Genes required for cellular UNC-6/Netrin localization in *Caenorhabditis elegans*

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Summary

UNC-6/Netrin is an evolutionarily conserved, secretory axon guidance molecule. In *C. elegans*, UNC-6 provides positional information to the axons of developing neurons, probably by establishing a concentration gradient from the ventral to the dorsal side of the animal. Although the proper localization of UNC-6 is important for accurate neuronal network formation, little is known about how its localization is regulated. Here, to examine the localization mechanism for UNC-6, we generated *C. elegans* expressing UNC-6 tagged with the fluorescent protein Venus, and identified thirteen genes required for the cellular Venus::UNC-6 localization. For example, in mutants of *unc-51*, *unc-14*, and *unc-104*, the neurons showed an abnormal accumulation of Venus::UNC-6 in the cell body, and little Venus::UNC-6 in the axon. An aberrant accumulation of Venus::UNC-6 in muscle cells was seen in mutants of *unc-18* and *unc-68*. *unc-51*, *unc-14*, and *unc-104* mutants also showed defects in the dorso-ventral axon guidance regulated by UNC-6, suggesting that the abnormal localization of this molecule also disturbed the positional information it provides. We propose that these genes regulate the process of UNC-6 secretion, including its expression, maturation, sorting, transport, or exocytosis. Our findings provide novel insight into the localization mechanism for the axon guidance molecule UNC-6/Netrin.
Introduction

A variety of axon guidance molecules and their receptors are critical for pathfinding axons to reach their precise targets (Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001; Dickson, 2002; Chilton, 2006; Killeen and Sybingco, 2008). Axon guidance molecules are expressed on the surface of cells or secreted into the extracellular space, and provide the positional information to axons. The axons express axon guidance receptors on their surface, and use them to receive positional information from the axon guidance molecules.

Netrin is an evolutionarily conserved axon guidance molecule that has both axonal attraction and repulsion activities (Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995). UNC-6 of Caenorhabditis elegans is a member of the Netrin family (Ishii et al., 1992). During C. elegans development, UNC-6 is expressed in ventral cells, including epidermoblasts, glia, neurons, muscle cells, and vulval precursor cells (Wadsworth et al., 1996; Asakura et al., 2007). UNC-6 is thought to establish a concentration gradient from the ventral to the dorsal side of the animal (Wadsworth, 2002), to provide ventral-dorsal positional information to attract some axons ventrally and to repel others, which extend dorsally (Hedgecock et al., 1990; McIntire et al., 1992; Wadsworth, 2002). In addition, UNC-6 provides positional information for cell migration (Hedgecock et al., 1990), synapse formation (Colón-Ramos et al., 2007; Poon et al., 2008), and cell polarity (Adler et al., 2006; Ziel et al., 2009). However, little is known about the molecular mechanisms of UNC-6 localization.

To examine the localization mechanisms of UNC-6, we generated C. elegans expressing UNC-6 tagged with the fluorescent protein Venus (Asakura et al., 2007), and identified thirteen genes required for the cellular Venus::UNC-6 localization, including unc-51, unc-14, unc-104, unc-18, and unc-68. In addition, unc-51, unc-14, and unc-104 mutants showed defects in UNC-6-mediated axon guidance, suggesting that the inappropriate UNC-6 localization also disturbed the positional information available to the axons. Our findings provide novel insight into the localization mechanisms of the axon guidance molecule UNC-6/Netrin.
Materials and methods

The general methods for growing and handling the *C. elegans* worms were described by Brenner (1974). The Bristol strain N2 was used as the standard wild-type strain.

Mutants used

Linkage group (LG) I: *unc-14(e57), unc-73(e936), unc-40(n324), unc-11(e47), unc-13(e51), and unc-101(m1).*

LG II: *unc-104(e1265), unc-53(e404), syd-1(ju82), rrf-3(pk1426), and unc-10(y250).*

LG III: *unc-25(e156), unc-36(e251), unc-64(e246), unc-116(rh24, e2310), hpl-2(ok917), and snt-1(md290).*

LG IV: *unc-5(e53), unc-44(e362), unc-33(mn407), egl-19(n582), osm-3(p802), and ghIs9(Venus::unc-6; str-3p::dsRed2).*

LG V: *unc-51(e369), unc-68(e540, r1162), snb-1(md247), unc-31(e169), nrx-1(ds1), and rpm-1(js410).*

LG X: *unc-6(ev400), unc-18(e234), nuIs9 (unc-5::GFP) and unc-10(e102).*

Imaging

To analyze the UNC-6 localization in vivo, we used *ghIs9(Venus::unc-6; str-3p::dsRed2)* (Asakura et al., 2007) as an integrant strain. Each animal was mounted on a 2.5% agarose pad in M9 buffer containing 5% sodium azide and observed using a fluorescence microscope (Axioplan2, Zeiss). Images were taken using a confocal microscope LSM510 (Zeiss).

