

UNC-18 Promotes Both the Anterograde Trafficking and Synaptic Function of Syntaxin

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The SM protein UNC-18 has been proposed to regulate several aspects of secretion, including synaptic vesicle docking, priming, and fusion. Here, we show that UNC-18 has a chaperone function in neurons, promoting anterograde transport of the plasma membrane soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein Syntaxin-1. In *unc-18* mutants, UNC-64 (*Caenorhabditis elegans* Syntaxin-1) accumulates in neuronal cell bodies. Colocalization studies and analysis of carbohydrate modifications both suggest that this accumulation occurs in the endoplasmic reticulum. This trafficking defect is specific for UNC-64 Syntaxin-1, because 14 other SNARE proteins and two active zone markers were unaffected. UNC-18 binds to Syntaxin through at least two mechanisms: binding to closed Syntaxin, or to the N terminus of Syntaxin. It is unclear which of these binding modes mediates UNC-18 function in neurons. The chaperone function of UNC-18 was eliminated in double mutants predicted to disrupt both modes of Syntaxin binding, but it was unaffected in single mutants. By contrast, mutations predicted to disrupt UNC-18 binding to the N terminus of Syntaxin caused significant defects in locomotion behavior and responsiveness to cholinesterase inhibitors. Collectively, these results demonstrate the UNC-18 acts as a molecular chaperone for Syntaxin transport in neurons and that the two modes of UNC-18 binding to Syntaxin are involved in different aspects of UNC-18 function.

INTRODUCTION

Neurotransmitter is released at synapses from a pool of recycling synaptic vesicles (SVs) in a highly regulated manner. SV exocytosis and endocytosis are mediated by several sequential steps, termed the SV cycle (Sudhof, 2004). SVs are first docked to the plasma membrane; subsequently, a subset of these vesicles become competent for calcium-evoked release through a process called priming. Several proteins that regulate SV docking and priming have been identified, including the plasma membrane soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein Syntaxin. SNARE proteins associated with the vesicle (*v*-SNAREs) and plasma membrane (*t*-SNAREs) form tightly associated complexes (termed SNARE complexes) that are thought to drive membrane fusion during exocytosis. Priming is thought to consist of SVs containing partially assembled SNARE complexes (Xu *et al.*, 1999). Recent studies suggest that Syntaxin is also required for docking of both secretory granules and SVs (de Wit *et al.*, 2006; Hammarlund *et al.*, 2007).

Syntaxin-1 adopts either an open or closed conformation. In the open conformation, the SNARE (H3) helix of Syntaxin-1 is available to associate with other *t*-SNARE (e.g., synaptosome-associated protein [SNAP]-25) and a *v*-SNARE (e.g., Synaptobrevin/vesicle-associated membrane protein

[VAMP]) proteins (Dulubova *et al.*, 1999). Expression of a constitutively open mutant form of Syntaxin (open-Syntaxin) promotes SV docking, priming, and fusion (Gracheva *et al.*, 2006; McEwen *et al.*, 2006; Hammarlund *et al.*, 2007). In the closed conformation, the SNARE helix of Syntaxin-1 interacts with the N-terminal regulatory domain of Syntaxin (the Habc helices), preventing formation of SNARE complexes, thereby decreasing SV priming and fusion rates.

Although SNARE complexes form spontaneously *in vitro*, and they can mediate fusion in reconstituted liposomes (Weber *et al.*, 1998), Syntaxin-binding proteins (e.g., Munc13 and Munc18) play critical roles in regulating SV priming and fusion *in vivo*. For example, mutants lacking Munc18 in worms, flies, and mice have a dramatic decrease in SV and secretory granule fusion, docking, and priming (Gengyo-Ando *et al.*, 1993; Harrison *et al.*, 1994; Verhage *et al.*, 2000; Voets *et al.*, 2001; Weimer *et al.*, 2003).

UNC-18, the *C. elegans* Munc18 orthologue, belongs to a family of proteins related to Sec1 in yeast and Munc18 in mammals known as SM proteins (Toonen and Verhage, 2003). SM proteins such as UNC-18 interact with Syntaxin family members through multiple independent binding modes. Munc18 forms a tight complex with closed-Syntaxin, which is proposed to inhibit priming and fusion (Hanson *et al.*, 1995; Dulubova *et al.*, 1999; Misura *et al.*, 2000). UNC-18 binding to closed-Syntaxin is not essential for secretion, because constitutively open-Syntaxin mutant proteins are able to mediate normal secretion (Richmond *et al.*, 2001).

SM proteins, e.g., Sly1 and Munc18, also interact with a short sequence near the N terminus of their cognate Syntaxins (Bracher and Weissenhorn, 2002; Dulubova *et al.*, 2007; Khvotchev *et al.*, 2007; Shen *et al.*, 2007). This N-terminal binding interaction allows SM proteins to interact with open-Syntaxin, thus permitting UNC-18/Munc18 to participate in SNARE complexes during SV priming and fusion.

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For the yeast SM protein Sly1, binding to the N terminus of its cognate Syntaxin (Sed5) is not required for Sly1 function (Peng and Gallwitz, 2004). This does not seem to be the case for the mammalian homologues of these proteins, because it has been shown that inhibition of rSly1 binding to the N terminus of Syntaxin-5 disrupts reconstituted endoplasmic reticulum (ER)-to-Golgi transport (Williams *et al.*, 2004). In addition, recent evidence suggests that the N-terminal binding mode may be important for the function of SM proteins regulating exocytosis. The SM proteins Sec1 and Munc18 stimulate SNARE-mediated fusion in reconstituted liposomes, and this stimulatory effect is greatly reduced by Syntaxin mutations that impair the N-terminal binding interaction (Scott *et al.*, 2004; Shen *et al.*, 2007). The functional importance of this N-terminal binding mode for Sec1 and Munc18 has not been addressed *in vivo*.

Munc18 has also been proposed to regulate Syntaxin delivery to the plasma membrane. When heterologously expressed in epithelial cells, neuronal Syntaxin-1 is retained in intracellular organelles, whereas delivery to the plasma membrane is greatly improved when Syntaxin-1 and Munc18 are coexpressed (Rowe *et al.*, 2001; Martinez-Arca *et al.*, 2003; Arunachalam *et al.*, 2007). These studies suggest that SM proteins may also function as chaperones, promoting delivery of Syntaxin to the plasma membrane. However, prior studies of worm and mouse mutants lacking Munc18 suggest that neuronal Syntaxin-1 is trafficked normally (Weimer *et al.*, 2003; Toonen *et al.*, 2005). Thus, it remains unclear whether UNC-18 regulates Syntaxin trafficking *in vivo*.

Here, we show that UNC-18 acts as a chaperone in neurons, promoting Syntaxin transport out of the ER. This chaperone function requires UNC-18 binding to Syntaxin-1; however, binding to either closed-Syntaxin or to the N terminus of Syntaxin was sufficient for proper trafficking. By contrast, UNC-18 binding to the N terminus of Syntaxin was required for normal locomotion behavior and neurotransmitter release.

MATERIALS AND METHODS

Strains

Strains were maintained at 20°C as described by Brenner (1974). The following strains were used in this study: N2 Bristol, KP4018 *unc-18(md299)*, CB0081 *unc-18(e81)*, CB02354 *unc-18(e234)*, RM0956 *ric-4(md1088)*, NM0467 *snb-1(md247)*, CB0102 *unc-10(e102)*, KP2410 *unc-13(s69)*, KP4078 *nuls159* [*Punc-129::SYD-2::GFP*], KP3928 *nuls165* [*Punc-129::UNC-10::GFP*], KP4491 *nuls226* [*Psnb-1::UNC-64::5N*], KP5390 [*Psnb-1::GFP::GOS28*], KP5416 [*Psnb-1::GFP::BET1*], KP5430 [*Psnb-1::GFP::VAMP-7*], KP5466 [*Psnb-1::GFP::VAMP-3*], KP5426 [*Psnb-1::GFP::ENDOBREVIN*], KP5456 [*Psnb-1::GFP::SNB-2*], KP5459 [*Psnb-1::GFP::VTI-1*], KP5472 [*Psnb-1::GFP::SNAP-23*], KP5387 [*Psnb-1::GFP::SNAP-29*], KP4495 *nuls227* [*Psnb-1::RFP::MEMBRIN*], KP4497 *nuls229* [*Psnb-1::RFP::SEC-22*], KP4498 *nuls230* [*Psnb-1::RFP::YKT6*], KP2380 *oxls34*, *unc64(js115)*, KP5396 [*Psnb-1::gfp::unc-64*], *unc-64(js115)*, KP5384 [*Psnb-1::gfp::unc-64*], *unc-64(js115)*; *unc-18(md299)*, KP5392 [*Psnb-1::gfp::unc-64(L9A)*]; *unc-64(js115)*, KP5425 [*Punc-129::gfp::unc-64*, *Punc-129::ssRFP-KDEL*], KP5397 [*Punc-129::gfp::unc-64(L9A)*, *Punc-129::ssRFP-KDEL*], KP5418 [*Punc-129::gfp::unc-64(Open)*, *Punc-129::ssRFP-KDEL*], KP5418 [*Punc-129::gfp::unc-64(L9A)*, *Open*], *Punc-129::ssRFP-KDEL*], KP5382 [*unc-18::FLAG*]; *unc-18(md299)*, KP5389 [*unc-18(R39C)::FLAG*]; *unc-18(md299)*, KP5394 [*unc-18(D34N)::FLAG*]; *unc-18(md299)*, KP5391 [*unc-18(L116K)::FLAG*], KP5460 [*unc-18(R39C, L116K)::FLAG*]; *unc-18(md299)*, and KP5461 [*unc-18(D34N, L116K)::FLAG*]; *unc-18(md299)*.

