrast, SNB-1::GFP still retains its punctate expression pattern in \textit{snt-1} animals, similar to wild type (Figure 5D). Therefore, SNT-1 is required for the SV localization of RBG-1. RBG-1 binds to its substrate RAB-3 (Figure 4-figure supplement 1, panel E). Thus, we also tested whether the SV localization of RBG-1 is controlled by RAB-3. In \textit{rab-3(js49)} mutants, we found that mCherry::RBG-1 retains its punctate expression and still co-localizes with SNB-1::GFP (Figure 5E), suggesting that the SV localization of RBG-1 does not rely on RAB-3 protein.

\textbf{Rab3GAP1/RBG-1 binds to the C2B domain of SNT-1}

The SNT-1-dependent SV association of RBG-1 suggests a direct association between SNT-1 and RBG-1. We co-expressed full-length RBG-1 and the cytosolic domain of SNT-1 (C2AB) in HEK293FT cells. After affinity purification, the RBG-1 protein was incubated with the SNT-1 C2AB fragment. In contrast to the mock-transfected sample, RBG-1 was effectively co-precipitated with the cytosolic region of SNT-1 (Figure 6A). The SNT-1 cytosolic portion was also co-precipitated by RBG-1 (Figure 6B). We next asked which domain of the SNT-1 cytosolic region is required for this binding. The cytosolic region of SNT-1 contains a C2A and a C2B motif. When the C2A domain was deleted, the remaining C2B motif retained the RBG-1 binding activity (Figure 6C). In contrast, when C2B was removed, the C2A domain alone could not bind to RBG-1 (Figure 6C). Therefore, the C2B domain is required for SNT-1 binding to RBG-1.
We further investigated whether the function of SNT-1 in regulating the RAB-3/SV association is mediated through the C2B domain. In comparison with full-length SNT-1, which fully rescues the GFP::RAB-3 mis-localization defect in \textit{snt-1} mutants, we found that SNT-1 without the C2B domain (deltaC2B) had no rescue effect (Figure 6D). In contrast, when the C2A domain is removed (deltaC2A) from SNT-1, the remaining protein still possesses the rescue activity (Figure 6D). Together, these data suggest that the C2B domain is critical for SNT-1 function.

SNT-1 lacking the trans-membrane domain (deltaTM) also failed to rescue the diffuse RAB-3 phenotype (Figure 6D), suggesting that the SV localization function of SNT-1 is required in addition to the C2B domain for regulating RAB-3/SV association \textit{in vivo}.

\textbf{Ca}^{2+} treatment decreases the binding between SNT-1 and Rab3GAP1/RBG-1

\textit{Ca}^{2+}-mediated exocytosis activates the dissociation of Rab3 from the SV membrane (Fischer von Mollard et al., 1994; Fischer von Mollard et al., 1991). Could synaptotagmin 1, as the \textit{Ca}^{2+} sensor for SV exocytosis, be the trigger to initiate the Rab3 SV dissociation process? We showed above that SNT-1 directly associates with Rab3GAP1/RBG-1. Therefore, we wondered whether the \textit{Ca}^{2+} level could affect the binding of SNT-1 to Rab3GAP1/RBG-1, and whether the inhibition of RAB-3 GAP by SNT-1 is relieved by \textit{Ca}^{2+} binding, thus allowing dissociation of RAB-3 from the SV during exocytosis. To test the ideas above, we asked whether the presence of \textit{Ca}^{2+}
disrupts the binding between SNT-1 and RBG-1. We purified RBG-1 and SNT-1
proteins and performed co-IP experiments with increasing concentrations of Ca\(^{2+}\). A 
Ca\(^{2+}\) concentration of 0.5 mM or 1 mM significantly compromised the 
RBG-1/SNT-1 interaction (Figure 7A and 7B). Thus, upon Ca\(^{2+}\) binding, SNT-1 
releases RBG-1.

Next, we asked whether SNT-1-RBG-1 binding is still affected by Ca\(^{2+}\) treatment 
if the Ca\(^{2+}\)-binding sites are removed from SNT-1. The amino acids critical for Ca\(^{2+}\) 
binding in the C2A domain (D248 and D250) and the C2B domain (D383 and D385) 
were mutated and the resulting SNT-1 mutant protein (C2A*B*) was purified and 
tested its ability to bind RBG-1. We found that the RBG-1-binding capability of 
C2A*B* was high in the absence or presence of Ca\(^{2+}\) (Figure 7C and 7D), suggesting 
that the Ca\(^{2+}\)-binding activity of SNT-1 is essential for attenuation of the 
SNT-1/RBG-1 interaction when Ca\(^{2+}\) is present.