Mutagenesis and genetic mapping

The *gh* alleles were isolated in a screen performed according to standard protocols (Anderson, 1995). Briefly, *ghIs9(Venus::unc-6)* was mutagenized with ethylmethane sulphonate (EMS), and the F2 generation was screened for animals that exhibited localization defects of Venus::UNC-6. We screened the approximately 3000 haploid genomes. The *gh36* mutation was dominant, and the other mutations were recessive. SNP (single nucleotide polymorphism) mapping was used for genetic mapping in the CB4856 strain (Wicks et al., 2001, Davis et al., 2005). The map position was further refined by a complementation test.

RNAi analysis

Analysis using *unc-6* RNAi was performed as described by Asakura et al. (2007). Experiments using RNAi against autophagy-related genes were performed as described by Ogura and
Goshima (2006). In this paper, the RNAi-hypersensitive double mutant \textit{rtf-3(pk1426); hpl-2(ok917)} was used (Wang et al., 2005).

\textbf{Molecular analysis}

We used KOD-Plus (Toyobo, KOD-201) for the PCR experiments. The \textit{unc-18} ORF was amplified from pPCR2.1F27D9#F1R1 (Gengyo-Ando et al., 1993), and inserted into the mCherry (McNally et al., 2006) expression vector pNW5 (\textit{myo-3} promoter::\textit{mCherry}) or pNW19 (H20 promoter::\textit{mCherry}), resulting in the \textit{myo-3p::unc-18::mCherry} (pNW7) and \textit{H20p::unc-18::mCherry} (pNW20) constructs.

\textbf{Transformation of \textit{C. elegans}}

Transformation was performed as described by Mello et al. (1991). \textit{myo-2p::mRFP} (Campbell et al., 2002) (pmy2P-mR) was used as the marker (10 ng/\mu l). pBluescript SK+ was used to equalize the amount of DNA in the transformations. Mixtures of [pBluescript SK+ (40 ng/\mu l), pmy2P-mR (10 ng/\mu l), and pNW7 (50 ng/\mu l)] or [pBluescript SK+ (40 ng/\mu l), pmy2P-mR (10 ng/\mu l), and pNW20 (50 ng/\mu l)] were injected into the YC81[\textit{unc-18(e234); ghIs9}] adult gonad, resulting in YC84[\textit{unc-18(e234); ghIs9; ghEx20(myo-3p::unc-18::mCherry; myo-2p::mRFP)}] or YC85[\textit{unc-18(e234); ghIs9; ghEx21(H20p::unc-18::mCherry; myo-2p::mRFP)}].
Results

Genetic screening to identify genes that regulate the Venus::UNC-6 localization.

To identify genes that regulate the UNC-6 localization, we used strain *ghIs9*, which expresses functional and visible Venus::UNC-6 (Asakura et al., 2007). On the wild-type background, Venus::UNC-6 was mainly detected in ventral cells, including epidermoblasts, glia, neurons, muscle cells, and vulval precursor cells (Fig. 1). Venus::UNC-6 was also detected in dorsal muscle cells in the tail (Fig. 1). In male worms, Venus::UNC-6 was expressed in the ray (data not shown). This distribution pattern of Venus::UNC-6 in a wild-type genetic background was similar to that of a previous report using immunostaining of 3xHA-tagged UNC-6 (Wadsworth, et al., 1996), except for our additional observation of Venus::UNC-6 expression in P6.p descendants, ventral muscle, dorsal muscle in the tail, and in the ray of the male tail (Fig. 1, data not shown). These differences were probably owing to the different fixation methods used, because our HA antibody staining of *urIs1* (3xHA::UNC-6, Wadsworth, et al., 1996) with Bouin’s fixative (Nonet et al., 1997) showed an identical pattern to that of *ghIs9* (data not shown). In addition, an *unc-6* promoter::mRFP fusion gene also showed the same pattern (data not shown).