Immunolabeling

For immunolabeling experiments wild-type, *unc-18 (md299)*, *unc-18 (e81)*, *unc-18 (e234)*, *ric-4 (md1088)*, *snb-1 (md247)*, *unc-10 (e102)*, and *unc-13 (s69)* worms were fixed using Bouin's fixative and labeled with either antibodies to UNC-64 Syntaxin (1:500), RIC-4 SNAP-25 (1:500), SNB-1 Synaptobrevin (1:500), or the FLAG epitope (1:500) (Sigma M2 antibody; Sigma-Aldrich, St. Louis, MO) (Saifee *et al.*, 1998; Koushika *et al.*, 2001; Weimer *et al.*, 2003). Cyanine 5-, Alexa Fluor 488-, or Alexa Fluor 598-conjugated secondary and tertiary antibodies were then applied at a concentration of 1:500, and animals were mounted and imaged.

Constructs and Transgenes

UNC-64 cDNA was generated by polymerase chain reaction (PCR) amplification of *unc-64a* from wild-type *Caenorhabditis elegans* cDNA with KpnI-flanked primers and inserted into pPD49.26. GFP::UNC-64 was generated by inserting a NotI site after the ATG of *unc-64a* cDNA and subcloning a NotI-flanked green fluorescent protein (GFP). The UNC-64::5N (5X N-glycosylation site) construct was made by first adding a BspEI endonuclease restriction site before the stop codon (TAA) of the *unc-64a* cDNA and cloning it into the KpnI site of pBluescript. The N-glycosylation sites were preceded by a flexible linker so that each added N-glycosylation site was coded with SG-4X(GGS)-DELYKYGGNGSGQHGYDQ. This was done by annealing the two oligonucleotides (oligos) encoding this fragment: 5'-ccggTGGTTCGGTTC-CGGTGGTTCGGTTCGGTTCgacgagctgtacaagtattgaggaaacggctccggtcagcaccagatgatcatg-3' and 5'-CCGGActgatcatcagctggtcagcggagcggcttctccatcctgtacagctcgtcGGAACCCACCGGAACCCACCGGAACCCACCGAACCACCA-3'.

After annealing, the oligos were cloned into the BspEI site added to the *unc-64a* cDNA. Although the annealed oligos contain overlaps compatible with BspEI ligation, the 5' site is rendered noncleavable by a base pair change encoded in the oligo and leaving the BspEI site at the 3' intact. This ligation was repeated five times to add five N-glycosylation sites to create UNC-64::5N. UNC-64::5N was then subcloned using flanking KpnI sites into pPD::49.26 that contained the SNB-1 promoter cloned into the SphI/BamHI sites. GFP was then added after the ATG of UNC-64::5N by using a NotI site that was added during the initial PCR amplification of the cDNA. The final *Psnb-1::GFP::UNC-64::5N* construct was injected into wild-type worms and integrated using a *uv* cross-linker to generate the strain *nuls226*.

SNARE GFP/red fluorescent protein (RFP) fusions were generated by PCR amplification from a whole worm cDNA library and cloned into pPD::49.26 containing the SNB-1 promoter cloned into the SphI/BamHI sites. The 5' primer for each SNARE added a NotI restriction site added in frame after the ATG of each gene. Each SNARE was cloned using either KpnI flanking both sides of the cDNA or KpnI/SacI. GFP or RFP was added in frame using the added NotI site. Each GFP/RFP SNARE was then injected into *unc-18(md299)* worms and backcrossed into wild type. Three VAMP-3 homologues, C30A5.5, ZK795.4, and T14D7.3 (Supplemental Table 1) share 99 and 88% identity in amino acid sequence, respectively, including identical SNARE interfaces, and they may have resulted from recent gene duplication events. Thus, just one member of this group (C30A5.5) was included in this analysis.

Imaging

Fluorescent imaging was performed using an Axiovert 100 microscope (Carl Zeiss, Thornwood, NY) and PlanApo 100× (numerical aperture [NA] = 1.4) objective (Olympus, Tokyo, Japan) equipped with FITC/GFP or RFP filters and an ORCA charge-coupled device camera (Hamamatsu, Bridgewater, NJ). Antibody-stained animals were mounted on agarose pads and imaged. Live animals were immobilized with 30 mg/ml 2,3-butanedione monoxime (BDM; Sigma-Aldrich). Image stacks were captured, and maximum intensity projections were obtained using MetaMorph 4.5 software (Molecular Devices, Sunnyvale, CA). Identical camera gain, exposure settings, and fluorescence filters were used for all live animal imaging.

Fluorescent imaging of neuronal cell bodies and rescue of *unc-18* trafficking defects were performed on an Olympus Fluoview FV1000 confocal laser scanning microscope by using an Olympus PlanApo N 60× objective (NA = 1.45). Animals imaged for neuronal cell bodies were immobilized with 30 mg/ml BDM (Sigma-Aldrich). Z-stacks were taken of the entire cell, and a representative single plane was selected. For colocalization analysis, images were analyzed with MetaMorph analysis software. Images were thresholded to remove background fluorescence. Antibody stained animals were mounted on agarose pads and imaged. Image stacks were captured and maximum intensity projections were obtained using Olympus Fluoview software.

Endoglycosidase H (Endo H) and Peptide-N⁺-(N-acetyl-β-glucosaminyl)asparagine Amidase (PNGase F) Treatment and Western Blot Analysis

For Endo H and PNGase F assays, lysates were made by boiling worms in 5× denaturing buffer (2.5% SDS and 5% beta mercaptoethanol) followed by sonication. Lysates were then diluted to 1× denaturing buffer in water plus protease inhibitors (10 μg/ml leupeptin, 5 μg/ml chymostatin, 3 μg/ml elastinal, 1 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). Digests were then performed as recommended by the enzyme manufacturer (New England Biolabs, Ipswich, MA). Treated and untreated lysates were run on SDS denaturing gels. Western blots were performed using an UNC-64 Syntaxin polyclonal antibody (gift from Mike Nonet, Washington University, St. Louis, MO). Quantitative imaging of Western blots was performed using a Typhoon Trio Plus variable mode imager and Image Quant TL v2005 software (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Each individual sample was analyzed by measuring the Endo H treated lane alone. Total protein detected by the UNC-64 antibody was measured for each Endo H-treated lane. Endo H sensitivity was then analyzed by measuring protein levels of the digested product as a percentage of total protein. The

digested product area was measured on the blot as an area equivalent to a PNGase sample loaded in an adjacent lane.

RESULTS

*UNC-64 Syntaxin-1 Is Localized to Neuronal Cell Bodies in *unc-18* Mutants*

When heterologously expressed in epithelial cells, Munc18 promotes anterograde delivery of neuronal Syntaxin-1 to the plasma membrane (Rowe *et al.*, 2001; Martinez-Arca *et al.*, 2003). These results suggest that Munc18 may act as a chaperone for Syntaxin trafficking. To determine whether UNC-18 plays a similar chaperone function in neurons of intact animals, we stained *unc-18* loss of function mutants with an antibody directed against the *C. elegans* Syntaxin-1 orthologue UNC-64. In wild-type animals, bright uniform anti-UNC-64 immunostaining was observed in all neuronal processes, with a small number of dimly stained neuronal cell bodies. By contrast, significantly increased cell body anti-UNC-64 immunostaining was observed in mutants carrying any of three *unc-18* alleles (*md299*, *e81*, and *e234*) (Figure 1, A and B; data not shown). To quantify this defect, we counted the number of cell bodies stained by anti-UNC-64 in the ventral cord. Fifty-eight cell bodies are present in the ventral nerve cord (White *et al.*, 1976). In both *unc-18(md299)* (48 cell bodies) and *unc-18(e81)* (49 cell bodies) there was a significant increase in the number of visible cell bodies compared with wild type (18 cell bodies) ($p = 1.45 \times 10^{-10}$ and $p = 3.91 \times 10^{-12}$, respectively) (Figure 1B). The cell bodies of *unc-18* mutants also had much brighter staining than was observed in wild-type controls. These results suggest that *unc-18* mutant neurons have a defect in anterograde transport of Syntaxin out of neuronal cell bodies.

