Genetic Interactions Between UNC-17/VACHT and a Novel Transmembrane Protein in *Caenorhabditis elegans*

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ABSTRACT The *unc-17* gene encodes the vesicular acetylcholine transporter (VACHT) in *Caenorhabditis elegans*. *unc-17* reduction-of-function mutants are small, slow growing, and uncoordinated. Several independent *unc-17* alleles are associated with a glycine-to-arginine substitution (G347R), which introduces a positive charge in the ninth transmembrane domain (TMD) of UNC-17. To identify proteins that interact with UNC-17/VACHT, we screened for mutations that suppress the uncoordinated phenotype of UNC-17(G347R) mutants. We identified several dominant allele-specific suppressors, including mutations in the *sup-1* locus. The *sup-1* gene encodes a single-pass transmembrane protein that is expressed in a subset of neurons and in body muscles. Two independent suppressor alleles of *sup-1* are associated with a glycine-to-glutamic acid substitution (G84E), resulting in a negative charge in the SUP-1 TMD. A *sup-1* null mutant has no obvious deficits in cholinergic neurotransmission and does not suppress *unc-17* mutant phenotypes. Bimolecular fluorescence complementation (BiFC) analysis demonstrated close association of SUP-1 and UNC-17 in synapse-rich regions of the cholinergic nervous system, including the nerve ring and dorsal nerve cords. These observations suggest that UNC-17 and SUP-1 are in close proximity at synapses. We propose that electrostatic interactions between the UNC-17(G347R) and SUP-1(G84E) TMDs alter the conformation of the mutant UNC-17 protein, thereby restoring UNC-17 function; this is similar to the interaction between UNC-17/VACHT and synaptobrevin.

COORDINATED muscle contraction in both vertebrates and nematodes requires release of the neurotransmitter acetylcholine (ACh) from motor neurons. ACh is synthesized in motor neurons by the enzyme choline acetyltransferase (ChAT) and transported into synaptic vesicles by the vesicular ACh transporter (VACHT). Following transmitter release, cholinergic signaling is terminated primarily through the action of acetylcholinesterase (AChE), which hydrolyses the released ACh into choline and acetyl-CoA. In the nematode *Caenorhabditis elegans*, the ChAT and VACHT proteins are encoded by the *cha-1* and *unc-17* genes, respectively (Alfonso *et al.* 1993, 1994b). Mutations that functionally

eliminate either protein are lethal (Rand 1989; Alfonso *et al.* 1993), while reduction-of-function mutations result in animals that are small, slow growing, uncoordinated, and resistant to AChE inhibitors such as aldicarb (Rand and Russell 1984; Rand 1989).

The *unc-17* missense mutation *e245* replaces a glycine with an arginine (G347R) in the ninth transmembrane domain (TMD9) of the VACHT protein (Alfonso *et al.* 1993). Genetic screens designed to identify mutations that improve the locomotion of *e245* homozygotes have identified several loci that suppress the deleterious effects of the VACHT G347R mutation. Sandoval *et al.* (2006) found that the *sup-8* locus corresponds to the previously characterized *snb-1* gene, which encodes the v-SNARE synaptobrevin. The single *sup-8* allele is associated with a missense mutation that results in a charge alteration (I97D) in the TMD of synaptobrevin. The Sandoval study suggests that synaptobrevin physically interacts with VACHT, implying a functional relationship between the synaptic vesicle fusion machinery and vesicular transport of ACh.

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In the present study, we show that the *sup-1* locus encodes a single-transmembrane protein that is a member of the DUF2650 subgroup of the STMC6 protein family (Pei and Grishin 2012). Like the *sup-8* mutation, the suppressing alleles of *sup-1* are associated with missense mutations that replace a neutral amino acid in the TMD with a negatively charged residue. We propose that this negative charge counterbalances the positive charge associated with the UNC-17(G347R) protein. Thus, suppression of *unc-17(e245)* mutants by *sup-1* would be mechanistically similar to suppression by *sup-8*. Using bimolecular fluorescence complementation (BiFC) analysis (Hu *et al.* 2002), we support this model by showing that the SUP-1 protein is closely associated with UNC-17 at cholinergic synapses.

Materials and Methods

Strains and strain maintenance

Standard laboratory methods for *C. elegans* were described by Brenner (1974). The wild-type strain is Bristol N2. Additional strains are listed in [Supporting Information, File S1](#). Worms were grown on NGM-L solid media (Sun and Lambie 1997), modified by the addition of streptomycin and mycostatin to reduce contamination, and use of a streptomycin-resistant bacterial strain OP50/1 as the food source (Johnson *et al.* 1988). The *pha-1(e2123) III*, *dpy-18(e364) III*, *unc-123(jd5) III*, and *unc-123(jd5jd10) III* mutants were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis). The sequence of the *unc-17(e245)* mutation was published in Alfonso *et al.* (1993). The amino acid substitutions associated with the other *unc-17* alleles are from Zhu *et al.* (2001), except that the amino acid substitutions associated with *e284* and *e464* were listed incorrectly in figure 1A of Zhu *et al.* (2001); the correct substitutions are *e284* = R60W and *e464* = E98K. Isolation of *sup-1* mutants is described in [File S2](#).

Sequencing of candidate genes and *sup-1* mutants

The *sup-1* gene was identified by amplification of specific genomic regions from *sup-1* mutant animals (Barstead and Waterston 1989), followed by sequencing of the purified PCR product with internal primers. All DNA sequencing was performed at the Oklahoma Medical Research Foundation DNA Sequencing Core Facility, using oligonucleotides obtained from Integrated DNA Technologies (Coralville, IA).

Protein sequence analysis

Putative signal sequences and TMDs were determined using SignalP 3.0 (Bendtsen *et al.* 2004) and TMHMM 2.0 (Krogh *et al.* 2001), respectively. Putative palmitoylation sites were determined using CSS-Palm 2.0 (Ren *et al.* 2008).

Transgene production and properties

All transgene constructs for injection were generated from amplified genomic sequences and/or plasmids using a PCR fusion method (Horton *et al.* 1989; Hobert 2002).

Reporters: Coding sequences for GFP and its color variants, including NLS-CFP::LacZ, were amplified from Fire expression vectors (Miller *et al.* 1999). The SUP-1::YFP fusion protein contained a 19-amino-acid linker between SUP-1 and YFP.

cDNAs: The *sup-1* cDNA clone *yk1353e09* was obtained from Yuji Kohara (National Institute of Genetics, Mishima, Japan); it includes a complete 22-bp SL1 *trans*-spliced leader sequence (Krause and Hirsh 1987; Bektesh *et al.* 1988), 14 bp of 5'-UTR, 309 bp of coding sequence, 321 bp of 3'-UTR, and a 20-bp polyA sequence. The *unc-17* cDNA RM#51p has been described previously (Alfonso *et al.* 1993). A cytochrome b5::YFP fusion (Rolls *et al.* 2002) was constructed from a DNA fragment amplified from genomic DNA corresponding to C31E19.7 (RM#962p).

BiFC constructs: The pET11a-link-NGFP and pMRBAD-link-CGFP clones were generously provided by the Regan lab (Magliery *et al.* 2005). The N-terminal portion of GFP (NGFP) was amplified from pET11a-link-NGFP and cloned into the Fire expression vector pPD95.77. The *unc-17* coding sequence from cDNA RM#51p was cloned downstream of the NGFP sequence to yield plasmid RM#935p. The G347R mutation was introduced into RM#935p using Stratagene's QuikChange procedure (RM#938p). The NGFP was converted to NYFP using a fragment amplified from a YFP-containing clone as the primer in a QuikChange reaction. The C-terminal portion of GFP (CGFP) was amplified from pMRBAD-link-CGFP and cloned into the Fire expression vector pPD95.77. The *sup-1* coding sequence from *yk1353e09* (without the termination codon) was cloned upstream of the CGFP sequence, with an 8-amino-acid linker between SUP-1 and CGFP (RM#934p). The G84E mutation was introduced into RM#934p to yield plasmid RM#939p, and the CGFP was then converted to CCFP using QuikChange.