**Dissociation of RAB-3 from SVs relies on the Ca\(^{2+}\)-binding activity of SNT-1**

Ca\(^{2+}\) treatment decreases the binding between SNT-1 and RBG-1. Is the inhibition of 
RAB-3 GAP by SNT-1 alleviated when the Ca\(^{2+}\) concentration rises? If so, SNT-1 that 
lacks Ca\(^{2+}\)-binding capability will fail to release RAB-3 GAP during Ca\(^{2+}\) influx, and 
thus the dissociation of RAB-3 from SVs will be inhibited. To test this hypothesis, we 
analyzed the GFP::RAB-3 pattern in transgenic animals that express different mutant 
forms of SNT-1. The two amino acids necessary for Ca\(^{2+}\) binding in the C2A domain 
of SNT-1 are D248 and D250. We created the SNT-1 mutant C2A* by replacing these 
two aspartic acids with alanines, and found that C2A* could rescue the GFP::RAB-3
mis-localization defect (Figure 7E). In addition to this rescue phenomenon, we noticed that the GFP::RAB-3 puncta were enlarged and the GFP signal was more enriched in the punctate regions in comparison to wild-type animals over-expressing *snt-1* (Figure 7E and 7F). A similar GFP::RAB-3 enrichment effect was observed in worms expressing the C2B* mutant form of SNT-1, which contains a C2B domain that cannot bind Ca$^{2+}$ (D383 and D385 were replaced with alanines) (Figure 7E and 7F). We further replaced the Ca$^{2+}$-binding sites in both C2A and C2B (C2A*B*).

When this construct was expressed, the GFP::RAB-3 signal was also increased in the punctate regions (Figure 7E and 7F).

Could the enhanced GFP::RAB-3 signal in the puncta indeed reflect the failure of exocytosis? Previous studies suggest that RAB3 dissociation from SV membranes is inhibited when SV exocytosis is disrupted by blocking either Ca$^{2+}$ influx or membrane fusion (Fischer von Mollard et al., 1994; Fischer von Mollard et al., 1991; Stahl et al., 1996). We examined two exocytosis mutants, *unc-2* and *unc-13*. *unc-2* encodes the alpha subunit of the voltage-gated Ca$^{2+}$ channel (Schafer and Kenyon, 1995). In *unc-2* mutants, we found that the GFP signal is significantly enriched within the punctate regions and the GFP::RAB-3 puncta are larger and brighter compared to wild type (Figure 7-figure supplement 1, panel A and B). *unc-13* encodes the Munc13 homolog in worms and loss of function of *unc-13* results in blockage of membrane fusion during SV exocytosis (Aravamudan et al., 1999; Richmond et al., 1999; Varoqueaux et al., 2002). We found that the GFP signal was enhanced in the punctate regions in *unc-13* mutants, as in *unc-2* mutants (Figure 7-figure supplement 1, panel A and B).
Blocking SV exocytosis resulted in failure of RAB-3 to dissociate from SVs; thus the enlarged GFP::RAB-3 puncta indeed indicate the decreased dissociation of RAB-3 protein from SVs. Taken together, the data above indicate that the Ca\(^{2+}\)-binding capability is essential for SNT-1 function in mediating the RAB-3 SV dissociation induced by Ca\(^{2+}\)-triggered exocytosis.

Interestingly, in *unc-13;snt-1* or *unc-2;snt-1* double mutants, the RAB-3::GFP signal is diffuse throughout whole neuronal cells (Figure 7-figure supplement 1, panel A and B), as seen in *snt-1* single mutants. Because the diffuse RAB-3::GFP signal suggests a dissociation of RAB-3 from SV membranes, the above observation implies that in the absence of SNT-1, the dissociation of RAB-3 from SVs can occur even when Ca\(^{2+}\)-induced exocytosis is blocked. As a consequence, the coupling between SV exocytosis and RAB-3 dissociation is disrupted when SNT-1 is missing. These data are consistent with the inhibition-of-inhibition role of SNT-1 on RAB-3/SV association. Together, these results suggest that SNT-1 plays a dual role in the RAB-3/SV cycle, inhibiting dissociation of RAB-3 from SVs during the resting state (no Ca\(^{2+}\) influx) and triggering dissociation of RAB-3 from SVs upon Ca\(^{2+}\) binding (Figure 7G).

**DISCUSSION**

Acute and precise neuronal activity requires precise coordination between the SV cycle and the Rab3 cycle. As the trigger of regulated vesicle secretion, synaptotagmin 1 is known to bind the membrane and the SNARE complex to give the final push for
complete assembly of the SNARE complex for membrane fusion (Chapman and Davis, 1998; Choi et al., 2010; Dai et al., 2007; Davletov and Sudhof, 1993; Vrljic et al., 2010). Here, we revealed that SNT-1/synaptotagmin 1 functions as an on-and-off switch to regulate Rab3 membrane association, thus facilitating repeated release. Our study has many important implications.