Since UNC-6 is a secreted protein, we expected that some Venus::UNC-6 would also be detected outside of cells. However, we could not detect any extracellular Venus::UNC-6, probably owing to its weak fluorescence intensity. Therefore, we focused our analysis on the cellular Venus::UNC-6 localization, and in this paper, the “localization” of Venus::UNC-6 refers to the cellular Venus::UNC-6 localization. We think that the observed cellular Venus::UNC-6 localization largely reflects process of its secretion.

To identify the genes responsible for the proper localization of Venus::UNC-6, we took two approaches: (1) we performed EMS mutagenesis screening with *ghIs9* to isolate mutant alleles in which the mislocalization of Venus::UNC-6 was observed, and (2) we examined the localization of Venus::UNC-6 in existing mutants of genes related to vesicular transport and secretion. From these experiments, we isolated or identified thirteen genes required for the proper localization of Venus::UNC-6 (Table 1). These mutants had no morphological defects on cell shapes except for axons and the penetrance of the localization phenotype in each mutant was 100% (data not shown).
Mutants showing an abnormal localization of Venus::UNC-6 in neurons.

In the neurons, Venus::UNC-6 on a wild-type background showed a punctate distribution pattern throughout the cytoplasm and axons, and was excluded from the nucleus (Fig. 2A). We identified four genes required for this localization of Venus::UNC-6 within neurons (Fig. 2B-E, Table 1). In mutant worms for these genes, the Venus::UNC-6 in other cell types showed a normal distribution pattern (data not shown), suggesting that its localization in neurons required a unique mechanism. In the \textit{unc-51(e369)} and \textit{unc-14(e57)} mutants, the Venus::UNC-6 in neurons accumulated in the cell body with an uneven distribution, and little was present in the axon (Fig. 2B, C). UNC-51 is a serine/threonine kinase homologous to yeast Atg1, which is required for autophagy (Ogura et al., 1994; Matsuura et al., 1997; Straub et al., 1997; Mizushima, 2007). UNC-14 is a RUN domain protein that is UNC-51’s binding partner (Ogura et al., 1997). Although their precise molecular functions are unknown, UNC-51 and UNC-14 have been implicated in membrane trafficking. Among their functions, UNC-51 and UNC-14 regulate the localization (trafficking) of UNC-5, a receptor for UNC-6 in neurons (Ogura and Goshima, 2006).

UNC-51 is also required for autophagy in \textit{C. elegans} (Meléndez et al., 2003). However, it is unlikely that defects in the traditional autophagy pathway caused the abnormal Venus::UNC-6 localization, because when the RNAis of other genes required for autophagy (\textit{bec-1/atg-6, atg-7, lgg-1/atg-8}, and \textit{atg-18}) were expressed in an RNAi-hypersensitive mutant strain, the Venus::UNC-6 localization was normal (Table 2).

In the \textit{unc-104(e1265)} mutant, the Venus::UNC-6 in the neurons accumulated evenly throughout the cell body, but little Venus::UNC-6 was present in the axon (Fig. 2D, F). \textit{unc-104} encodes a kinesin motor protein homologous to KIF1A (Otsuka et al., 1991; Hirokawa and Noda, 2008). These findings therefore suggest that UNC-6 is transported by the motor protein UNC-104 from the neural cell body to the axon. In the \textit{gh23} mutant, the Venus::UNC-6 in neurons also accumulated evenly in the cell body, with very little appearing in the axon (Fig. 2E, F), similar to the \textit{unc-104(e1265)} mutant (Fig. 2D). Complementation analysis revealed that \textit{gh23} was not an allele of \textit{unc-104}. These similar patterns indicated that the responsible gene product of \textit{gh23} might be involved in UNC-104’s function in UNC-6 transport.

Mutants showing an abnormal localization of Venus::UNC-6 in muscle cells.