Promoters: The *sup-1* promoter (*Psup-1*) included 3360 bp upstream of the start codon. The *unc-17* promoter (*Punc-17*) included 3180 bp upstream of the SL1 *trans*-splice site (Alfonso *et al.* 1994a); it drives expression exclusively in cholinergic neurons (Rand *et al.* 2000). A derivative of this promoter, designated *Punc-17β*, drives expression in the AS, DA, DB, VA, and VB cholinergic motor neurons of the ventral nerve cord (Charlie *et al.* 2006).

DNA injections: Transgenic nematodes were generated by microinjection of DNA (plasmids and/or PCR products), essentially as described by Mello and Fire (1995). The final DNA concentration was adjusted to ~50 ng/μl by the addition of pBluescript. For BiFC experiments, we found that the optimal concentration of DNA for fluorescence was higher than for behavioral suppression. Therefore, we used an intermediate amount of DNA to achieve a balance between BiFC fluorescence and suppression. Transformation markers included the pBX plasmid, which rescues the temperature-

sensitive lethality of *pha-1(e2123)* mutants (Granato *et al.* 1994).

Behavioral assays

All behavioral measurements were performed at ~22° on NGM-L plates. Swimming rates were measured using hermaphrodites raised at 20° as described previously (Miller *et al.* 1996). Acute response to 2 mM aldicarb (2-methyl-2-[methylthio]proprionaldehyde-O-[methylcarbamoyl]oxime; Chem Service, West Chester, PA) was measured as described previously (Lackner *et al.* 1999; Mullen *et al.* 2007). Statistical significance tests used the Student's *t*-test.

Immunofluorescence staining

Nematodes were stained using a modified freeze-fracture procedure as previously described (Duerr *et al.* 1999; Mullen *et al.* 2006). Antibodies used in this study include chicken polyclonal (C96) and mouse monoclonal (mAb1403) α -UNC-17 antibodies (Duerr *et al.* 2008). Secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

Microscopy and imaging

Live worms were immobilized in acrylamide with 0.05% sodium azide. Confocal images were collected on a Leica TCS NT confocal microscope. Lower-resolution images were collected with a 40 $\times$ Plan Fluotar 1.0 numerical aperture (NA) oil immersion objective, at 512 $\times$ 512 or 1024 $\times$ 1024 pixels, with 0.5 micrometer Z-steps. Higher-resolution images were collected with a 63 $\times$ Plan APO 1.4 NA oil immersion objective, at 512 $\times$ 512 pixels, with 4 $\times$ zoom, and 0.2- μ m Z-steps. Images were cropped to size, assembled, and annotated using Adobe Photoshop CS2. Digital manipulations were limited to rotating and cropping (Photoshop Bicubic) of images, as well as minor level adjustments.

Quantification of anti-UNC-17 staining was performed on confocal images of N2 and mutant worms that were grown under the same conditions, fixed and stained together, and imaged on the same day with the same imaging parameters. A maximum projection of each confocal series was used for analysis. Images were opened in Photoshop CS2 and a fixed size rectangular marquee tool was used to select the nerve ring in each image. Images were cropped with no change in resolution and saved in *tif* format. Cropped images were imported into ImageJ and the analyze > histogram function was used to create histograms of intensity values. The list function was then used to create lists of pixel intensities and counts; values were copied to Microsoft Excel 2010. Each intensity value was multiplied by the count (number of pixels with that intensity value) and these weighted values were summed to obtain a summation of the pixel intensity for each image. These summed values were then normalized against the appropriate wild-type (N2) values. Similar results were obtained by simply determining the mean pixel intensity of each cropped image and normalizing against the appropriate wild-type value.

Results

Allele-specific suppression of *unc-17* mutations by a dominant *sup-1* mutation

To identify proteins that interact with UNC-17/VACHT, we performed genetic screens for mutations that suppress the growth and movement deficits of *unc-17(e245)* mutants (see Supporting Information, File S2 for details). *e245* mutant animals grow slowly and are extremely uncoordinated; therefore, suppressed animals are readily identifiable by their larger size and improved movement. We identified several extragenic suppressors, and genetic analysis revealed that most of the suppressor mutations were dominant, unlinked to *unc-17*, and had no obvious phenotypes in the absence of the *unc-17* mutation. A number of the suppressor mutations mapped to a region of LG III approximately three map units from *dpy-18*. Although the dominant suppression and lack of other phenotypes made complementation testing impractical, these mutations appeared to represent alleles of the same gene, designated *sup-1*.

Two of the most obvious phenotypes associated with the *unc-17(e245)* mutation are uncoordinated locomotion (both crawling and swimming) and resistance to AChE inhibitors (Brenner 1974). To quantify the effects of one of the *sup-1* alleles (*e995*) on *unc-17(e245)* phenotypes, we measured the swimming behavior as well as the onset of paralysis following exposure to the AChE inhibitor aldicarb. For both phenotypes, the suppressor mutation restored ~70% of the wild-type function (Figure 1). We then characterized the allele specificity of the *sup-1(e995)* mutation; these results are summarized in Table 1 and details are provided in Table S1. We found that only *e245* and other alleles conferring the same G-to-R protein sequence change (*e359* and *p300*) were suppressed by *sup-1(e995)*. As noted earlier, the suppression by *sup-1* is dominant; *sup-1/+*; *unc-17* animals are nearly wild type in appearance and behavior (Figure 1).

The dominance of the *sup-1* suppressor mutations suggested that they were gain-of-function mutations. To examine the possible loss-of-function phenotype for this gene, a screen for loss of the dominant *e245* suppression by *sup-1(e995)* was performed (see File S2 for details). This screen yielded a putative second site mutation of *sup-1*, *e2636* in *cis* to *e995*. The double mutant *sup-1(e995e2636)* exhibited no obvious abnormalities as a homozygote and had no suppressive effect on *unc-17(e245)* (Figure 1).

The *sup-1* gene encodes a small transmembrane protein

We mapped the *e995* mutation to the right arm of chromosome III (12.08 $\pm$ 0.598 cM). We then used a candidate gene sequencing approach to identify sequence alterations associated with three independent *sup-1* mutations (see File S2). We found that the *sup-1* gene corresponds to the open reading frame Y41C4A.13; the molecular identity of the gene was confirmed by transgenic expression experiments (see below). The *sup-1* gene has three exons and spans 3949

Figure 1 Suppression of *unc-17* mutant phenotypes by *sup-1* alleles. (A) Swimming behavior. Bars indicate the mean number of body bends per minute for 20 individuals of each strain; error bars indicate SD. (B) Aldicarb-induced paralysis. Bars indicate the mean percentage of paralyzed animals for each strain after a 60-min exposure to 2 mM aldicarb; error bars indicate SD (four trials of 20 worms each).

base pairs (bp) of genomic sequence (Figure 2A); *sup-1* transcripts are *trans*-spliced to the SL1 leader sequence and are ~650 bp in length.

The predicted **SUP-1** protein is a small type I membrane protein (103 amino acids) with a putative 16-amino-acid signal peptide, a cysteine-rich N-terminal extracellular or luminal domain, a transmembrane segment, and a small C-terminal cytoplasmic region (Figure 2B). The luminal domain contains a pattern of cysteines followed by a tryptophan and a transmembrane segment [C(X)₁₀CC(X)₆CC(X)₅W + TM]; this corresponds to the “nematode-specific” DUF2650 protein family (DUF is a PFAM designation denoting “domain of unknown function”). In addition to the pattern of luminal cysteines, **SUP-1** has a short (7 amino acid) cytoplasmic C-terminal tail with 3 basic amino acids and three cysteines (Figure 2B and Figure S1); each of these cysteines matches a consensus for palmitoylation (Ren *et al.* 2008).

Several other genes in the *C. elegans* genome encode small DUF2650 proteins with varying degrees of similarity to **SUP-1** (Figure S2); some of these proteins also possess short basic C-terminal tails containing potentially palmitoylated cysteines. Genes encoding similar “**SUP-1**-like” proteins and DUF2650 domains are found in most or all nematode genomes (and in the planarian *Schmidtea mediterranea*). Although genes encoding DUF2650 protein domains have not been identified in mammalian genomes, DUF2650 proteins represent a subfamily of a large group designated STMC6 proteins, and different STMC6 subfamilies are found in most animal phyla (Pei and Grishin 2012).