The apparent defect in UNC-64 trafficking observed in *unc-18* mutants could be a secondary consequence of decreased neurotransmitter secretion. To test this possibility, we analyzed UNC-64 localization in mutants lacking UNC-13 Munc13, UNC-10 RIM1, RIC-4 SNAP-25, and SNB-1 Synaptobrevin-1, all of which have severe neurotransmitter secretion defects (Nonet *et al.*, 1998; Richmond *et al.*, 1999; Koushika *et al.*, 2001). In all of these mutants, UNC-64 distribution was indistinguishable from wild-type controls, i.e., bright diffuse fluorescence in nerve cords and weak fluorescence in a few neuronal cell bodies (Figure 1, A and B). Therefore, the UNC-64-traffic defect was specific to *unc-18* mutants.

In previous studies, the cell body retention of UNC-64 Syntaxin-1 in *unc-18* mutants was not reported (Weimer *et al.*, 2003). To confirm that our result was not an artifact of differing fixation or staining protocols, we also analyzed the localization of GFP-tagged UNC-64 Syntaxin-1 (GFP::UNC-64), expressed in the cholinergic DA motor neurons. Bright GFP::UNC-64 fluorescence was distributed along the ventral and dorsal nerve cord processes, and dim fluorescence was observed in DA neuron cell bodies. In *unc-18(md299)* mutants, GFP::UNC-64 fluorescence was greatly increased in DA cell bodies, forming a fluorescent ring encircling the nucleus (Figure 1C). This change in GFP::UNC-64 localization was specific to *unc-18* mutants as cell body accumulation was not seen in other synaptic mutants (data not shown). Thus, *unc-18* mutants had decreased anterograde trafficking of both endogenously expressed UNC-64 (in fixed animals) and transgenically expressed GFP-tagged UNC-64.

*Other SNARE Proteins Are Properly Localized in *unc-18* Mutants*

The UNC-64 trafficking defect seen in *unc-18* animals may disrupt the distribution of other SNARE proteins that associate with UNC-64, e.g., the v-SNARE Synaptobrevin-1

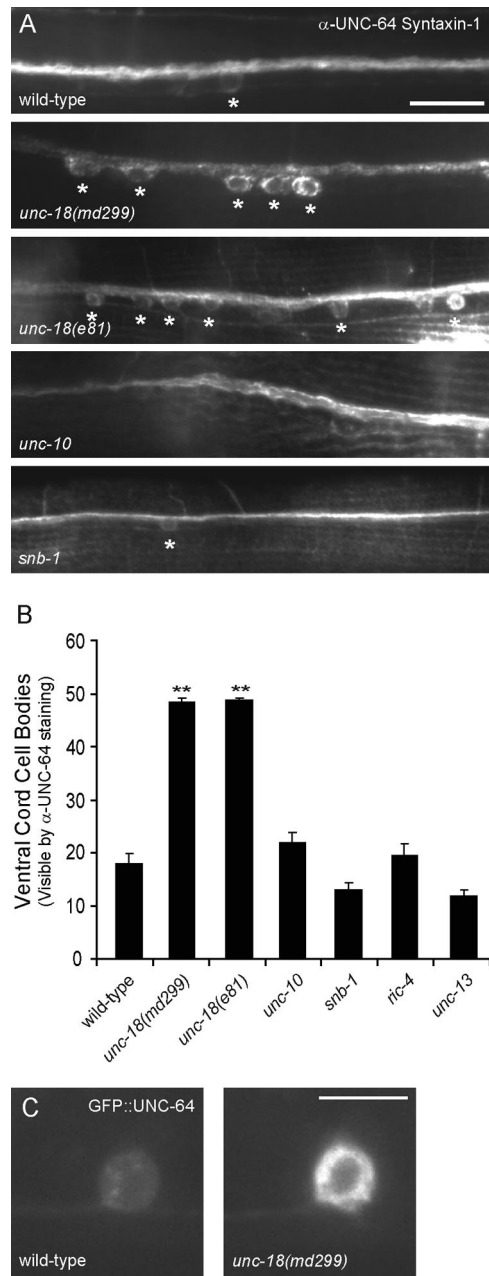


Figure 1. Syntaxin-1 accumulates in cell bodies of *unc-18* mutants. Animals were Bouin's fixed and stained with anti-UNC-64 Syntaxin-1 antibodies. (A) In wild-type animals, all neuronal processes are labeled and a small number of cell bodies can be seen. In *unc-18(md299)* and *unc-18(e81)* mutants, cell bodies accumulate UNC-64 brightly and can be easily visualized. Other synaptic mutants, *unc-10(e102)* and *snb-1(md247)* have similar staining patterns as wild type. (B) Quantification of the number of visible cell bodies along the ventral nerve cord of animals ($n = 20$ for each genotype). (C) GFP::UNC-64 Syntaxin-1 expressed under the UNC-129 promoter in the DA motor neurons. In wild-type animals, GFP::UNC-64 is diffuse. In *unc-18(md299)* GFP::UNC-64 accumulates in cell bodies. Bars, 10 μ m. Values that differ significantly from wild type based on the Student's *t* test, ** $p < 0.001$. Error bars represent SEM.

(SNB-1) and the t-SNARE SNAP-25 (RIC-4). To test this possibility, we examined trafficking of these proteins by immunostaining with antibodies directed against SNB-1 Synaptobrevin-1 and RIC-4 SNAP-25. In wild-type animals,

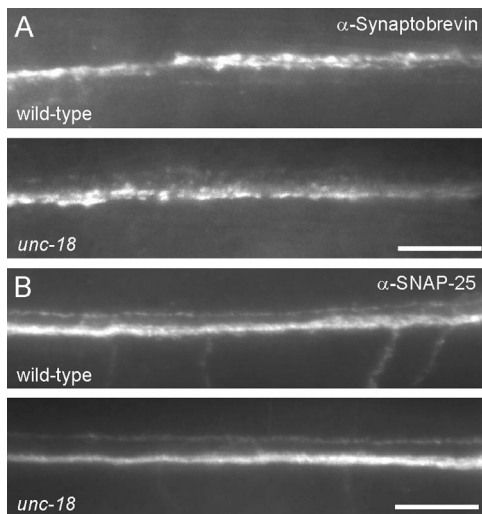


Figure 2. Localization of other presynaptic SNAREs is UNC-18 independent. Wild-type and *unc-18(md299)* animals were Bouin's fixed and stained with antibodies against SNB-1 Syntaptobrevin-1 (A) and RIC-4 SNAP-25 (B). The distribution of both SNARE proteins was unaffected in *unc-18 (md299)* animals. Bars, 10 μ m.

SNB-1 Syntaptobrevin-1 staining was punctate throughout all neuronal processes with little cell body staining (Figure 2A). RIC-4 SNAP-25 was diffusely localized in the nerve cords, and was also absent from cell bodies (Figure 2B). In *unc-18(md299)* mutants, both SNB-1 Syntaptobrevin-1 and RIC-4 SNAP-25 were localized normally, showing no notable cell body staining (Figure 2, A and B). These results demonstrate that UNC-64 Syntaxin-1 is the only presynaptic SNARE protein that is trafficked in an UNC-18-dependent manner.

SM proteins have been proposed to regulate the specificity of SNARE pairing (Martinez-Arca *et al.*, 2003). Thus, in *unc-18* mutants, other SNAREs may bind to UNC-64, forming noncognate SNARE complexes. In this scenario, the distribution of other SNARE proteins would be altered in *unc-18* mutants. To test this possibility, we constructed tagged proteins containing GFP or RFP fused to the N terminus of 12 other SNARE proteins (Supplemental Table 1). Each tagged SNARE was expressed in all neurons by using the *snb-1* promoter.

The GFP/RFP::SNARE fusions varied in their localization, consistent with the localization of the orthologous SNARE proteins in other organisms. Punctate localization in cell bodies was seen for Vti-1, Sec-22, Ykt-6, Gos-28, and Membrin, consistent with their predicted localization to the Golgi (Hay *et al.*, 1996; Volchuk *et al.*, 2004). The Bet-1 orthologue was primarily restricted to cell bodies, consistent with the predicted intermediate compartment localization. The VAMP-8 Endobrevin homologue was punctate in nerve cords, consistent with the localization of other endosome markers (Advani *et al.*, 1998; Sieburth *et al.*, 2005). The SNB-2 orthologue showed punctate localization similar to what is seen for the close homologue SNB-1. None of these tagged SNARE proteins accumulated in cell bodies of *unc-18* mutants (Figure 3). Thus, *unc-18* mutants did not have a generalized defect in the trafficking of SNARE proteins.