In the resting state, Rab3 protein is associated with SVs in the GTP-bound form, and Rab3 GTP, together with its corresponding effectors, docks the vesicles at the active zone region (Sudhof, 2004). However, Rab3 GAP, the negative regulator of Rab3, is also enriched in the synaptic fraction and is localized on SVs (this study) (Oishi et al., 1998). How therefore is the active form of Rab3, and hence the proper docking complex, maintained when GAP is close by? Based upon our study, it is entirely possible that synaptotagmin 1 prevents the hydrolysis of Rab3 GTP by directly sequestering or inhibiting Rab3 GAP before Ca^{2+} influx. In fact, the SV localization of Rab3GAP1/RBG-1 is particularly interesting, given the fact that the Rab3 cycle must be spatially regulated so that Rab3 is kept in close proximity to SVs for repeated neurotransmitter release. Indeed, when synaptotagmin 1/SNT-1 binds Ca^{2+}, the inhibition on RAB-3 GAP is alleviated, so the locally enriched Rab3 GAP can freely and quickly access GTP-Rab3 and hydrolyze Rab3 GTP to GDP. Together with the functional Ca^{2+} switch, synaptotagmin 1 can therefore efficiently coordinate the Rab3 cycle with the SV cycle. The concentration of Ca^{2+} needed for exocytosis ranges from ~10-200 μM in the nerve terminals, so the Ca^{2+} concentration that is required to release RBG-1 from SNT-1 is relatively high (500 μM). However, it is
known that the rather low affinity of synaptotagmin 1 for Ca^{2+} (Fernandez et al., 2001; Ubach et al., 1998) can be strongly affected by the presence of synaptotagmin-binding partners, especially membrane lipids (Chapman, 2002). Therefore, it will be interesting to test whether plasma membrane-enriched lipids such as PIP2 (Lee et al., 2010) can act synergistically with Ca^{2+} to regulate the binding of SNT-1 to RBG-1.

At the molecular level, synaptotagmin 1 is known to bind the plasma membrane and the SNARE complex in the presence of Ca^{2+} (Chapman and Davis, 1998; Choi et al., 2010; Dai et al., 2007; Davletov and Sudhof, 1993; Fernandez et al., 2001; Vrljic et al., 2010). The interaction of synaptotagmin with membranes and SNARE proteins has well-documented consequences, including creating local positive membrane curvature and displacing the clamping factor complexin from the SNARE complex (Hui et al., 2009; Martens et al., 2007). Combining these previous reports with data presented in this study, we think that synaptotagmin-Ca^{2+} may not only give the final push for complete assembly of the SNARE complex for membrane fusion, but also play a role in terminating vesicle docking by indirectly deactivating Rab3, thus facilitating repetitive transmitter release.

Among more than 60 Rabs in humans and mice, Rab3 and Rab27 seem to be specifically involved in stimulated secretion in a variety of secretory cells (Fukuda, 2008, 2013). Synaptotagmins have also evolved specifically to regulate secretion. Interestingly, the regulatory role of SNT-1 on RAB-3 does not extend to RAB-27, which is functionally closely related to RAB-3 (Mahoney et al., 2006). Simultaneous knockdown of Rab3 and Rab27 causes secretion defects more severe than single
knockdown in worms and PC12 cells (Mahoney et al., 2006; Tsuboi and Fukuda, 2006). Both Rab27 and Rab3 are localized on SVs and can bind to RIM, thereby docking the vesicles (Fukuda, 2003; Wang et al., 1997). Rab3 and Rab27 share the same GEF, which is AEX-3 in worms and DENN/MADD in mammals (Coppola et al., 2002; Levivier et al., 2001; Mahoney et al., 2006). In contrast, Rab3 GAP serves as a specific GAP for Rab3 (Fukui et al., 1997; Itoh and Fukuda, 2006; Nagano et al., 1998). Here, SNT-1 apparently only affects the RAB-3 cycle and this action is mediated by inhibition of the Rab3-specific GAP. Therefore, although Rab3 and Rab27 play redundant roles in SV exocytosis, they can be differentially controlled through their specific regulators. We would like to point out that humans and worms have multiple synaptotagmins, and it remains to be determined whether any of the other synaptotagmins play a similar regulatory role on Rab27.

Rab3 GAP consists of the catalytic subunit Rab3GAP1 and the noncatalytic subunit Rab3GAP2 (Fukui et al., 1997; Nagano et al., 1998). Rab3GAP1 and Rab3GAP2 form a complex in vitro and co-immunoprecipitate in vivo (Nagano et al., 1998). Although Rab3GAP2 does not affect the in vitro GAP activity of Rab GAP1, it may act to stabilize, regulate or localize Rab3GAP1 correctly in cells. The functional characteristics are consistent with their close biochemical interactions.