Sequence analysis of *sup-1* mutations

We identified the sequence changes associated with the three *sup-1* mutant alleles (Figure 2B). The suppressor alleles *e995* and *md44* are associated with identical G-to-A transitions that change a glycine residue to a glutamic acid (G84E) in the **SUP-1** TMD. The putative null allele *e995e2636* contains both the original G84E mutation and

Table 1 Allele-specific suppression of *unc-17* mutations by *sup-1(e995)*

<i>unc-17</i> allele	Suppression by <i>sup-1</i>	Protein change
<i>e245</i>	+	G347R
<i>e283</i>	—	R60W
<i>e284</i>	—	R60W
<i>e327</i>	—	S211F
<i>e334</i>	—	G103R
<i>e335</i>	—	A206V
<i>e359</i>	+	G347R
<i>e464</i>	—	E98K
<i>e795</i>	—	A206V
<i>p300</i>	+	G347R

The amino acid substitutions associated with the *unc-17* alleles are from Alfonso *et al.* (1993) and Zhu *et al.* (2001), except as noted in *Materials and Methods*. See Table S1 for details. Suppression specificity experiments were performed before the sequence alterations associated with the mutations had been determined.

a second G-to-A transition that introduces a nonsense mutation in exon 2 (W58*). For convenience, we will refer to *sup-1(e995e2636)* as *sup-1(null)*.

Expression and localization of the *sup-1* gene product

Using a *sup-1* transcriptional reporter (FRM466, Figure 2A), we found that *sup-1* is expressed in a subset of *C. elegans* neurons and muscles (Figure 3). Most or all of the **SUP-1**⁺ neurons are cholinergic, including cholinergic motor neurons in the ventral nerve cord, as well as a subset of cholinergic head neurons (Figure 3). Expression was also noted in the anterior sublateral (SAB) neurons and at least a subset of the body sublateral neurons. Nonneuronal expression of *sup-1* appeared to be limited to the body wall muscles.

To examine the subcellular localization of the **SUP-1** protein, we took advantage of the suppressor activity associated with the G84E mutation and generated a transgenic **SUP-1** (G84E)::YFP fusion protein under the control of the *sup-1* promoter. This construct (FRM260, Figure 2A) was introduced into an *unc-17(e245)* mutant background. The **SUP-1** (G84E)::YFP mutant fusion protein significantly suppressed the *unc-17(e245)* mutant phenotype (swimming rate improved from 1.5 ± 1.4% of wild type to 76.9 ± 7.4%; *n* = 20), suggesting that the fusion protein is functional. We did not observe any significant differences in distribution between the wild-type and G84E mutant **SUP-1**::YFP fusion proteins. Wild-type **SUP-1**::YFP fluorescence in cholinergic neurons and body wall muscles was first observable in embryos and persisted throughout development (Figure 4). In cholinergic neurons, we observed enrichment of **SUP-1** in synaptic regions (Figure 4, A and B), although fluorescence was also observed in processes and cell bodies.

The presence of **SUP-1**::YFP in both neurons and muscles made it difficult to determine the precise subcellular localization of the protein. Therefore, we expressed **SUP-1**::YFP specifically in the cholinergic motor neurons of the ventral nerve cord, and we determined that the distribution of the **SUP-1** fusion protein in cell bodies did not have any significant overlap with cytochrome b5, a marker for the

Figure 2 The *sup-1* gene and SUP-1 protein. (A) *sup-1* gene, exon structure, and reporter constructs. Orange regions are coding sequences, dark gray regions are untranslated regions, and SL1 is the *trans*-spliced leader sequence. Also shown are the structures of reporter constructs FRM466, FRM260, FRM408, FRM401, and FRM409; the promoter portion of each construct is 3380 bp. FRM401 and FRM408 contain the wild-type SUP-1 coding sequence; FRM260 and FRM409 encode the SUP-1(G84E) variant. FRM260 includes GFP and FRM408 includes CFP; the fluorescent protein tags (XFP and CCFP) are not shown to scale. (B) Sequence and predicted structure of the SUP-1 protein. The putative N-terminal signal

peptide is shown in green. The predicted SUP-1 mature protein is a single-pass transmembrane protein (transmembrane segment shown in blue), with a larger amino terminal luminal domain and a smaller carboxyl terminal cytoplasmic domain. The luminal cysteines characteristic of the PFAM DUF2650 domain are shaded yellow, and the cytoplasmic cysteines predicted to be palmitoylated are shown in red. Also shown are the positions of the e995, *md44*, and e2636 mutations.

endoplasmic reticulum (Rolls *et al.* 2002). Rather, the SUP-1 fusion protein in cell bodies appeared to be restricted to the plasma membrane (Figure 4, D–F). In the body wall muscles, SUP-1::YFP also appeared to be localized to the plasma membrane (Figure 4A).

sup-1 loss-of-function mutants

sup-1(null) mutants are viable and superficially wild type in appearance, development, and behavior. We found that *sup-1* null mutants have only a modest (~23%) deficit in swimming behavior (Figure 1A) and they do not differ appreciably from wild-type animals in their response to aldicarb (Figure 1B). Therefore, although *sup-1* is expressed in many cholinergic neurons, *sup-1*(null) mutants have at most a minor deficit in cholinergic neurotransmission.

We considered the possibility that the mild phenotype of *sup-1* null mutants might reflect some level of overlapping function between SUP-1 and similar “SUP-1-like” proteins (Figure S2). However, although some of these proteins are expressed in restricted subsets of neurons, none of the SUP-1-like proteins has an expression pattern consistent with acting redundantly with SUP-1 (details in File S2).

Effects of *sup-1* mutations on UNC-17 abundance and trafficking

We previously demonstrated by immunostaining that UNC-17 protein is less abundant in *e245* mutants (Sandoval *et al.* 2006). Additional experiments demonstrated that in *unc-17*(*e245*); *sup-1*(*e995*) double mutants, both the levels and localization of UNC-17 protein appeared normal (Figure 5). The restoration of UNC-17 levels in *unc-17*(*e245*); *sup-1*(*e995*) double mutants raised the possibility that *sup-1* might suppress the *unc-17* mutant phenotype simply by increasing the abundance of the G347R mutant protein. We tested this hypothesis in two ways. First, we overexpressed the G347R protein, with or without an N-terminal YFP tag, in an *unc-17*(*e245*) mutant background. We found that overexpression of the G347R protein increased the amount of UNC-17 immunoreactivity, but did not improve the behavioral deficits of the *unc-17*(*e245*) animals (Figure 6). Next, we examined the behavior of *unc-17*(*md1447*) mutants; the *md1447* mutation is a deletion in the 3'-UTR of the *unc-17* gene that results in a severe reduction in UNC-17 immunostaining (Alfonso *et al.* 1994a). Although the decrease in UNC-17 immunoreactivity in this mutant is more

Figure 3 SUP-1-expressing cells. Animals carrying the *Psup-1::NLS-CFP-LacZ* (green) and *Punc-17::NLS-YFP* (magenta) transgenes were imaged with a confocal microscope. An L1 larva is shown in A–C. The head region of an adult hermaphrodite is shown in D–F. SUP-1 is expressed in a subset of cholinergic neurons in the head and body (note that all of the green neurons in A and D are also magenta in C and F, respectively), as well as the body wall muscles. The nerve ring (nr), a ventral nerve cord motor neuron nucleus (vnc), and a body wall muscle cell nucleus (m) are indicated with arrows. Anterior is to the left, ventral is down. Bars, ~10 μm.

Figure 4 SUP-1::CFP fusion is expressed in neurons and muscles. (A–C) Confocal images of a transgenic animal expressing wild-type SUP-1::CFP (green, A and B) and YFP::UNC-17 (magenta, B and C) fusion proteins under the control of their respective promoters. Shown is the head of a young adult hermaphrodite. The positions of the nerve ring (nr), dorsal nerve cord (dnc), and muscles are indicated. Bar, ~10 μ m. (D–F) Confocal images of a transgenic animal expressing SUP-1::CFP (green, D and E) and cytochrome b5::YFP (an ER marker; magenta, E and F) in the ventral cord cholinergic motor neurons. SUP-1 is largely absent from the ER. Bar, ~5 μ m. In all panels, anterior is to the left and ventral is down.

profound than in *unc-17(e245)* (Figure S3), we found that the behavioral defects are considerably less severe (Figure 6C). Based on these results, it is unlikely that increasing the abundance of the mutant UNC-17 is responsible for the suppression of the *e245* behavioral phenotype. We also determined that *sup-1(null)* mutants have approximately wild-type levels and localization of UNC-17 by immunostaining (data not shown). We conclude that SUP-1 is not required to maintain UNC-17 levels or localization in a wild-type genetic background.