Active Zone Proteins Were Properly Localized in *unc-18* Mutants

Syntaxin-1 is delivered to presynaptic elements in transport vesicles that contain other active zone components

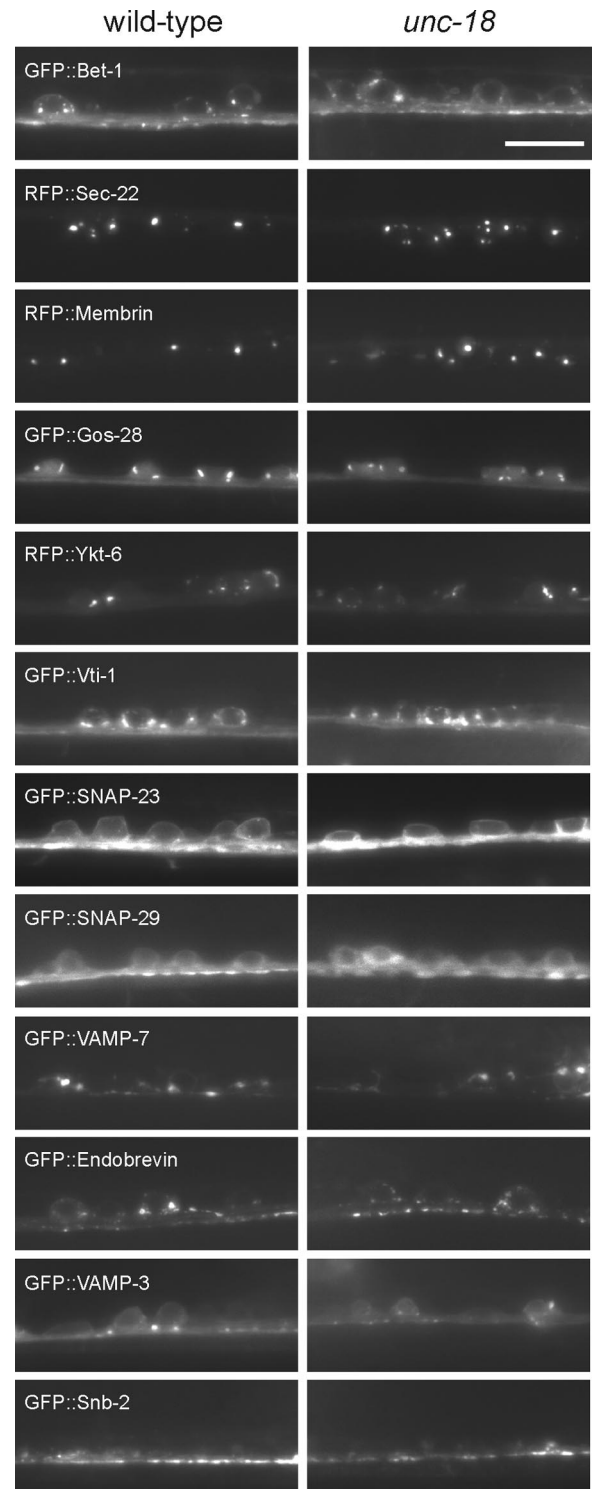


Figure 3. Most *C. elegans* SNARE proteins are trafficked normally in *unc-18* mutants. SNARE proteins were tagged with either GFP or RFP and then expressed under the pan-neuronal *snb-1* promoter. Fluorescently tagged SNARE proteins were then compared in wild-type versus *unc-18(md299)* mutant animals. There was no obvious trafficking defect for the 12 SNAREs analyzed in *unc-18* mutants. Bar, 10 μ m.

(Zhai *et al.*, 2001); consequently, we wondered whether trafficking of active zone proteins was altered in *unc-18* mutants. To test this idea, we analyzed the distribution of

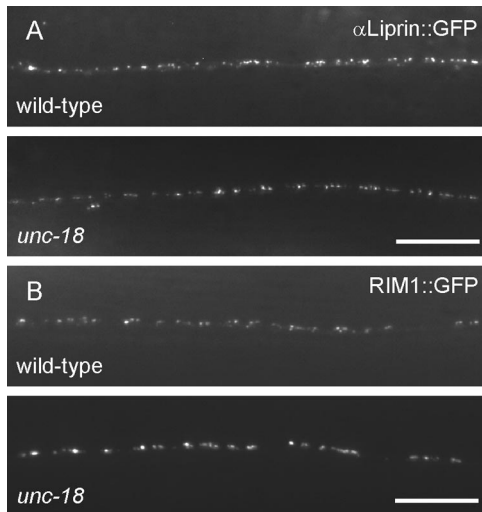


Figure 4. Active zone proteins α Liprin and RIM1 are localized normally in *unc-18* mutants. GFP tagged SYD-2 α Liprin and UNC-10 RIM1 were expressed under the UNC-129 promoter in a subset of motor neurons. (A) SYD-2 α Liprin::GFP is localized to presynaptic active zones along the dorsal nerve cord and is not changed in *unc-18* (*md299*) compared with wild-type animals. (B) UNC-10 RIM1::GFP has a similar presynaptic distribution and is not changed in *unc-18* (*md299*) compared with wild-type animals. Bars, 10 μ m.

GFP-tagged SYD-2 α Liprin and UNC-10 RIM1 (Zhen and Jin, 1999; Koushika *et al.*, 2001). Both proteins were expressed in a subset of motor neurons and imaged in the dorsal cord to look for changes in presynaptic distribution and in the ventral cord to assess cell body accumulation. The distribution of SYD-2::GFP and UNC-10::GFP in the nerve cords and in cell bodies of *unc-18*(*md299*) mutants was similar to wild-type animals (Figure 4, A and B; data not shown). These results suggest that active zone proteins are localized properly in *unc-18* mutants, consistent with previous ultrastructural studies that found morphologically normal presynaptic densities in *unc-18* mutants (Weimer *et al.*, 2003).

Syntaxin-1 Accumulates in the Endoplasmic Reticulum of *unc-18* Animals

In heterologous cells, Syntaxin-1 accumulates in either the ER or Golgi (Martinez-Arca *et al.*, 2003; Liu *et al.*, 2004). We wanted to determine where in the secretory pathway the UNC-64 Syntaxin-1 trafficking defect occurs in *unc-18* mutants. The susceptibility of N-glycans to hydrolysis by Endo H is often used to assess the distribution of secreted proteins in the ER and Golgi. Simple carbohydrate side chains found in the ER are sensitive to Endo H, whereas complex chains found in the Golgi are Endo H-resistant. UNC-64 has a short luminal domain lacking N-glycosylation sites. To assay Endo H sensitivity, we constructed a chimeric UNC-64 protein containing five luminal N-glycosylation sites (GFP::UNC-64::5N). When expressed in all neurons with the *snb-1* promoter, GFP::UNC-64::5N rescued the lethality of the *unc-64*(*js115*) null mutant; however, the locomotion defects were only partially rescued (data not shown). Thus, addition of the five glycosylation sites did not eliminate the UNC-64 function.

An integrated transgene expressing GFP::UNC-64::5N (*nuls226*) was expressed in *unc-18*(*md299*) mutants and wild-type controls. Heterogeneous GFP::UNC-64::5N bands were detected in Western blots, whereas a single \sim 80-kDa band

was observed after pretreatment with the glycosidase PNGase F, which removes both simple and complex N-glycans (Figure 5). The \sim 80-kDa band observed after PNGase F treatment had the expected mobility for the unmodified GFP::UNC-64::5N protein. In wild-type animals, $26 \pm 6.3\%$ of GFP::UNC-64::5N was Endo H sensitive. Similar results were observed in *ric-4*(*md1088*) mutants ($29 \pm 2.5\%$ Endo H sensitive). By contrast, in *unc-18*(*md299*) mutants, a significantly higher fraction of GFP::UNC-64::5N was Endo H sensitive ($49 \pm 2.7\%$), consistent with an approximately twofold increase in the amount of GFP::UNC-64::5N retained in the ER.