Loss-of-function mutations in Rab3GAP1 and Rab3GAP2 produce clinically almost indistinguishable conditions, Warburg Micro syndrome and Martosolf syndrome, characterized by brain, eye, and endocrine abnormalities (Aligianis et al., 2005; Aligianis et al., 2006). We have now revealed that both Rab3GAP1/rbg-1 and
Rab3GAP1/rbg-2 mutations can suppress the RAB-3 mis-localization phenotype in snt-1 mutants, implying that Rab3GAP does indeed function as a complex to participate in the SNT-1-mediated regulation of the RAB-3 cycle. However, we noticed that loss of function of rbg-2 alone leads to additional synaptic or axonal defects (data not shown) compared to loss of rab-3 or rbg-1. This suggests that RBG-2 may play roles in nervous system development other than forming the Rab3GAP complex with RBG-1 during SV exocytosis.

The GTP-bound active form of Rab promotes membrane trafficking by interacting with specific effectors. In contrast with Rab effectors that function in secretory vesicle trafficking, relatively little is known about the specific Rab GEFs and GAPs and how they are regulated during vesicle secretion. Evidence that the sub-cellular localization and activity of RAB-3 GAP can be regulated by synaptotagmin/SNT-1 strongly hints that regulators of Rabs could be subjects for active manipulation during various types of intracellular membrane trafficking. A recent intriguing study is in agreement with our notion. In the amoeba Dictyostelium discoideum, vacuolar Ca\(^{2+}\) release activates the Rab GAP CnrF, thus subsequently down-regulating Rab11a (Donato et al., 2013). Taken together, current data suggest a vital role of Ca\(^{2+}\) as the functional switch for regulated secretion processes. Because the key principles and regulatory components of different intracellular vesicle trafficking events are broadly conserved, the mechanism that we have uncovered is likely to represent a conserved mode of action.

Understanding how the sequential activation of Rab GTPases is achieved during
vesicle trafficking is a central theme of cell biology. This in turn raises the question of
how regulatory Rab GEFs or Rab GAPs are activated in the right place and at the
right time. Synaptotagmins act as the primary Ca\(^{2+}\)-sensors in most forms of
Ca\(^{2+}\)-induced exocytosis, as exemplified by synaptic transmission. Our studies have
proposed the elegant molecular machinery by which Rab GAP can be temporally and
spatially regulated in response to acute cellular signals, so that correctly activated
Rabs can perform their appropriate functions and allow vesicle fusion to occur in an
orderly fashion.

**EXPERIMENTAL PROCEDURES**

**Worm strains and mutagenesis**

Strain maintenance and genetic manipulations were performed as described (Brenner,
1974). Strains used in this study are: **LG I**: EG2710 [unc-57(ok310)], CB450
[unc-13(e450)], VC2481 [rgb-2(ok3195)], CB47 [unc-11(e47)]. **LGII**: NM204
[snt-1(md290)], CB1265 [unc-104(e1265)], MT6977 [snt-1(n2665)], RM1606
[snt-1(md172)], RM1603 [snt-1(md125)], RM1620 [snt-1(md220)], NM791
[rab-3(js49)]. **LGIII**: XD1366 [rep-1(xd56)], NM1278 [rbf-1(js232)]. **LGIV**:
EG3027 [unc-26(s1710)], CB169 [unc-31(e169)]. **LGV**: XD1925 [hmgs-1(xd145)],
CB268 [unc-41 (e268)], NM467 [snb-1(md247)], RM956 [ric-4(md1088)]. **LGX**:
XD1199 [aex-3(xd58)], RB1453 [rgb-1(ok1660)], CB81 [unc-18(e81)], CB55
[unc-2(e55)], CB102 [unc-10(e102)], CX51 [dyn-1(ky51)]. Additional strains are:
XD2188 [xdEx1380; Paex-3::AEX-3::GFP], XD2702 [xdEx1214;
Punc-25::mCHERRY::RBG-1], XD3132 [\textit{xdEx1461}; Phmr-1b::SNT-1FL], XD3017 [\textit{xdEx1419}; Phmr-1b::SNT-1 deltaTM], XD3134 [\textit{xdEx1463}; Phmr-1b::SNT-1 deltaC2A], XD3034 [\textit{xdEx1398}; Phmr-1b::SNT-1 deltaC2B], XD3032 [\textit{xdEx1396}; Phmr-1b::SNT-1 C2A*], XD3033 [\textit{xdEx1397}; Phmr-1b::SNT-1C2B*], XD3133 [\textit{xdEx1462}; Phmr-1b::SNT-1C2A*B*]. Mutagenesis was carried out in the \textit{xdIs7} (\textit{Phmr-1b::GFP::RAB-3}) strain treated with ethylmethane sulfonate. From 5,000 genomes, 13 mutations were isolated. Subsequent genetic and molecular analysis revealed that we had isolated four alleles of \textit{rep-1}(\textit{xd56, xd138, xd139, and xd142}), six alleles of \textit{aex-3}(\textit{xd58, xd60, xd137, xd143, xd148, and xd149}), and three alleles of \textit{hmgs-1}(\textit{xd128, xd129, and xd145}). \textit{rep-1(xd138)}, \textit{rep-1(xd139)}, \textit{hmgs-1(xd128)} and \textit{hmgs-1(xd129)} animals are larval lethal. The rest of the identified mutants are fertile.