The SUP-1 and UNC-17 proteins are closely associated in synaptic vesicles

The genetic interactions between *sup-1* and *unc-17* are very similar to the interactions between *snb-1* and *unc-17* reported by Sandoval *et al.* (2006), and we therefore speculated that the UNC-17 and SUP-1 proteins might also be in close physical proximity, or perhaps interact, in synaptic vesicles. To test this hypothesis, we used BiFC analysis to look at association between SUP-1 and UNC-17 in the *C. elegans* cholinergic nervous system. This approach has been used in a number of organisms, including *C. elegans*, to vi-

sualize protein–protein interactions (Hu *et al.* 2002; Shyu *et al.* 2008). For these experiments, an N-terminal partial peptide from YFP (NYFP) was fused to the N terminus of UNC-17, and a C-terminal partial peptide from CFP (CCFP) was fused to the C terminus of SUP-1. We expressed the NYFP::UNC-17 fusion protein under the control of the *unc-17* promoter and the SUP-1::CCFP under the control of the *sup-1* promoter. Neither fusion protein by itself gave rise to detectable fluorescence, but when the two wild-type fusion proteins were coexpressed, fluorescence was observed in synapse-rich regions of the cholinergic nervous system, including the nerve ring and dorsal and ventral nerve cords (Figure 7A). Coexpression of the SUP-1(G84E)::CCFP and NYFP::UNC-17(G347R) fusion proteins gave similar results (Figure 7B), although more fluorescence was observed in cell bodies than was observed with the wild-type combination.

These results, together with the genetic data, suggest that UNC-17 and SUP-1 are close together at the synapse and may physically interact. Potentially, the G347R mutation may disrupt physical interactions between UNC-17 and SUP-1, and the compensatory SUP-1(G84E) mutation may then restore these interactions. We tested this hypothesis by coexpressing NYFP::UNC-17(G347R) and SUP-1(WT)::CCFP. We note that Morell *et al.* (2008) have shown that inhibiting protein–protein interactions between BiFC partners results in a significant reduction in fluorescence. However, we observed strong BiFC fluorescence (Figure 7C), which was qualitatively similar to that observed when the two mutant proteins were coexpressed (Figure 7B). We conclude that the G347R mutation does not significantly disrupt the ability of UNC-17 and SUP-1 fusion proteins to produce BiFC fluorescence.

The transmembrane domain of SUP-1 mediates interaction with UNC-17/VACHT

To identify the specific regions of the SUP-1 protein that facilitate interaction with UNC-17, we generated deletion derivatives of the SUP-1(G84E)::CCFP construct. These were co-injected with the NYFP::UNC-17(G347R) construct and crossed into an *unc-17(e245)* mutant background. The resultant strains were assayed for suppression of the *unc-17* mutant swimming phenotype and for BiFC; these results are summarized in Figure 8. We found that deletion of the C-terminal (cytoplasmic) domain had little effect on suppression or BiFC fluorescence. In contrast, deletion of the luminal region between the signal sequence and the TMD eliminated suppression and most of the BiFC fluorescence. The little remaining fluorescence was restricted to the cell bodies, presumably due to improper trafficking of the SUP-1 protein. To test this hypothesis, we replaced the signal peptide and N-terminal domain of SUP-1 with the analogous region from SNT-1/synaptotagmin (Nonet *et al.* 1993; Mathews *et al.* 2007), an unrelated synaptic vesicle protein with the same (type I) membrane topology. We found that this chimeric protein suppressed the *unc-17(e245)* mutant

Figure 5 The *sup-1(e995)* mutation restores the synaptic abundance of UNC-17 in *unc-17(e245)* mutants. (A–D) Confocal images of heads of young adult hermaphrodites stained with a monoclonal antibody to UNC-17 (mAb1403; see *Materials and Methods*). (A) wild type (N2); (B) *unc-17(e245)*; (C) *sup-1(e995); unc-17(e245)*; and (D) *sup-1(e995)*. Positions of the nerve ring (nr), dorsal nerve cord (dnc), and ventral nerve cord (vnc) are indicated. The square in A indicates the size and location of the region that was analyzed quantitatively for each of the images.

Representative examples of groups of animals stained for each strain are shown. Anterior is to the left, ventral is down. Bar, ~10 μ m. (E) Quantitation of fluorescence intensity. Fluorescence intensity was quantified as described in *Materials and Methods* and normalized against the appropriate wild-type values.

phenotype and restored BiFC fluorescence (Figure 8). Although the luminal domain of SUP-1 appears to be required for proper trafficking to synaptic vesicles, neither the luminal nor the cytoplasmic domains are required for interaction with UNC-17. It therefore appears that the interaction with UNC-17 is mediated entirely through the SUP-1 TMD, and simply trafficking the TMD to synaptic vesicles permits the SUP-1–UNC-17 suppressive interaction.

Discussion

sup-1 mutants

We show here that alleles of the *sup-1* gene strongly suppress specific *unc-17* mutations (Figure 1). The suppressor alleles are associated with a G-to-E substitution (G84E) in the SUP-1 TMD and suppress mutations associated with a G-to-R substitution (G347R) in the ninth TMD of UNC-17/VACHT (Figure S4). Other *unc-17* alleles, including a G-to-R substitution elsewhere in the protein, are not suppressed (Table 1). The suppression is dominant (Figure 1A), and transgenic expression of SUP-1(G84E) restores locomotion and swimming behavior in *unc-17(e245)* mutant animals.

We note that additional *unc-17* mutant phenotypes have been reported, including reduced growth rate, small adult size, decreased pharyngeal pumping, reduced defecation rate, and constitutive egg laying (Rand and Russell 1984; Rand 1989; Avery 1993; Nguyen *et al.* 1995; Bany *et al.* 2003), and these phenotypes are also suppressed by *sup-1* mutations (not shown).

The SUP-1 protein

SUP-1 is a member of the DUF2650 subfamily of STMC6 (single-transmembrane proteins with conserved 6 cysteines) family of proteins; STMC6 proteins are single-transmembrane proteins that have a conserved pattern of cysteines in their N-terminal domains (Pei and Grishin 2012). In addition, many members of the family have cysteine residues predicted to be palmitoylated near the cytoplasmic face of the transmembrane segment. Like other members of the STMC6

family, SUP-1 is predicted to be a type I transmembrane protein (extracellular or luminal N terminus, and cytoplasmic C terminus). This predicted topology is consistent with our BiFC data: we observed fluorescence with the CCFP moiety on the C terminus of SUP-1 and the NYFP moiety on the cytoplasmic N terminus of UNC-17.

As noted earlier, SUP-1 is also a member of the DUF2650 subfamily and is the first DUF2650 family member to be characterized in any detail. Although the PFAM description for this domain suggests that it is found only in nematodes, we found several apparent DUF2650 proteins in the genome of planarian *S. mediterranea* (Figure S1).

Our reporter studies indicate that SUP-1 is expressed in a subset of neurons and in body wall muscles (Figure 3); most or all of the SUP-1⁺ neurons are cholinergic, including the ventral nerve cord and sublateral motor neurons. In

Figure 6 Overexpression of the UNC-17(G347R) protein does not rescue *unc-17(e245)* swimming deficits. (A and B) Confocal images of heads of young adult hermaphrodites stained with mAb1403 monoclonal antibody to UNC-17. Anterior is to the left, ventral is down. Bar, ~10 μ m. (A) Wild-type control. (B) *unc-17(e245)* mutant overexpressing the UNC-17(G347R) protein. (C) Swimming behavior. Bars represent the mean number of body bends per minute for each strain; error bars indicate SD (four trials of 20 worms each). The overexpression (O/E) transgenic lines G347R O/E and YFP::G347R O/E are in an *unc-17(e245)* genetic background. Complete genotypes are described in File S1.