To confirm that UNC-64 accumulates in the ER in *unc-18* mutants, we measured colocalization of GFP::UNC-64 (lacking N-glycosylation sites) with an ER marker, secreted RFP tagged with an ER retention signal (ssRFP::KDEL). Secreted proteins containing the KDEL sequence are constitutively recycled back to the ER by COPI-mediated retrograde transport (Munro and Pelham, 1987; Pidoux and Armstrong, 1992; Terasaki *et al.*, 1996). We expressed both GFP::UNC-64 and ssRFP::KDEL in the cholinergic DA motor neurons, by using the *unc-129* promoter. In wild-type animals, GFP::UNC-64 was localized primarily to the periphery of the cell bodies, with only $30 \pm 1.7\%$ colocalization with ssRFP::KDEL (Figure 5, C and E). These results suggest that GFP::UNC-64 was efficiently delivered to the plasma membrane in wild-type neurons. In *unc-18*(*md299*) mutants, significantly more GFP::UNC-64 colocalized with ssRFP::KDEL ($67 \pm 1.2\%$, $p < 0.001$) (Figure 5, C–E), and this was accompanied by a reduction in GFP::UNC-64 fluorescence at the cell periphery. To determine whether there was a corresponding decrease in GFP::UNC-64 levels in axons, we imaged wild-type and *unc-18*(*md299*) animals in the dorsal nerve cord (DNC) in which DA motor neurons presynapses are formed. We found that there was an accompanying decrease in DNC fluorescence in *unc-18*(*md299*) mutants ($63 \pm 3\%$; $p < 0.01$) (Figure 5, F–H). Thus, the ER accumulation of UNC-64 in *unc-18* mutants was accompanied by a significant decrease in the abundance of UNC-64 in dorsal cord axons. Together, these studies indicate that UNC-64 accumulates in the ER in *unc-18* mutants, resulting in decreased anterograde trafficking of UNC-64 to axons.

UNC-18 Binding to UNC-64 Is Required for Anterograde Transport Out of the ER

Thus far, our results suggest that UNC-18 is required for efficient trafficking of UNC-64 out of the ER. A simple explanation for these results would be that UNC-18 associates with UNC-64 at the ER, and promotes its anterograde transport. To test this idea, we examined the effects of *unc-18* point mutations that are predicted to disrupt UNC-64 binding. Munc18 proteins bind to Syntaxin-1 via two modes: binding to closed-Syntaxin through extensive contacts with all four helices, and binding to a short N-terminal sequence on Syntaxin (Misura *et al.*, 2000; Shen *et al.*, 2007). To disrupt binding to closed-Syntaxin, we used two previously described missense mutants (R39C and D34N) (Supplemental Table 2), which disrupt Munc18 binding to closed Syntaxin-1 (Wu *et al.*, 1998; Ciufo *et al.*, 2005). To disrupt the second binding mode, we introduced a charged residue into a hydrophobic pocket (L116K) that mediates binding to the N terminus of Syntaxin. An analogous mutation in the SM protein Sly1 disrupted N-terminal binding to its cognate Syntaxin (Sed5) (Peng and Gallwitz, 2004). Each UNC-18 transgene was tagged with the FLAG epitope at the C terminus, to control for expression levels. All three UNC-18 mutants (R39C, D34N, and L116K) were able to rescue the

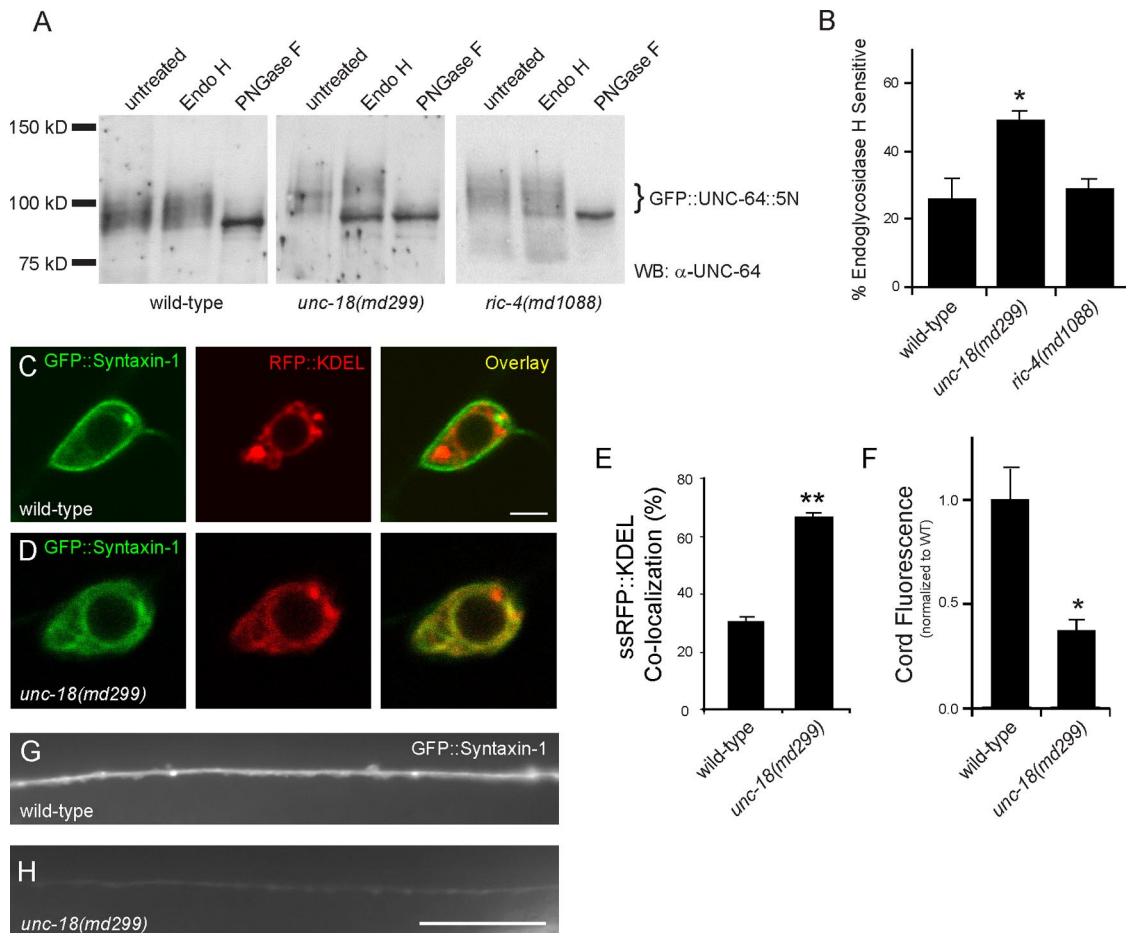


Figure 5. An N-glycosylated Syntaxin-1 accumulates in the ER in *unc-18* mutants. (A) Lysates of GFP::UNC-64::5N expressing worms were untreated or treated with either Endo H or PNGase F and analyzed by Western blot with anti-UNC-64 Syntaxin-1 antibodies. In all untreated lysates, GFP::UNC-64::5N occurs as a series of bands ranging from 80 to 100 kDa. PNGase treatment results in a single band near 80 kDa, demonstrating efficient N-glycosylation of GFP::UNC-64::5N. In *unc-18(md299)* animals, there is an increase in the amount of GFP::UNC-64::5N that is sensitive to the enzyme Endo H, indicating a larger fraction of GFP::UNC-64::5N present in pre-Golgi compartments compared with wild type and *ric-4(md1088)* controls. (B) Summary data of quantitative Western blots for each genotype indicated (n = 5 per genotype) shows that in *unc-18* mutants there is a twofold increase in the Endo H-sensitive fraction of GFP::UNC-64::5N. (C and D) GFP::UNC-64 was expressed under the UNC-129 promoter in a subset of motor neurons along with ssRFP::KDEL to determine colocalization with the ER compartment. (C) GFP::UNC-64 expressed in wild-type animals is mostly localized to the cell periphery separate from ssRFP::KDEL. (D) In *unc-18(md299)* mutant animals, GFP::UNC-64 has increased colocalization with ssRFP::KDEL and decreased abundance at the cell periphery. Bars, 2 μ m. (E) Quantification of colocalization of GFP::UNC-64 and the amount of overlap with ssRFP::KDEL is shown (n = 10). (F) Quantification of GFP::UNC-64 fluorescence levels along the dorsal nerve cord is shown (n = 10). (G and H) GFP::UNC-64 imaged along the dorsal nerve cord. (G) Mostly diffuse GFP::UNC-64 is seen along the nerve cord in wild-type animals. (H) In *unc-18(md299)* animals, GFP::UNC-64 fluorescence is decreased along the nerve cord. Bar, 10 μ m. Values that differ significantly from wild type, *p < 0.01, Student's *t* test. Values that differ significantly from wild-type animals, **p < 0.001, Student's *t* test. Error bars represent SEM.

UNC-64-trafficking defect of *unc-18(md299)* mutants (Figure 6, A–D). By contrast, two double mutants predicted to disrupt both binding modes (R39C/L116K and D34N/L116K) failed to restore normal trafficking of endogenously expressed UNC-64. Both double mutant UNC-18 proteins were well expressed, as documented by anti-FLAG immunostaining. These results suggest that anterograde trafficking of endogenously expressed UNC-64 was dependent upon binding to UNC-18 and either mode of binding was sufficient for normal trafficking.