\textbf{DNA constructs and transgenic animals}

Promoters, GFP, mCherry, and various cDNA or genomic DNA fragments were cloned into the deltapSM vector through standard procedures. Site-directed mutagenesis was performed using standard PCR-based methods. Transgenic animals were produced as previously described (Song et al., 2010). Integrated strains were obtained by UV irradiation. All integrated transgenic animals were out-crossed at least 3 times.

\textbf{GTP-RAB-3 pull-down assay}

DNA fragments containing the RBD domains of rat RIM2 were inserted into the pGEX-4T-3 vector. Expression and purification of GST fusion proteins in \textit{E. coli} were carried out according to standard procedures. Worms with different genotypes were
collected and washed in M9 buffer. 800 μl of homogenizing buffer (50mM Tris-Cl pH8.0, NaCl 150mM, 0.5% sodium deoxycholate, 1% Triton-X 100) was added and samples were disrupted with a Dounce homogenizer (Cheng-He Company, Zhuhai, China) (Chen et al., 2010) on ice for 5 minutes. Debris was removed by centrifuging at 12000 rpm for 10 minutes at 4°C. The amount of GFP-RAB-3 input in each experiment was equalized before the pull-down assay. The worm lysates were incubated for 4 hours at 4°C with GST-tagged RBD RIM2 coupled to glutathione-Sepharose 4B (GE Healthcare). After washing three times, the GFP-RAB-3 level was analyzed by 10% SDS-PAGE followed by standard western blotting with an anti-GFP antibody (1:5000 dilution) (Santa Cruz Biotechnology).

**Co-immunoprecipitation**

To express proteins in HEK293FT cells, cDNA fragments were amplified and cloned into modified pcDNA™3.1/myc-HIS(-) or pFLAG-CMV-2 vectors through standard procedures. HET293FT cells were cultured in DMEM medium supplemented with 12% FBS. Plasmid transfections were carried out using Lipofectamine 2000 (Invitrogen). 24 hours after transfection, cells were harvested and lysed for 10 min at 4°C. After centrifugation, the supernatants were incubated with anti-FLAG or anti-myc beads at 4°C for 4 hr. Samples were resolved by standard immunoblotting techniques. For co-immunoprecipitation experiments with purified proteins, the immunoprecipitated samples were eluted with elution buffer (Thermal Scientific) and neutralized with Tris buffer (pH 9.0). For GFP-tagged proteins, anti-GFP antibody (Abcam) was incubated with the protein supernatant.
Cell fractionation

Worms with different genotypes were collected and washed in M9 buffer. 500 µl of lysis buffer (250mM sucrose, 50mM Tris-HCl with pH6.8, 1mM EDTA) were added and worm samples were homogenized with a Dounce homogenizer (Cheng-He Company, Zhuhai, China) (Chen et al., 2010) on ice for 15 minutes. The nuclear pellet was removed by centrifuging at 3,000 rpm for 10 minutes at 4°C. The supernatant was further centrifuged at 40,000 rpm for 1 hour. The new supernatant was collected as the cytosolic fraction. The pellet was further washed and centrifuged at 40,000 rpm for 45 minutes. All samples were mixed with 2xSDS loading buffer before 10% SDS-PAGE gel analysis. The GFP-RAB-3 and tubulin levels in each fraction were analyzed by standard western blotting procedures.

Microscopy and image analysis

Images were captured using a Plan-Apochromat 40X/1.4 objective on an Olympus confocal microscope. All images were taken at the young adult stage unless specifically indicated. Images were analyzed with custom Image J software. Two main parameters were determined: puncta number (PN) and synaptic enrichment (SE) (Ch'ng et al., 2008). These were calculated from the punctal fluorescence (PF), which measures the signal intensity at the pre-synaptic specialization, and the inter-punctal fluorescence (IPF), which measures the signal intensity in axons between synapses. An individual punctum is defined when the peak PF/average peak IPF is ≥2. Synaptic enrichment is defined as total PF/total IPF within a 100 µm length of middle dorsal cord region. All data are shown as mean ± SD. Statistical analyses were performed
with Student’s t-test. For each genotype, more than 20 animals were imaged and analyzed.

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Figure Legends

Figure 1. RAB-3 synaptic vesicle association requires SNT-1

(A) Punctate distribution of GFP::RAB-3 in C. elegans motor neurons in wild-type animals (top). The GFP::RAB-3 puncta become diffuse in aex-3, rep-1, hmg-1, and snt-1 mutants (lower panels). Yellow arrows indicate the cell bodies along the ventral cord. A representative line-scanning image for each genotype is shown in the right panel. (B) Quantification of the synaptic enrichment in wild-type, aex-3, rep-1, hmg-1, and snt-1 animals. Data are presented as mean ± SD; ** P<0.01. (C) Schematic representation of the RAB-3/SV association and dissociation cycle. Scale bar, 5 μm.