Figure 7 BiFC analysis of UNC-17–SUP-1 protein interactions. Confocal images of the nerve rings (nr) of young adult hermaphrodites expressing different combinations of NYFP::UNC-17 and SUP-1::CCFP. The wild-type UNC-17 and SUP-1 fusion constructs are identical to those used in Figure 4, except that the full YFP and CFP proteins were used in Figure 4. (A) Interaction between wild-type UNC-17 and wild-type SUP-1. (B) Interaction between mutant UNC-17(G347R) and mutant SUP-1(G84E). (C) Interaction between mutant UNC-17(G347R) and wild-type SUP-1. Comparable levels of BiFC fluorescence were observed in the three combinations. Anterior is to the left, ventral is down. Bar, ~10 μ m.

these cholinergic neurons, SUP-1 is present in processes, and based on the genetic and BiFC interactions between SUP-1 and UNC-17, at least some SUP-1 is localized to synaptic vesicles.

The UNC-17(G347R) mutant protein

We propose that the primary effect of the G347R amino acid substitution is a disruption of UNC-17 protein conformation; this could be a consequence of the increased bulk of the arginine residue and/or the presence of an additional charged amino acid in TMD9. The altered conformation leads to decreased protein stability and reduced transport of ACh; collectively, these confer the G347R mutant phenotype. The reduced loading of ACh into synaptic vesicles leads to the obvious behavioral phenotypes, including uncoordinated crawling, decreased swimming rate, and aldicarb resistance (Figure 1).

The reduced level of UNC-17 immunostaining in *unc-17(e245)* mutants (Figure 5) suggests that the mutant protein is turned over faster than the wild-type protein; however,

our results do not address the specific subcellular site of UNC-17 protein degradation. One possibility is that the G347R protein is correctly trafficked to synaptic vesicles, but is less stable than the wild-type protein. Another possibility is that the aberrant conformation of the mutant protein is recognized by a cellular quality control mechanism in the ER, where the G347R protein would be targeted for degradation. However, since a complete lack of synaptic UNC-17 protein is lethal (Alfonso *et al.* 1993), at least some of the mutant protein must be properly trafficked. We suggest that the cellular, rather than synaptic, localization of overexpressed UNC-17(G347R) protein (Figure 6B) is consistent with the latter model. Presumably, the aberrant conformation of the overexpressed mutant protein still targets it for degradation, but the sheer amount of mutant protein exceeds the capacity of the protein degradation pathway, and the UNC-17(G347R) protein accumulates in the ER. In contrast, when wild-type UNC-17 is overexpressed, most of the protein traffics normally to synapses and is functional, resulting in increased ACh release and hypersensitivity to aldicarb (Sandoval *et al.* 2006). Although G347R mutants have reduced synaptic levels of UNC-17 protein and severely impaired behavior, and the SUP-1(G84E) protein suppresses both of these phenotypes (Figures 1 and 5), we show that the decreased abundance of UNC-17 is not the primary cause of the behavioral phenotypes; it is therefore unlikely that the increase in protein abundance in animals expressing SUP-1(G84E) is responsible for suppression of the behavioral phenotypes.

Interaction between UNC-17 and SUP-1 proteins

According to current structural models of VACHT proteins (Vardy *et al.* 2004; Law *et al.* 2008), TMD9 is an “external” TMD and is therefore in a position to interact with TMDs of other proteins. Our genetic data suggest that the TMD of SUP-1 can interact with TMD9 of UNC-17/VACHT. Using BiFC, we show that the wild-type UNC-17 and SUP-1 proteins are closely associated at synapses, as are the two mutant proteins (Figure 7, A and B). We also show that the G347R mutation in TMD9 of UNC-17 does not prevent close association with the wild-type SUP-1 protein at synapses (Figure 7C).

BiFC data are normally interpreted as a measure of specific protein–protein interactions (Hu *et al.* 2002; Shyu *et al.* 2008). Alternatively, the observed BiFC interactions may actually reflect protein proximity and not necessarily protein interaction (Bhat *et al.* 2006). If two proteins are able to diffuse freely, then prolonged proximity would require protein interaction; however, when both proteins are spatially constrained in the synaptic vesicle membrane (Takamori *et al.* 2006), then proximity-based BiFC interactions may be passive and would not necessarily reflect functional interactions. Molecular modeling of the NYFP::UNC-17 and SUP-1::CCFP proteins suggests that fluorescence complementation could occur in synaptic vesicles without direct physical contact between the fusion partners (J. Manjarrez,

Figure 8 Structure–function analysis of SUP-1. Deletions were generated in the SUP-1(G84E)::CCFP expression construct to identify regions of the SUP-1 protein that facilitate interaction with the UNC-17(G347R) mutant protein. The SUP-1 signal peptide, luminal domain, transmembrane segment (TM, with a yellow diamond indicating the location of the G84E substitution), and cytoplasmic region (Cy) are indicated. Also indicated are the vector-

derived spacer segment (Sp) and the C-terminal peptide of CFP (CCFP). ΔN terminus, Δ20–71; ΔC terminus, Δ97–103. For the SNT-1::SUP-1 chimera, the N-terminal domain of SUP-1 (including signal peptide) was replaced with the analogous domain from SNT-1 (residues 1–72). Constructs were co-injected with the NYFP::UNC-17(G347R) expression construct, and the resulting extrachromosomal arrays were crossed into an *unc-17(e245)* mutant background. Complete genotypes are described in File S1. Constructs were assayed for suppression of the *unc-17(e245)* swimming deficit and for BiFC fluorescence. Suppression was defined as a >20-fold increase in swimming rate (body bends per minute) relative to the *unc-17(e245)* control. For BiFC assays, +, bright synaptic fluorescence; –, faint/undetectable synaptic fluorescence.

personal communication). Therefore, although the BiFC data indicate that UNC-17 and SUP-1 are in close proximity in synaptic vesicle membranes, we cannot determine at this point whether there are direct protein–protein interactions between the wild-type UNC-17 and SUP-1 proteins. More generally, we suggest that even though BiFC studies with synaptic vesicle membrane proteins may not necessarily reflect functional interactions, they can nevertheless be useful for assessment of protein trafficking and colocalization.

We currently favor the model that the interaction between wild-type UNC-17 and wild-type SUP-1 is passive, and the observed BiFC reflects the physical proximity of the two proteins. The wild-type SUP-1 protein does not appear to be involved in the trafficking or localization of UNC-17 protein, because there is not a significant reduction in synaptic levels of wild-type UNC-17 in *sup-1* null mutants (not shown). Similarly, since the *sup-1* null mutant does not have a strong cholinergic phenotype, wild-type SUP-1 is unlikely to have a significant effect on vesicular ACh transport, although we cannot exclude the possibility that other proteins function redundantly with SUP-1.

However, in addition to this proposed passive interaction, there is the charge interaction that occurs between mutant forms of the two proteins. It is likely that the formation of the arginine–glutamic acid ion pair (Figure S4) helps restore the conformation of UNC-17, either by reorienting the bulky arginine or by neutralizing the additional charge in TMD9, and this interaction offsets most of the adverse G347R-mediated effects on UNC-17 stability and ACh transport. Presumably, for the UNC-17(G347R) protein to be stabilized and to be trafficked properly, the two mutant proteins would need to “encounter” each other during an early stage of trafficking, and they would likely remain associated all the way to the synapse.

We have shown that the suppressive interaction between the mutant SUP-1 and UNC-17 proteins does not require either the SUP-1 luminal or cytoplasmic domains and presumably requires only that the SUP-1 TMD containing the G-to-E substitution be trafficked to synaptic vesicles (Figure 8). Furthermore, we note that the SNB-1(I97D) mutant protein also provides essentially complete phenotypic suppression of UNC-17(G347R) mutant phenotypes (Sandoval *et al.*

2006). Since SUP-1 is a type I transmembrane protein and SNB-1 is a type II protein, it appears that the relative orientation of the interacting transmembrane helices does not affect the effectiveness of the suppressive charge interaction. One might therefore conclude that the only requirements for extragenic suppression of the G347R mutant are: (1) a transmembrane protein correctly trafficked to synaptic vesicles in cholinergic neurons and (2) an aspartate or glutamate residue approximately in the middle of the TMD. However, transgenic expression of an engineered synaptotagmin [SNT-1(I85D)] containing an isoleucine-to-aspartate substitution in the middle of the TMD provided only a small level of suppression of UNC-17(G347R) phenotypes (Sandoval *et al.* 2006). It therefore appears that there are additional requirements for the suppressing TMDs.