These results do not exclude the possibility that the defective UNC-64 trafficking observed in the *unc-18* mutants was caused by defects in other aspects of UNC-18 function (including failure to bind proteins other than UNC-64). To address this possibility, we constructed a parallel set of UNC-64 mutants predicted to disrupt both binding modes

to UNC-18. A double mutation, L166A/E167A, produces a constitutively open-UNC-64 protein, thereby preventing high-affinity UNC-18 binding to closed-Syntaxin (Dulubova *et al.*, 1999; Richmond *et al.*, 2001). The N terminus of UNC-64 contains a putative UNC-18 binding motif (KDRXXXL) that is conserved in all Syntaxin-1 orthologues (Rickman *et al.*, 2007; Shen *et al.*, 2007). In Syntaxin-4, the conserved leucine residue of this motif packs tightly into a hydrophobic pocket of Munc18c (Hu *et al.*, 2007). To disrupt UNC-18 binding to the N terminus of UNC-64, we mutated this leucine to alanine [UNC-64(L9A)], a mutation that has been shown to disrupt binding of Munc18 to the N terminus of Syntaxin-1 (Shen *et al.*, 2007; Burkhardt *et al.*, 2008). The cell body fluorescence and colocalization with ssRFP-KDEL of GFP::UNC-64(open), and GFP::UNC-64(L9A) alone, was similar to that observed for wild-type GFP::UNC-64 (33.9 \pm

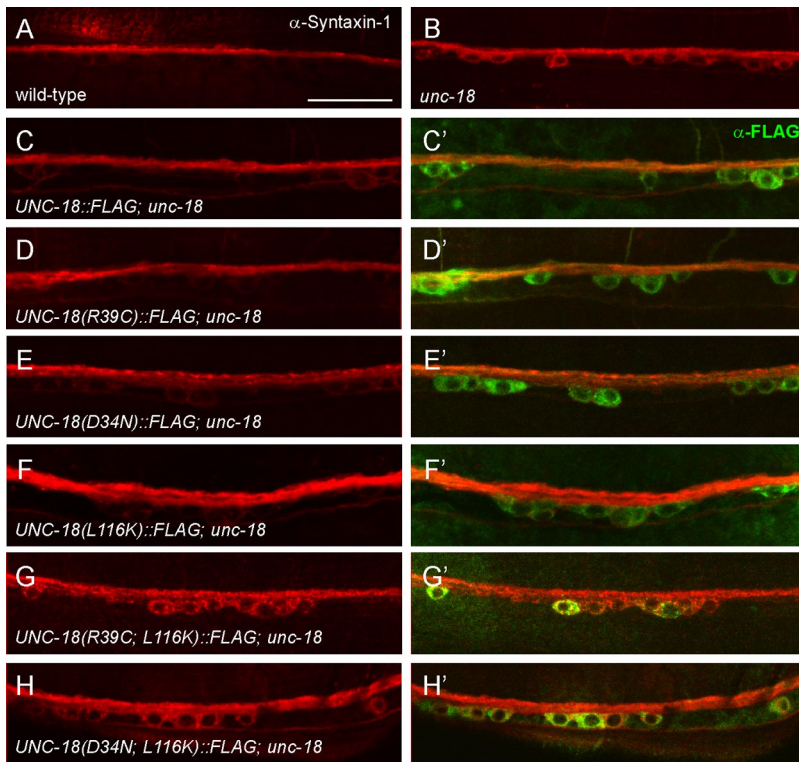


Figure 6. Rescue of endogenous UNC-64 trafficking with UNC-18::FLAG and UNC-18::FLAG mutants. Fixed animals were immunostained with anti-UNC-64 Syntaxin-1 antibodies (Red). (A) Wild-type animals stained with anti-UNC-64 show very few visible cell bodies. (B) *unc-18(md299)* mutant animals show an accumulation of UNC-64 in the cell bodies. (C–H) *unc-18(md299)* mutant animals carrying a transgenic array expressing either wild-type UNC-18::FLAG or point mutants. (C'–H') Anti-FLAG staining to show expression of UNC-18::FLAG constructs (Green). UNC-64 transport was rescued by transgenes expressing wild-type UNC-18 (C), UNC-18(R39C) (D), UNC-18(D34N) (E), and UNC-18(L116K) (F). Rescue was not observed with transgenes expressing the double mutants UNC-18(R39C; L116K) (G), and UNC-18(D34N; L116K) (H). Bars, 10 μ m.

2.8 and $32.9 \pm 1.6\%$, respectively) (Figure 7, A, C, and F). However, when both binding modes were disrupted using the GFP::UNC-64(open; L9A) double mutant protein, significantly increased colocalization with ssRFP::KDEL was observed ($65.2 \pm 4.4\%$) (Figure 7, E and F). These results suggest that binding of UNC-18 to Syntaxin-1 promotes ER exit, but either binding mode is sufficient.

The N-terminal Binding Mode Is Required for UNC-18 Function in Neurotransmitter Secretion

Which binding modes are required for UNC-18 function in neurotransmitter secretion? To answer this question, we analyzed the effect of mutations disrupting the two binding modes on rescue of locomotion behavior, and on rescue of the aldicarb resistance phenotype. Sensitivity of animals to paralysis induced by the cholinesterase inhibitor aldicarb has been used as a measure of acetylcholine release (ACh) at neuromuscular junctions (Miller *et al.*, 1996). For example, the decrease in SV fusion that occurs in *unc-18* mutants is accompanied by increased resistance to aldicarb-induced paralysis (Hosono *et al.*, 1992). To test the importance of UNC-18 binding to closed Syntaxin, we used the UNC-18(R39C) and UNC-18(D34N) mutations and UNC-64(open). To test the importance of the N-terminal binding mode, we used the UNC-18(L116K) and UNC-64(L9A) mutations. Each mutant UNC-18 construct was expressed in *unc-18(md299)* null mutants, and each UNC-64 construct was expressed in *unc-64(js115)* null mutants. The locomotion behavior and aldicarb sensitivity of the resulting transgenic strains were then analyzed (Figure 8). Although the *unc-64(js115)* null mutants are inviable, this lethal phenotype was rescued by expression of the mutant UNC-64(L9A) transgene, allowing us to assay the locomotion and aldicarb responsiveness of these transgenic strains.

All three mutations predicted to disrupt UNC-18 binding to closed-Syntaxin restored normal locomotion rates and

significantly increased sensitivity to aldicarb-induced paralysis (Figure 8, A and C–E). This is consistent with prior studies showing that expression of open-Syntaxin restore relatively normal patterns of synaptic transmission to the *unc-64* null mutants (Richmond *et al.*, 2001). These results suggest that UNC-18 binding to closed-Syntaxin is not required to maintain a normal level of neurotransmitter secretion as measured in these assays. By contrast, the UNC-64(L9A) mutation caused locomotion and aldicarb resistance defects that were similar to those observed in *unc-18* single mutants (Figure 8, B and E). The UNC-18(L116K) mutation produced relatively normal locomotion rates, and it caused a more modest decrease in aldicarb-induced paralysis, perhaps due to a less significant disruption of the N-terminal binding mode (Figure 8, A and D). The locomotion and aldicarb defects observed for UNC-18 double mutants containing mutations disrupting both modes of binding (i.e., R39C, L116K and D34N, L116K) were similar to those observed in *unc-18* null mutants, as would be predicted if disrupting binding to closed-Syntaxin can enhance defects caused by weak mutations in the N-terminal binding mode (Figure 8, A and D). Collectively, these results suggest that the N-terminal binding mode is required for UNC-18 function in ACh secretion, whereas binding to closed-Syntaxin may have a more subtle function.