Figure 1-figure supplement 1. snt-1 is require for RAB-3 synaptic vesicle localization

(A) Multiple snt-1 alleles show the diffuse GFP::RAB-3 phenotype. White arrows
indicate the cell bodies. (B) GFP::RAB-3 expressed pan-neuronally under the control
of the Prab-3 promoter displays punctate distribution. (C) GFP::RAB-3 is diffuse in
all Prab-3-expressing cells. Red boxed areas are enlarged in panels underneath the
image taken from a whole animal. (D) The diffuse GFP::RAB-3 phenotype in snt-1
mutants is rescued by expressing wild-type snt-1 gene pan-neuronally using the
Psnt-1 promoter or in DD, VD, and AS neurons using the Phmr-1 promoter. Scale bar,
5 μm.

Figure 2. Synaptic vesicle clustering is unaffected by loss of snt-1 function
(A) SNB-1::GFP puncta distribution in wild type and snt-1 mutants. (B) The synaptic
enrichment of SNB-1::GFP puncta is indistinguishable in wild type and snt-1. Data
are presented as mean ± SD; NS, not significant. In both wild type (C) and snt-1 (D),
the SNB-1::GFP puncta are present in the synaptic area on the ventral cord, which is
outside of the cell body (C” and D”). In unc-104 (E) or snt-1 unc-104 double mutants
(F), SNB-1::GFP accumulates in the cell bodies on the ventral cord (E” and F”). (G)
In wild type, GFP::RAB-3 is distributed in a punctate pattern in the pre-synaptic
regions on the ventral cord (G”). (H) GFP::RAB-3 is diffuse throughout the whole
axon including both dorsal (H’) and ventral (H’’) processes. (I) GFP::RAB-3
accumulates in ventral cell bodies (I”). (J) In snt-1 unc-104 double mutants,
GFP::RAB-3 is diffuse throughout the whole axon in both dorsal (L’) and ventral (L’’)
regions. Yellow boxes indicate part of the dorsal cord, which is enlarged in the lower
left panels. Red boxes indicate part of the ventral cord, which is enlarged in the lower
right panels (white arrows indicate DD cell bodies in the ventral cord). A schematic
drawing of a DD neuron during the L1 stage is presented underneath the fluorescence
images of each genotype, with the SNB-1::GFP or GFP::RAB-3 signal shown in
green. Small green dots represent the pre-synaptic areas. Individual DD cell bodies
are indicated as large ovals at the bottom right of each diagram. Scale bars, 5 μm.

Figure 3. The GTP-bound form of RAB-3 is decreased in snt-1 mutants

(A) A GST-fused RBD domain of RIM2 binds active GTP-RAB-3. The amount of
GTP-RAB-3 pulled down by RBD is decreased in both aex-3 and snt-1 animals. (B)
Quantification of the GTP-RAB-3 level in wild type, aex-3, and snt-1. Data are
presented as mean ± SD; ** P<0.01. (C) The amount of GFP-RAB-3 in the cytosolic
fraction is increased in snt-1 mutants. (D) Localization of GFP::RAB-27 puncta is
affected by mutation of aex-3, but not by mutation of snt-1. The white arrows indicate
the cell bodies. (E) Over-expression of aex-3 does not rescue the snt-1 mutant
phenotype. Yellow arrows indicate the cell bodies. (F) The AEX-3::GFP level is
unchanged in snt-1 mutants compared to wild type. Scale bars, 5 μm.

Figure 4. RAB-3 GAP mutations suppress the snt-1 mutant phenotype

(A) The punctate distribution of GFP::RAB-3 is restored in rbg-1;snt-1 animals, while
the aex-3 phenotype could not be suppressed by mutation of rbg-1. Yellow arrows
indicate the cell bodies along the ventral cord. Scale bar, 5 μm. A representative
line-scanning image for each genotype is shown in the right panel. (B) Quantification
of the synaptic enrichment of GFP::RAB-3 signal in the genotypes shown in (A). Data
are represented as mean ± SD. ** P<0.01; NS, not significant. (C) The amount of
GTP-RAB-3 pulled down by RBD is increased in \textit{rbg-1;snt-1} animals compared to
\textit{snt-1}.

**Figure 4-figure supplement 1. \textit{rbg-2} suppresses the \textit{snt-1} mutant phenotype**
(A) Schematic representation of the \textit{rbg-1}(\textit{ok1660}) deletion mutation. (B) Schematic
representation of the \textit{rbg-2}(\textit{ok3195}) deletion mutation. Solid boxes indicate exons and
thin lines indicate introns. The bar below the gene indicates the deleted region. (C)
The enlarged GFP::RAB-3 puncta phenotype in \textit{rbg-1} mutants is rescued by
expressing a wild-type copy of the \textit{rbg-1} gene. (D) Quantification of the synaptic
enrichment of GFP::RAB-3 signal in the genotypes shown in (C). Data are
represented as mean ± SD; ** P<0.01. (E) Loss of \textit{rbg-2} function suppresses the \textit{snt-1}
mutant phenotype. White arrows indicate the cell bodies. (F) RAB-3 is co-precipitated
with RBG-1. Scale bars, 5 μm.