The function of SUP-1

Although the gain-of-function *sup-1* alleles have been useful in understanding some features of a specific type of UNC-17 alteration, the *sup-1* loss-of function phenotypes have not been very informative about normal SUP-1 function. In particular, we have no information about the role of SUP-1 in the body muscles: muscle structure and function appear normal in *sup-1(null)* mutants. In the nervous system, it is possible that SUP-1 plays a role in trafficking, sorting, or stabilization of UNC-17, but in that case, there must be other proteins whose functions overlap those of SUP-1.

The STMC6 family of proteins is large and heterogeneous, and it is difficult to infer much about SUP-1 function based on its membership in this group. The STMC6 family includes the Shisa, Shisa-like, WBP1/VOPP1, TMEM92, CYR1, CX, and DUF2650 protein subfamilies, and family members are implicated in a variety of biological processes and pathological conditions, including embryonic development, synaptic plasticity, apoptosis, and cancer; the current mechanistic interpretation is that many STMC6 proteins are adaptors that regulate membrane proteins such as cell surface receptors (Pei and Grishin 2012). We also note that SUP-1 also displays some intriguing structural similarities to progranulins, cysteine-rich proteins that regulate a variety of cellular processes (Bateman and Bennett 2009).

In summary, SUP-1 is a member of the DUF2650 subfamily of STMC6 proteins, and a glycine-to-glutamic acid substitution

(G84E) in the TMD of SUP-1 suppresses the behavioral and protein abundance phenotypes associated with a glycine-to-arginine amino acid substitution (G347R) in the ninth TMD of UNC-17/VACHT. We propose that electrostatic interactions between the UNC-17(G347R) and SUP-1(G84E) TMDs (Figure S4) restore UNC-17 stability, and function; this form of suppression is mechanistically similar to a previously characterized interaction between UNC-17/VACHT and synaptobrevin.

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GENETICS

Supporting Information

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Genetic Interactions Between UNC-17/VACHT and a Novel Transmembrane Protein in *Caenorhabditis elegans*

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Y41C4A.13 -----MSYTLAACTCLANANVDHDKSAVNEVTTTKDGTYPVPLVGTCTSSIFHYWKCGELNKECFNLQTNWVTLALFGVIFTASVTSVRCICCRK
F52A8.1 -----MSYTLPLFLAMALLACAPSVSAEGDDLIDYVKSIEPLTIIRSPACPLPVVGQAPENVFVHYFKCGQTADQCFRLQDWTVTLFLAVCTLSIIINCVRCCCA
F58F12.4 MWFSLILLCTTISLKAVPALTKAIAESSDLANFINETDSEMTSSQIESSTQIPSSTMNPSTKLPSMRGKDLHCPSTENAVSSCPKDSKWVYTCGQANMYCEHIQTNLSFLAVIIVFLSIFLIGCCVRCCKSYKRRKTSQSYSFDDK
F42C5.6 MLESHRKTFELNSCFSTLTQELTAEYFNSLLAONSEETQTEGSIQTVTSFKVLGFEILKDIQTVVKIESDAIWCPTHRVGNOCPEETLLSFYKCGHLNKECFHLRWVIVIMIVLPICLVPASTFLIRRLCSSSQPTGRAPSAADRVRQL/
C08H9.15 -----MARTATLLLLLPVAYAMVWPVLDNRNTRSTIVRPNMAEKNLGQWCRNFTVNEHTCPOASAFHQFDCGLHETECFAIQGWVIVITIVGTGAILIFSVLLKMNLCMPYTTKQTPSTYTGIOE
R10E11.9 -----MHFLFKLFVLTLNLTLSNVKSSQSYNDVICRRPWYFNSPRPAPMRCPTASFFTYYECGFELENCWRFQEPITIGVILLVLLLVCCCCVSWIFVGRKKKSSNIEPEKKPTKKDS/
ZK353.2 -----MSYCGGYGNCGGILGVLGMAGMAGGYGGYGGYGGYGRGADNVFYRWRCDYSPYECIQLETNWVFLVIFITIGFEVCLCACLACVWSARNRE

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Figure S2 Aligned protein sequences of SUP-1 (Y41C4A.13) and *C. elegans* SUP-1-like proteins (labeled with their cosmid designations). As in the previous figure, predicted signal sequences are shaded green, predicted transmembrane domains are shaded blue, the DUF2650 conserved pattern of luminal cysteines is shaded yellow, and cytoplasmic cysteines predicted to be palmitoylated are in red. The C-terminal 18 amino acids of F42C5.6 and the C-terminal 45 amino acids of R10E11.9 have been truncated for ease of presentation.

Figure S3 Comparison of UNC-17 immunofluorescence in different *unc-17* mutants. Shown are confocal images of heads of young adult hermaphrodites stained with mAb1403 monoclonal antibody to UNC-17. (A) wild type (N2); (B) *unc-17(md1447)*; (C) *unc-17(e245)*. The insets in Panels B and C have their brightness amplified 3-fold, to permit visualization of the faint fluorescence. Note that animals homozygous for *md1447* have considerably less UNC-17 protein than *e245* homozygotes, even though their behavioral phenotype is much milder (Figure 6C). Anterior is to the left, ventral is down, and the scale bars are ~10 μ m. Positions of the nerve ring (nr), dorsal nerve cord (dnc), and ventral nerve cord (vnc) are indicated in panel A.

FIGURE S4 Proposed interactions between the UNC-17 and SUP-1 proteins. UNC-17 is a twelve-pass transmembrane protein; the *e245*, *e359*, and *p300* alleles are associated with identical missense mutations (G347R) that introduce a positive charge in the ninth TMD. SUP-1 is a Type I single-transmembrane protein; suppressing alleles of *sup-1* are associated with identical missense mutations (G84E) that introduce a negative charge in the transmembrane segment. We propose that the TMDs of the two proteins interact (inset), and the negatively charged glutamic acid of SUP-1(G84E) neutralizes the positively charged arginine of UNC-17(G347R), facilitating proper conformation and function of the transporter. The N- and C-termini of each protein are indicated, and the predicted SUP-1 signal peptide is shown in green. The curved arrows in the inset represent the direction of translation of each protein; note that the interacting TMDs are anti-parallel.

File S1.

Strains used in these studies

The reference strain for *unc-17(e245)* IV (outcrossed 6x) is RM908.

The reference strain for *sup-1(e995)* III (outcrossed 7x) is RM3670.

The reference strain for *sup-1(md44)* III (outcrossed 3x) is RM3277.

The reference strain for *sup-1(e995 e2636)* III (outcrossed 6x) is RM3571.

The *unc-17* strains used for the isolation of suppressor mutations were:

CB933: *unc-17(e245)* IV

PR1443: *unc-17(p300) dpy-13(e184)* IV

The strain used for DNA sequencing to identify the *sup-1* gene was RM921: *dpy-18(e364) sup-1(e995)* III; *unc-17(e245)* IV

The strains used to assess behavioral suppression and protein abundance (Figures 1 and 5) were:

N2: wild type

RM908: *unc-17(e245)* IV

RM3660: *sup-1(e995)* III; *unc-17(e245)* IV

RM3670: *sup-1(e995)* III

RM3643: *sup-1(e995 e2636)* III; *unc-17(e245)* IV

RM3571: *sup-1(e995 e2636)* III

The strains used to determine allele-specific suppression by *sup-1* (Tables 1 and S1) were:

CB2108: *dpy-18(e364) sup-1(e995)* III

CB933: *unc-17(e245)* IV

CB283: *unc-17(e283)* IV

CB284: *unc-17(e284)* IV

CB327: *unc-17(e327)* IV

CB334: *unc-17(e334)* IV

CB335: *unc-17(e335)* IV

CB356: *unc-17(e359)* IV

CB464: *unc-17(e464)* IV

CB795: *unc-17(e795)* IV

PR1170: *unc-17(p300)* IV

PR1158: *cha-1(b401)* IV

PR1154: *cha-1(p1154)* IV

CB81: *unc-18(e81)* X

The strain used to compare the expression patterns of *sup-1* and *unc-17* reporters (Figure 3) was:

RM3549: *pha-1(e2123)* III; *mdls139* [FRM186 = *Punc-17::NLS-YFP*; pBX]; *mdEx1044* [FRM466 = *Psup-1::NLS-CFP::LacZ*; pBX; pBS]

The strains used to assess behavioral suppression of *unc-17(e245)* phenotypes by transgenic expression of SUP-1(G84E) were:

RM3483: *pha-1(e2123)* III; *unc-17(e245)* IV; *mdEx995* [FRM260 = *Psup-1::SUP-1(G84E)::GFP*; pBX; pBS]

RM2086: *pha-1(e2123)* III; *mdEx186* [pBX] (control: pBX rescue of *pha-1*)

RM2501: *pha-1(e2123)* III; *unc-17(e245)* IV; *mdEx186* [pBX] (control: pBX rescue of *unc-17*; *pha-1*)

The strains used to determine the subcellular localization of SUP-1, UNC-17, and cytochrome b5 fusion proteins (Figure 4) were:

RM3480: *pha-1(e2123)* III; *mdEx992* [FRM408 = *Psup-1::SUP-1(+)::CFP*; FRM403 = *Punc-17::YFP::UNC-17(+)*; pBX]

RM3623: *pha-1(e2123)* III; *mdEx1099* [FRM489 = *Punc-17b::cytochrome b5::YFP*; FRM491 = *Punc-17b::SUP-1(+)::CFP*; pBX; pBS]

The strains used to analyze overexpression of UNC-17(G347R) and YFP::UNC-17(G347R) (Figure 6) were:

RM3220: *pha-1(e2123)* III; *unc-17(e245)* IV; *mdEx791* [RM#349p = *Punc-17::NLS-GFP*; RM#883p = *Punc-17::UNC-17(G347R)*; pBX]

RM3674: *pha-1(e2123)* III; *unc-17(e245)* IV; *mdEx1112* [FRM419 = *Punc-17::YFP::UNC-17(G347R)*; pBX; pBS]

RM2086: *pha-1(e2123)* III; *mdEx186* [pBX] (control: pBX rescue of *pha-1*)

RM2501: *pha-1(e2123)* III; *unc-17(e245)* IV; *mdEx186* [pBX] (control: pBX rescue of *unc-17*; *pha-1*)

The *unc-17(md1447)* IV strain (Figures 6 and S3) is RM580.

The strains used for BiFC analysis of UNC-17 and SUP-1 proteins (Figure 7) were:

RM3478: *pha-1(e2123)* III; *mdEx990* [FRM394 = *Punc-17::NYFP::UNC-17(+)*; FRM401 = *Psup-1::SUP-1(+)::CCFP*; pBX; pBS]

RM3482: *pha-1(e2123)* III; *mdEx994* [FRM387 = *Punc-17::NYFP::UNC-17(G347R)*; FRM409 = *Psup-1::SUP-1(G84E)::CCFP*; pBX; pBS]

RM3689: *pha-1(e2123)* III; *mdEx1139* [FRM387 = *Punc-17::NYFP::UNC-17(G347R)*; FRM401 = *Psup-1::SUP-1(+)::CCFP*; pBX; pBS]

RM3465: *pha-1(e2123)* III; *mdEx978* [FRM394 = *Punc-17::NYFP::UNC-17(+)*; pBX; pBS]

RM3496: *pha-1(e2123)* III; *mdEx1001* [FRM401 = *Psup-1::SUP-1(+)::CCFP*; pBX; pBS]

The interaction of NYFP::UNC-17(G347R) with full-length SUP-1(G84E)::CCFP and the Δ C-terminus, the Δ N-terminus, and SNT-1::SUP-1 chimeric versions of SUP-1(G84E)::CCFP (Figure 8) were studied in strains RM3482, RM3634, RM3636, and RM3673, respectively:

RM3482: *pha-1(e2123) III; unc-17(e245) IV; mdEx994* [FRM387 = *Punc-17::NYFP::UNC-17(G347R)*; FRM409 = *Psup-1::SUP-1(G84E)::CCFP*; pBX; pBS]

RM3634: *pha-1(e2123) III; unc-17(e245) IV; mdEx1038* [FRM387 = *Punc-17::NYFP::UNC-17(G347R)*; FRM437 = *Psup-1::SUP-1(G84E) Δ 97-103::CCFP*; pBX; pBS]

RM3636: *pha-1(e2123) III; unc-17(e245) IV; mdEx1007* [FRM387 = *Punc-17::NYFP::UNC-17(G347R)*; FRM420 = *Psup-1::SUP-1(G84E) Δ 20-71::CCFP*; pBX; pBS]

RM3673: *pha-1(e2123) III; unc-17(e245) IV; mdEx1116* [FRM387 = *Punc-17::NYFP::UNC-17(G347R)*; FRM513 = *Psup-1::SNT-1(1-72)::SUP-1(73-103,G84E)::CCFP*; pBX; pBS]

RM2086: *pha-1(e2123) III; mdEx186* [pBX] (control: pBX rescue of *pha-1*)

RM2501: *pha-1(e2123) III; unc-17(e245) IV; mdEx186* [pBX] (control: pBX rescue of *unc-17; pha-1*)

The *unc-123* mutant strains were:

ER10: *unc-123(jd5) III*

ER50: *unc-123(jd5jd10) III*

Supporting methods and results

Isolation of suppressors of *unc-17(e245)*:

We performed several genetic screens for mutations that suppress the growth and movement deficits of *unc-17(G347R)* mutants. As described previously, the *e245* and *p300* alleles are associated with the same G347R sequence alteration (Alfonso *et al.* 1993; Zhu *et al.* 2001). *unc-17(e245)* or *unc-17(p300) dpy-13(e184)* L4 homozygotes were mutagenized with 50 mM ethyl methanesulfonate (EMS) and plated onto 100 mm NGM plates. *unc-17* mutant animals grow slowly and are highly uncoordinated; suppressed progeny were identified by their larger size and improved movement, and we identified several dominant extragenic suppressors. In particular, the *e995* mutation (isolated against *e245*) and the *md44* mutation (isolated against *p300*) both mapped to LGIII.

Isolation of a putative *sup-1(null)* mutation:

The *e995e2636* allele was isolated by mutagenizing a strain with genotype *sup-1(e995); unc-17(e245) him-8(e1489)*. Mutagenized males were then crossed with females with the genotype *dpy-18(e499); unc-17(e245) fem(hc17); xol-1(y9)*. The only progeny of this cross are hermaphrodite cross-progeny, which are homozygous for *unc-17(e245)* and heterozygous for *sup-1(e995)*. Therefore, the cross progeny are non-Unc, unless the *sup-1(e995)* mutation has been over-ridden, in which case the animal will be Unc. About 4000 progeny were screened for Unc progeny. Seven candidate Uncs were picked, of which six produced some wild-type self-progeny, suggesting that they were mosaic or carried a dominant Unc mutation. The seventh bred true, with a standard Unc-17 phenotype. Single Unc non-Dpy progeny were picked, of which 2/9 segregated no Dpy Uncs, and these two were therefore presumed to be homozygous with the genotype *sup-1(e995e2636); unc-17(e245)*. One of these was used to establish the strain CB5265. This strain was then outcrossed six times to remove possible background mutations; the outcrossed strain was designated RM3571.

Further experiments suggested that *e2636* is indeed a *sup-1* null or severe loss of function allele, rather than a revertant to the wild-type allele. An outcrossed strain CB5285 with genotype *sup-1(e995e2636); unc-17(e245)* was mutagenized with EMS to select for suppressors. None was found, apart from a partial same-site revertant of *unc-17(e245)* itself, whereas a parallel control experiment with *unc-17(e245)* yielded numerous suppressors (0/16 experimental versus 16/16 control 100 mm plates). This result suggested that *sup-1* locus had lost function, and therefore could no longer be mutated to create a dominant *e245* suppressor. After the *sup-1* locus was cloned (see below), the *e2636* allele was sequenced, and was found to be associated with both the original G84E mutation and a nonsense mutation in exon 2 (W58*).