The locomotion and aldicarb responsiveness defects caused by the UNC-64(L9A) mutation were similar to those observed in *unc-18* mutants. These results suggest that UNC-18 binding to the N terminus of UNC-64 is required for ACh secretion; however, these results do not exclude the idea that the L9A mutation disrupts UNC-64 interactions with other proteins required for synaptic function. To address this issue, we analyzed UNC-64(L9A); *unc-18* double mutants. If the synaptic defects observed in the UNC-64(L9A) mutants are caused by disrupting interaction with UNC-18, we would expect that the L9A and *unc-18* muta-

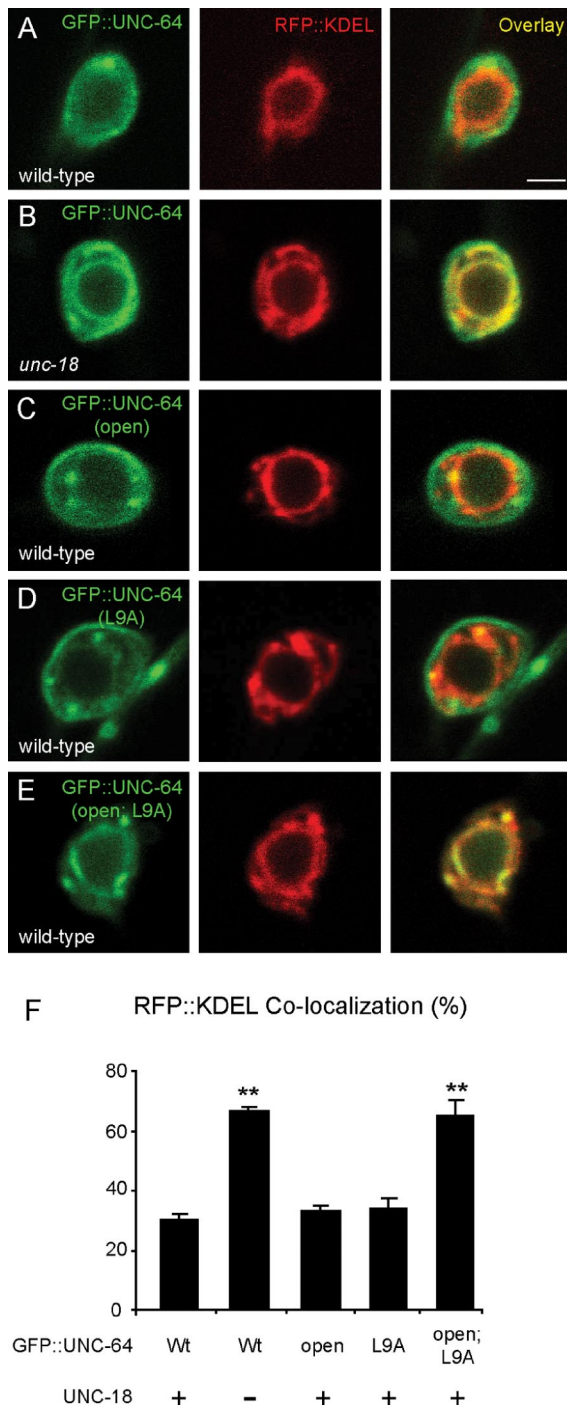


Figure 7. Effect of UNC-64 mutations on anterograde trafficking. (A–E) GFP::UNC-64 variants and an ER marker (ssRFP::KDEL) were expressed in the cholinergic DA motor neurons (using the *unc-129* promoter). Wild-type GFP::UNC-64 is primarily found at the cell periphery in wild-type neurons (A), but they had increased colocalization with ssRFP::KDEL and decreased abundance at the cell periphery in *unc-18(md299)* mutants (B). A wild-type distribution was observed for UNC-64(open) (C) and UNC-64(L9A) (D), whereas increased colocalization with ssRFP::KDEL and decreased abundance at the cell periphery was observed for UNC-64(open; L9A) (E). (F) Quantification of colocalization of GFP::UNC-64 variants with ssRFP::KDEL is shown ($n = 10$). The wild-type GFP::UNC-64 data in wild-type and *unc-18(md299)* mutants are the same as shown in Figure 5E. Bars, 2 μm . Values that differ significantly from GFP::UNC-64 in wild-type controls based on the Student's *t* test, ** $p < 0.001$. Error bars represent SEM.

tions would not have additive effects in double mutants. Consistent with this idea, we found that the locomotion and aldicarb defects observed UNC-64(L9A); *unc-18* double mutants were indistinguishable from those observed in the corresponding single mutants (Figure 8, B and E). These results suggest that disrupting UNC-18 binding to the N terminus of UNC-64 is sufficient to account for the synaptic defects observed in UNC-64(L9A) mutants.

DISCUSSION

The SM protein UNC-18 plays several critical roles in pre-synaptic function. In *unc-18* Munc18 mutants, SV docking, priming, and fusion are all significantly decreased (Gengyo-Ando *et al.*, 1993; Harrison *et al.*, 1994; Voets *et al.*, 2001; Weimer *et al.*, 2003). Here, we demonstrate that UNC-18 also has a chaperone function, specifically promoting trafficking of Syntaxin-1 out of the ER. This chaperone function is mediated by UNC-18 binding to Syntaxin, but either of the two UNC-18/Syntaxin binding modes is sufficient to restore chaperone function. By contrast, UNC-18 binding to the N terminus of Syntaxin is specifically required to restore normal behavior and aldicarb responses. These results suggest that the two binding modes participate in different aspects of UNC-18 function.

UNC-18 as a Chaperone for Syntaxin-1 Transport

In *unc-18* mutants, UNC-64 Syntaxin-1 accumulated in the cell bodies of neurons. This effect was seen with multiple *unc-18* alleles, with endogenously expressed UNC-64, and with GFP::UNC-64 transgenes. This UNC-64 trafficking defect was specific for *unc-18* mutants, because it was not seen in other presynaptic mutants (e.g., *unc-13* mutants). The decreased UNC-64 trafficking was accompanied by a significantly increased retention in the ER, as documented both by Endo H sensitivity and by colocalization with an ER marker, and by decreased delivery of UNC-64 to the plasma membrane of neuronal cell bodies. This trafficking defect was specific for UNC-64. No significant changes were observed in the distribution of 14 other SNARE proteins. Finally, UNC-18 binding to UNC-64 was required to promote transport out of the ER, but transport was disrupted only when both binding modes (i.e., binding to closed-Syntaxin and to the N terminus of Syntaxin) were disrupted. Similar trafficking defects were observed previously when Syntaxin-1 was heterologously expressed in epithelial cells (Rowe *et al.*, 2001; Martinez-Arca *et al.*, 2003).

Previous studies of worm and mouse *unc-18* Munc18 knockouts concluded that Syntaxin delivery to the plasma membrane was normal in these mutants. Both studies document significant levels of Syntaxin in axons (consistent with our results), although there was a 50 to 70% decrease in total Syntaxin abundance (Weimer *et al.*, 2003; Toonen *et al.*, 2005). Therefore, these authors concluded that UNC-18/Munc18 proteins do not play an essential role in anterograde transport of Syntaxin to the plasma membrane. However, neither study directly analyzed the abundance of Syntaxin in neuronal cell bodies. Our results indicate that the post-Golgi component of UNC-64 was reduced 33% in *unc-18* mutants. Taking into account both the decreased total abundance and the decreased trafficking, we estimate that the plasma membrane abundance of UNC-64 is ~35% wild type levels in *unc-18* mutants, which is consistent with the decreased GFP::UNC-64 abundance that we observed in dorsal cord axons.