**Figure 5. Localization of RBG-1 on synaptic vesicles requires SNT-1**
(A and B) mCherry::RBG-1 has a punctate distribution in wild type (A) but
accumulates in cell bodies (yellow arrows) in \textit{unc-104} mutants (B). (C)
mCherry::RBG-1 (red) is co-localized with SNB-1::GFP puncta (green). (D) In \textit{snt-1}
mutants, mCherry::RBG-1 loses its punctate localization and becomes diffuse in
axons, while SNB-1::GFP retains its punctate pattern. (E) mCherry::RBG-1 retains its
punctate distribution and is co-localized with SNB-1::GFP in *rab-3* mutants. Scale bars, 5 μm.

**Figure 6. RBG-1 associates with the C2B domain of SNT-1**

(A) RBG-1 is precipitated by the intracellular domain (C2AB) of SNT-1. (B) The SNT-1 intracellular domain is precipitated by RBG-1. (C) The C2B domain of SNT-1 binds to RBG-1. (D) SNT-1 without the C2B domain fails to rescue the *snt-1* mutant phenotype. Yellow arrows indicate cell bodies. The schematic diagram shows the transmembrane (TM) and intracellular calcium-binding domains (C2A and C2B) of SNT-1. Scale bar, 5 μm.

**Figure 7. Dissociation of RAB-3 from synaptic vesicles requires the Ca$^{2+}$-binding activity of SNT-1**

(A) Ca$^{2+}$ treatment diminishes the binding between RBG-1 and the intracellular domain (C2AB) of SNT-1. (B) Quantification of the relative binding between RBG-1 and the C2AB domain upon Ca$^{2+}$ treatment. (C) SNT-1 without Ca$^{2+}$-binding sites (C2A*B*) still binds to RBG-1 in the presence of Ca$^{2+}$. (D) Quantification of the relative binding between RBG-1 and the C2A*B* domain upon Ca$^{2+}$ treatment. (E) Mutant SNT-1 proteins without Ca$^{2+}$-binding activity stabilize RAB-3 on SVs. *Ex snt-1*, over-expression of SNT-1; *Ex snt-1 C2A* and *Ex snt-1 C2B*, over-expression of SNT-1 with mutant C2A domain or C2B domain respectively; *Ex snt-1 C2A*B*, over-expression of SNT-1 with mutant C2A and C2B domains. Scale bar, 5 μm. (F)
Quantification of the synaptic enrichment in the genotypes shown in (E). (G) SNT-1 functions as a molecular switch controlling RAB-3/SV association and disassociation during SV exocytosis. Data are represented as mean ± SD. * P<0.05; ** P<0.01; NS, not significant.

Figure 7-figure supplement 1. Exocytosis is uncoupled from RAB-3 synaptic vesicle dissociation in snt-1 mutants

(A) The failure of RAB-3/SV dissociation caused by exocytosis mutants, including unc-2 and unc-13, is bypassed by mutation of snt-1. Yellow arrows indicate the cell bodies along the ventral cord. Scale bar, 5 μm. (B) Quantification of the synaptic enrichment in the genotypes shown in (A). Data are presented as mean ± SD. *

P<0.05; NS, not significant.
**Figure 1**

(A) GFP::RAB-3

Dorsal Ventral

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Fluorescence intensity at dorsal (AU)

(B) Synaptic enrichment

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(C) Schematic diagram

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Figure 2

(A) SNB-1::GFP

(B) Synaptic enrichment

(C) wt C' C'' snt-1 D' D''

(D) snt-1 unc-104 E' E'' F' F''

(E) unc-104 G' G''

(F) snt-1 unc-104 H' H''

(G) wt I' I''

(H) snt-1 unc-104 J' J''

(IS) SNB-1::GFP

(JS) GFP::RAB-3
Figure 3

A

<table>
<thead>
<tr>
<th>GST</th>
<th>GST-RBD</th>
<th>GFP-RAB-3</th>
<th>GFP-RAB-3</th>
<th>Pull-down</th>
<th>5% input</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aex-3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>snt-1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aex-3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>snt-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

GTP-RAB-3 level

wt | aex-3 | snt-1

**

C

Cyto Mem Cyto Mem

GFP-RAB-3 Tubulin

wt | snt-1

D

GFP::RAB-27

Dorsal Ventral

wt

aex-3

snt-1

E

GFP::RAB-3

Dorsal Ventral

wt

snt-1

snt-1; Paex-3::aex-3

F

AEX-3::GFP

Dorsal Ventral

wt

snt-1
Figure 4

A

GFP::RAB-3

Dorsal

Ventral

Fluorescence intensity at dorsal (AU)