Candidate gene strategy to identify the *sup-1* gene:

Mapping experiments indicated that *sup-1* mapped to the right arm of chromosome III (12.09 ± 0.60 cM). This placed *sup-1* (with 95% confidence) in a region between 11.49 and 12.69 cM, which corresponds to a physical distance of 149 kb and includes 24 protein-coding genes. Genes were excluded as candidates if the predicted protein did not include a transmembrane domain, if there were RNAi data suggesting that null alleles were lethal, or if there were data indicating that the protein was not expressed in the nervous system. Genomic DNA was isolated from the RM921 strain (*dpy-18(e364) sup-1(e995); unc-17(e245)*) and used as a template for the PCR amplification of 1-2 kb regions, which were then sequenced and compared to the canonical wild-type *C. elegans* genome. We identified a sequence alteration associated with the *e995* allele in the Y41C4.13 open reading frame. We then sequenced the *md44* and *e995e2636* alleles, and identified sequence alterations in the same open reading frame. The identity of the *sup-1* gene was confirmed by transformation experiments: microinjection of a genomic fragment from RM921 animals containing the Y41C4.13 open reading frame with the *e995* mutation suppressed the *unc-17(245)* phenotype.

PCR-based method for identification of point-mutants:

For outcrossing and generation of double mutants, the presence of mutant and wild-type alleles was determined in parallel reactions using a PCR-based point-mutant detection method (Qiang *et al.* 2002). For each reaction, either the mutant-specific primer or the wild-type-specific primer was paired with the "common" primer as indicated.

unc-17(e245) or *unc-17(p300)*

Mutant-Specific Primer: 5'-TGGTTGGGTTGGCTATGGAAA-3'

WT-Specific Primer: 5'-TGGTTGGGTTGGCTATGGAAG-3'

"Common" Primer: 5'-ATCACTACAGTAGCCCGACA-3'

Annealing temperature: 65°C (mutant) or 66°C (wild type)

Size: 1077 bp.

sup-1(e995) or *sup-1(md44)*

Mutant-Specific Primer: 5'-GGTAACCCTCGCCTTGTTCAA-3'

WT-Specific Primer: 5'-GGTAACCCTCGCCTTGTTTCA-3'

"Common" Primer: 5'-TGGGCAGCAGCTAAAGTTAC-3'

Annealing temperature: 57°C (mutant or wild type)

Size: 721 bp.

sup-1(e2636)

Mutant-Specific Primer: 5'-CTCTAGCATCTTCCACTACTAA-3'

WT-Specific Primer: 5'-CTCTAGCATCTTCCACTACTAG-3'

"Common" Primer: 5'-CTTAGTGGCCTATACGTCTC-3'

Annealing temperature: 62°C (mutant or wild type)

Size: 624 bp.

***sup-1* is not *unc-123*:**

A previous report (Walthall *et al.* 1993) suggested that the *unc-123* and *sup-1* loci might correspond to the same gene. We sequenced the *sup-1* region in *unc-123(jd5)* and *unc-123(jd5jd10)* mutants, and neither of the *unc-123* strains had any changes in the *sup-1* coding sequence or splice sites. We conclude that *unc-123* and *sup-1* are separate genes.

Transcriptional reporters and expression patterns of *C. elegans* SUP-1-like proteins:

F52A8.1: The reporter had 5082 bp of upstream sequence driving expression of NLS-CFP. We did not observe any expression in transgenic animals. Meissner *et al.* (2009) observed expression in muscles.

F58F12.4: The reporter had 2374 bp of upstream sequence driving expression of NLS-CFP. We observed bright fluorescence in the vulva, a few head neurons, and lateral lines down the body; these may be the lateral hypodermal (seam) cells.

F42C5.6: The reporter had 1020 bp of upstream sequence driving NLS-CFP expression. We observed fluorescence in two cell bodies with processes, at the level of the vulva. The posterior cell sends a process to the posterior, and another process anterior, terminating at the pharynx. The anterior cell body sends a process anterior.

C08H9.15: The reporter had 3405 bp of upstream sequence driving expression of NLS-CFP. We observed fluorescence in the intestine, hypodermis, and vulva.

R10E11.9: The reporter had 3466 bp of upstream sequence driving expression of NLS-CFP. We observed very faint fluorescence.

ZK353.2: The reporter had 2130 bp of upstream sequence driving expression of NLS-CFP. We observed fluorescence in the vulva and in the tail; the tail fluorescence may be the anal or intestinal muscles. In addition, we also observed fluorescence in a few cells in the head; these may be interlabial neurons, or sheath and socket cells.

K06B4.3: The reporter had 1122 bp of upstream sequence driving expression of NLS-CFP. We observed extremely faint fluorescence, including in cells around the pharynx; these may be pharyngeal cells or neurons.

Table S1. Phenotypic progeny classes segregating from *dpy-18 sup-1/+ +; unc-17/+* heterozygotes

<i>unc-17</i> Allele	Protein Change	Phenotype Data				Total	Segregation Ratios***				Suppress?
		WT	Unc	Dpy	UncDpy		WT	Unc	Dpy	UncDpy	
<i>e245</i>	G347R	100	9	33	0	142	11.3	1.0	3.7	0.0	Yes
<i>e283</i>	R60W	130	34	42	8	214	9.7	2.5	3.1	0.6	No
<i>e284</i>	R60W	55	19	23	7	104	8.5	2.9	3.5	1.1	No
<i>e327*</i>	S211F	47	16	17	10	90	8.4	2.8	3.0	1.8	No
<i>e334</i>	G103R	98	27	34	10	169	9.3	2.6	3.2	0.9	No
<i>e335</i>	A206V	41	15	16	4	76	8.6	3.2	3.4	0.8	No
<i>e359</i>	G347R	87	12	30	0	129	10.8	1.5	3.7	0.0	Yes
<i>e464</i>	E98K	40	10	11	6	67	9.6	2.4	2.6	1.4	No
<i>e795</i>	A206V	47	15	9	5	76	9.9	3.2	1.9	1.1	No
<i>p300</i>	G347R	210	18	84	0	312	10.8	0.9	4.3	0.0	Yes
<i>cha-1</i> alleles**											
<i>b401</i>	<i>cha-1</i>	56	23	21	3	103	8.7	3.6	3.3	0.5	No
<i>p1154</i>	<i>cha-1</i>	47	23	21	6	97	7.8	3.8	3.5	1.0	No
<i>unc-18</i> allele**											
<i>e81</i>	<i>unc-18</i>	59	15	25	8	107	8.8	2.2	3.7	1.2	No

dpy-18(e364) sup-1(e995) males were crossed to hermaphrodites homozygous for the *unc-17* allele to be tested. The resulting (non-Unc) F₁ hermaphrodite cross progeny will have a *dpy-18 sup-1/+ +; unc-17/+* genotype; five such F₁ hermaphrodites were transferred individually to plates, and allowed to lay eggs for ~24 hr at 20°. Two days later, 1 - 4 plates were selected (based on the total number of animals) and phenotypes were scored for all F₂ progeny on these plates.

*Hard to score (mild Unc)

**Two alleles of *cha-1* (*b401* and *p1154*) and the *unc-18* allele *e81* were included as additional controls. *cha-1* and *unc-17* transcripts are derived from a single complex locus (Rand & Russell 1984; Rand 1989); *unc-18* mutants, like *unc-17* mutants, are strongly resistant to cholinesterase inhibitors such as aldicarb (Hosono *et al.* 1987; Miller *et al.* 1996).

***Segregation Ratios: The number of animals in each phenotypic class was divided by the total number of animals, and the result was multiplied by 16; this permits comparison with predicted dihybrid ratios. We expect these ratios to be **11:1:4:0** for **dominant suppression**, **9:3:4:0** for **recessive suppression**, and **9:3:3:1** for **no suppression** (ignoring recombination between *dpy-18* and *sup-1* in each case). The *dpy-18* and *sup-1* loci are ~3.2 cM apart (map positions on LG III are +8.85 for *dpy-18* and +12.09 for *sup-1*), so recombination between them will have very little effect on the segregation ratios.

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