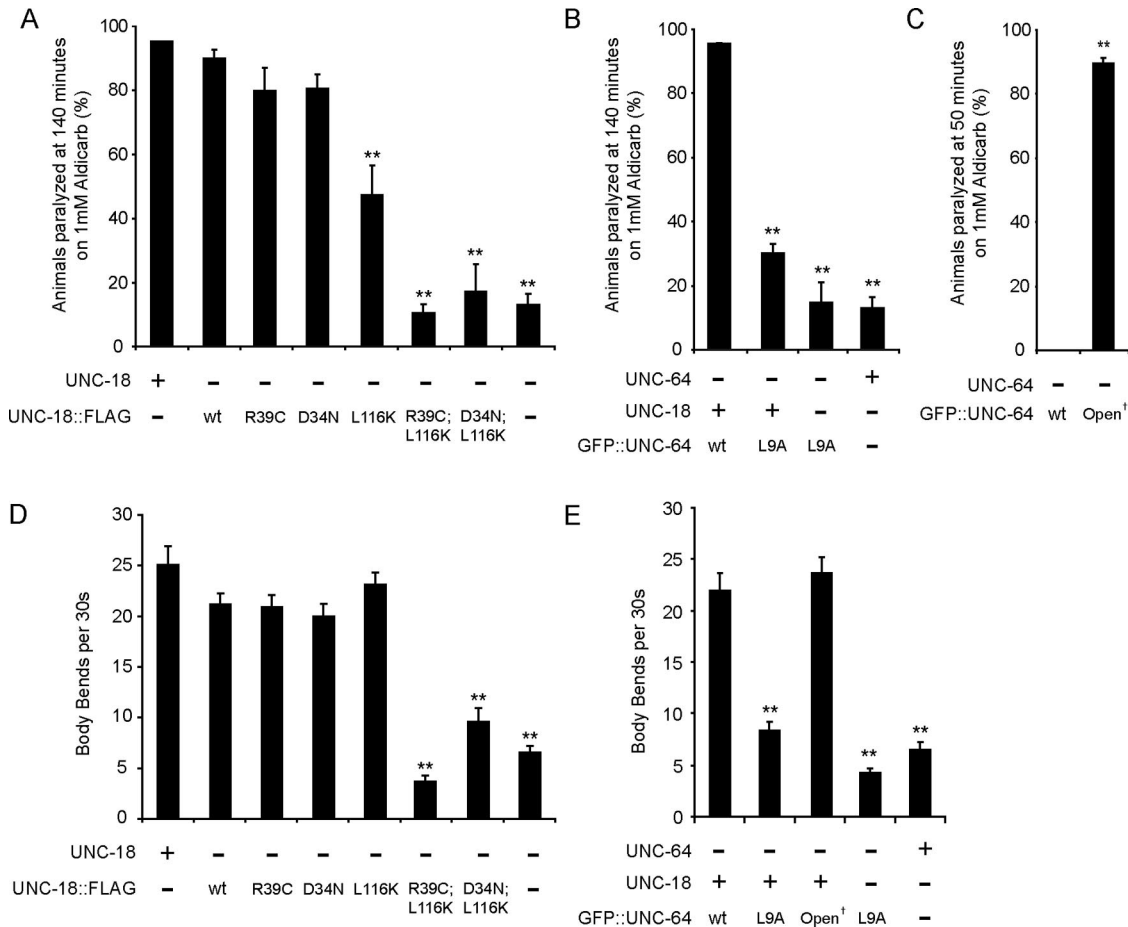


Figure 8. Mutations that disrupt UNC-18 binding to the N terminus of UNC-64 have behavioral defects. (A–E) UNC-18 constructs were expressed in *unc-18(md299)* mutants, UNC-64 constructs were expressed in the *unc-64(js115)* background as indicated. (A–C) Animals were placed on NGM plates containing 1 mM aldicarb and assayed over time for percentage of paralysis. The percentage of animals paralyzed at 140 min (A and B) and 50 min (C) are summarized. (D and E) The number of body bends per 30 s on NGM plates without food as a measure of locomotion for indicated transgenes expressed in *unc-18(md299)* mutants (D) and *unc-64(js115)* mutants (E). R39C, D34N, and L116K are indicators for UNC-18::FLAG transgenes containing the indicated point mutations in the UNC-18 protein. L9A and Open represent UNC-64 transgenes containing the indicated point mutations in the UNC-64a protein. Values that differ significantly from animals rescued with wild-type constructs based on the Student’s *t* test, ***p* < 0.001). Error bars represent SEM. Daggers (†) indicates use of the *oxIs34* [open-UNC-64] strain that does not contain N-terminal GFP.

A Potential Role for UNC-18 in ER Quality Control?

The ER quality control system surveys several aspects of protein structure and function. Misfolded, misassembled, and inactive proteins are actively retained in the ER, thereby preventing anterograde transport of functionally impaired proteins. We speculate that the UNC-18 chaperone function plays an important role in ER quality control for Syntaxin. Misfolded proteins retained in the ER are often substrates for ER-associated protein degradation (ERAD). The mechanism underlying the 50% decrease in total Syntaxin abundance in *unc-18* Munc18 knockouts has not been determined; however, it seems plausible that this decrease is caused by ERAD. Consistent with this idea, the yeast Syntaxin, Ufe1 is degraded by ERAD in mutants lacking the SM protein Sly1 (Braun and Jentsch, 2007). In addition, we showed that both binding modes to Syntaxin contribute to the UNC-18 chaperone function. Thus, UNC-18 surveys multiple aspects of Syntaxin structure in the ER, ensuring that only those molecules that are competent to bind to UNC-18 are able to undergo transport out of the ER. The ability of UNC-64 to interact with UNC-18 plays critical

roles in later aspects of secretion, e.g., SV docking, priming, and fusion; therefore, the UNC-18 dependence for anterograde trafficking out of the ER provides a simple mechanism for ensuring quality control for Syntaxin function.

What Is the Role of the UNC-18 Chaperone Function in Secretion?

It is plausible that the decreased delivery of UNC-64 to the plasma membrane contributes to the decreased neurotransmitter secretion observed in *unc-18* mutants. In *unc-18* mutants, stimulus-evoked neurotransmitter secretion was reduced to ~25% wild-type levels (Weimer *et al.*, 2003), whereas the abundance of UNC-64 in axons is ~35% the wild-type level. Syntaxin participates in several aspects of secretion, including SV docking, priming, and fusion. Therefore, neurotransmitter release is likely sensitive to plasma membrane Syntaxin levels.

Nonetheless, several results suggest that the UNC-64 trafficking defect is not sufficient to account for the secretion defects that occur in *unc-18* mutants. First, overexpression of UNC-64 did not rescue the secretion or behavioral defects of

unc-18 mutants (Weimer *et al.*, 2003); however, other studies suggest that Syntaxin overexpression fragments the Golgi and inhibits secretion, which could account for the failure to observe rescue (Rowe *et al.*, 2001; Mitchell and Ryan, 2005). In *Saccharomyces cerevisiae*, overexpression of the Syntaxins Sso1p and Sso2p suppressed the growth defects of mutants lacking the UNC-18 homologue *sec1*, implying that Syntaxin abundance may be limiting when SM protein function is impaired (Aalto *et al.*, 1993). Second, mutations disrupting UNC-18 binding to the N terminus of Syntaxin restored UNC-64 trafficking but did not rescue the behavioral and aldicarb-resistance phenotypes of *unc-18* mutants. Together, these results suggest that although UNC-64 trafficking may contribute to the secretion defects, other aspects of UNC-18 function (e.g., promoting SV docking and fusion) are likely to play a more prominent role.

Role of the Different Modes of UNC-18 Binding to Syntaxin-1

Several recent studies have shown the SM proteins, including UNC-18, have several independent modes of binding to their target Syntaxin. These modes include binding to closed-Syntaxin, binding to the N terminus of Syntaxin, and binding to fully assembled *cis*-SNARE complexes (Misura *et al.*, 2000; Dulubova *et al.*, 2007; Khvotchev *et al.*, 2007; Shen *et al.*, 2007). This diversity of binding mechanisms has led to the question of which binding modes contribute to UNC-18 function. Our results suggest that these binding modes play distinct roles in UNC-18 function. Binding to either closed-Syntaxin, or to the N terminus of Syntaxin is sufficient to mediate the UNC-18 chaperone function. In particular, single mutants predicted to disrupt either mode of binding restored Syntaxin transport out of the ER, whereas double mutants disrupting both modes caused retention in the ER. By contrast, mutations predicted to disrupt binding to the N terminus of Syntaxin significantly impaired rescue of the behavioral and aldicarb-resistance defects of *unc-18* mutants, whereas mutations disrupting binding to closed-Syntaxin did not. These results suggest that the N-terminal binding mode plays the predominant role in UNC-18 function promoting secretion. The N-terminal binding mode has also been shown to play a critical role in the function of other SM proteins. For example, in the yeast SM protein VPS45p, the N-terminal mode plays the primary role in regulating the stability of its cognate Syntaxin (Tlg2p); however, in this case, disrupting the N-terminal binding mode has no effect on Tlg2p-dependent vesicle traffic (Carpp *et al.*, 2007).

These results are consistent with recent studies examining the role of Munc18 in promoting SNARE-mediated fusion of reconstituted liposomes (Scott *et al.*, 2004; Khvotchev *et al.*, 2007; Shen *et al.*, 2007). In these studies, Munc18 accelerated the rate of SNARE-mediated liposome fusion, and this stimulatory effect was observed with both neuronal and yeast SNAREs. The stimulatory effect of Munc18 was eliminated by a mutation disrupting the N-terminal binding mode [Syntaxin(L8A)] but not by a mutation disrupting binding to closed-Syntaxin (open-Syntaxin) (Shen *et al.*, 2007). Thus, the critical importance of the N-terminal binding mode for UNC-18 promoted secretion *in vivo* reported here mirrors its importance in reconstituted SNARE-mediated liposome fusion *in vitro*.

Previous studies proposed that UNC-18 binding to closed-Syntaxin negatively regulates secretion (Wu *et al.*, 1998; Dulubova *et al.*, 1999). Expressing constitutively open-Syntaxin causes hypersensitivity to aldicarb and increases the pool of primed SVs. These results were interpreted to mean that UNC-18 binding to the closed-Syntaxin may negatively regulate secretion, perhaps by inhibiting docking, priming, or

fusion. However, the open-Syntaxin mutations may also alter the function of other SV priming factors (e.g., UNC-13 and UNC-10 RIM1) (Koushika *et al.*, 2001; Richmond *et al.*, 2001; McEwen *et al.*, 2006).

Our results suggest that the diverse binding modes for SM proteins have been conserved across phylogeny because they serve distinct cell biological functions. Both modes contribute to UNC-18 chaperone function: the closed-Syntaxin binding mode contributes to negative regulation of secretion, and the N-terminal binding mode contributes to stimulation of SNARE-mediated secretion. Mutations selectively disrupting these binding modes will provide critical reagents for further dissecting the mechanisms underlying these diverse aspects of SM protein function.

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