B

Synaptic enrichment

C

Pull-down

Input

GST-RBD

GST

wt  snt-1  rbg-1

aex-3  ;snt-1

aex-3  rbg-1
Figure 5

mCherry::RBG-1

A Dorsal Ventral

wt

unc-104

B

C RBG-1

SNB-1

SNB-1 RBG-1

wt

D RBG-1

SNB-1

SNB-1 RBG-1

snt-1

E RBG-1

SNB-1

SNB-1 RBG-1

rab-3
Figure 6

A

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<th>IP: anti-Myc</th>
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<td>++</td>
</tr>
<tr>
<td>C2AB-Myc</td>
<td>-</td>
<td>++</td>
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<tr>
<td>anti-GFP</td>
<td>++</td>
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<tr>
<td>anti-Myc</td>
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B

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<tr>
<th></th>
<th>1% Input</th>
<th>IP: anti-Flag</th>
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</thead>
<tbody>
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<td>C2AB-Myc</td>
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<td>+</td>
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<tr>
<td>Flag-RBG-1</td>
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<td>+</td>
</tr>
<tr>
<td>anti-Myc</td>
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<td>-</td>
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<tr>
<td>anti-Flag</td>
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<td>+</td>
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</tbody>
</table>

C

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<th>5% Input</th>
<th>1% Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flag-RBG-1</td>
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<td>+</td>
</tr>
<tr>
<td>C2A-Myc</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C2B-Myc</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

D

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<th>Rescue</th>
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<tr>
<td>+</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

GFP::RAB-3

snt-1; Ex SNT

Dorsal

Ventral

snt-1; Ex SNT-1 deltaTM

snt-1; Ex SNT-1 deltaC2A

snt-1; Ex SNT-1 deltaC2B
Figure 7

A. 

\[
\begin{array}{c|c|c|c|c|c}
\text{Ca}^{2+} \text{(mM)} & - & - & 0.5 & 1 \\
\text{C2AB-Myc} & + & + & + & + \\
\text{Flag-RBG-1} & - & + & + & + \\
\end{array}
\]

IP: anti-Flag

1% Input

5% Input

B.

GFP::RAB-3

\[
\begin{array}{c|c|c|c}
0 & 0.5 & 1 \\
\end{array}
\]

RBG-1 and C2AB binding

C.

\[
\begin{array}{c|c|c|c|c|c}
\text{Ca}^{2+} \text{(mM)} & - & - & 0.5 & 1 \\
\text{C2A*B*-Myc} & + & + & + & + \\
\text{Flag-RBG-1} & - & - & + & + \\
\end{array}
\]

IP: anti-Flag

1% Input

5% Input

D.

GFP::RAB-3

\[
\begin{array}{c|c|c|c}
0 & 0.5 & 1 \\
\end{array}
\]

RBG-1 and C2A*B* binding

E.

\begin{tabular}{|c|c|}
\hline
\text{GFP::RAB-3} & \text{Dorsal} \\
\hline
\text{wt} & \text{---} \\
\hline
\text{snt-1} & \text{---} \\
\hline
\text{snt-1; Ex snt-1} & \text{---} \\
\hline
\text{snt-1; Ex snt-1 C2A*} & \text{---} \\
\hline
\text{snt-1; Ex snt-1 C2B*} & \text{---} \\
\hline
\text{snt-1; Ex snt-1 C2A*B*} & \text{---} \\
\hline
\end{tabular}

\begin{tabular}{|c|c|}
\hline
\text{GFP::RAB-3} & \text{Ventral} \\
\hline
\text{wt} & \text{---} \\
\hline
\text{snt-1} & \text{---} \\
\hline
\text{snt-1; Ex snt-1} & \text{---} \\
\hline
\text{snt-1; Ex snt-1 C2A*} & \text{---} \\
\hline
\text{snt-1; Ex snt-1 C2B*} & \text{---} \\
\hline
\text{snt-1; Ex snt-1 C2A*B*} & \text{---} \\
\hline
\end{tabular}

F.

Synaptic enrichment

\[
\begin{array}{c|c|c|c|c|c}
\text{wt} & 30 & 23 & 25 & 22 & 20 \\
\text{snt-1} & 30 & 23 & 25 & 22 & 20 \\
\text{Ex snt-1 C2A*} & 30 & 23 & 25 & 22 & 20 \\
\text{Ex snt-1 C2B*} & 30 & 23 & 25 & 22 & 20 \\
\text{Ex snt-1 C2A*B*} & 30 & 23 & 25 & 22 & 20 \\
\end{array}
\]

G.

Diagram showing Ca\(^{2+}\) influx and synaptic fusion events.