In the muscle cells, Venus::UNC-6 on a wild-type background was distributed throughout the
cytoplasm and was excluded from the nucleus (Fig. 3A). We identified seven genes required for this Venus::UNC-6 localization within muscle cells (Table 1). In worms mutant for these genes, Venus::UNC-6 accumulated abnormally in the muscle cells (Fig. 3B-D), but showed a normal distribution pattern in other cell types in these mutants (data not shown), suggesting that, like neurons, muscle cells have a specific mechanism for controlling UNC-6 localization. Complementation analysis with existing mutants revealed that gh21, gh22, gh28, gh29, gh32, and gh37 were alleles of unc-68. The reference alleles unc-68(e540) and unc-68(r1162) exhibited the same phenotype (Fig. 3B). UNC-68 is homologous to ryanodine receptors (RyRs), which regulate body-wall muscle contraction by controlling Ca\(^{2+}\) release from the endoplasmic reticulum (ER) (Maryon et al., 1996; Sakube et al., 1997; Zalk et al., 2007). This Ca\(^{2+}\) release from the ER mediated by UNC-68 is also required for regulating both spontaneous and evoked neurotransmitter release (Liu et al., 2005). These results therefore suggest that the Ca\(^{2+}\) release from the ER mediated by UNC-68 is required for proper UNC-6 localization in muscle.

Complementation analysis revealed that gh27 and gh38 were allelic mutants. gh27 and gh38 mapped to LGV, in which unc-68 is located (Fig. S1); therefore, we performed complementation analysis with unc-68(e540) to observe the effect on the accumulation of Venus::UNC-6 in muscle. Venus::UNC-6 accumulated in the muscle cells of the heterologous strain e540/gh27, but the level was clearly lower than in the homologous strains e540/e540, gh27/gh27, and gh38/gh38 (data not shown). Since unc-68(e540) is thought to be a null allele (Sakube et al., 1997), inter-allelic complementation of the sort observed in hypomorphic alleles, such as for unc-5 (Merz et al. 2001), was unlikely. In addition, unc-68(e540) shows Unc phenotype, however, gh27 and gh38 didn’t. These findings indicated that gh27 and gh38 interacted genetically with unc-68, but were not allelic to it. Complementation analysis revealed that gh33 and gh26 were alleles of unidentified genes (Table 1).

Venus::UNC-6 also accumulated in the muscle cells of the unc-18(e234) mutant (Fig. 3C). UNC-18, which is homologous to the SM (Sec1/Munc18-like) proteins and regulates a multi-step vesicle exocytosis process in neurons, in cooperation with SNARE proteins (Gengyo-Ando et al., 1993; Malsam et al., 2008; Südhof and Rothman, 2009). Therefore, we next examined whether neuronal UNC-18 regulated the localization of Venus::UNC-6 in muscle.

UNC-18 in neurons acts non-cell autonomously to regulate the localization of Venus::UNC-6 in muscle.
Although Venus::UNC-6 was accumulated abnormally in the muscle cells of the unc-18(e234) mutant, UNC-18 is expressed by the neurons, not by the muscles (Gengyo-Ando et al., 1993). To analyze the cell autonomy of this molecule, we examined whether muscle- or neuron-specific expression of unc-18 could rescue the Venus::UNC-6 localization defect in unc-18(e234) mutants. In these rescue experiments, we used the myo-3 promoter for muscle-specific expression (Okkema et al., 1993) and the H20 promoter for neuron-specific expression (Shioi et al., 2001).

The muscle-specific expression of unc-18 did not rescue the abnormal Venus::UNC-6 accumulation in the muscle cells of unc-18(e234) mutants (Fig. 4A), but its neuron-specific expression did (Fig. 4B). These results suggested that neuronal UNC-18 cell-non-autonomously regulates the localization of UNC-6 in muscle. The possible involvement of presynaptic input in the regulation of UNC-6's localization in muscle is also supported by the phenotypic defects we observed in the syd-1(ju82); rpm-1(ju44) double mutant, in which Venus::UNC-6 accumulated in the muscle cells (Fig. 3D). The syd-1(ju82); rpm-1(ju44) mutant shows a severe defect in locomotion, since the number of synapses is reduced and presynaptic components are disrupted (Nakata et al., 2005). Our findings therefore suggest that synaptic activity is required for the proper localization of UNC-6 in the muscle.

As unc-18 and unc-68 regulate the exocytosis of synaptic vesicles in neurons, we examined the localization of Venus::UNC-6 in the mutants of other exocytosis-related genes, such as snt-1/synaptotagmin, snb-1/synaptobrevin, unc-64/syntaxin, and unc-13/Munc13 (Nonet et al., 1993; Nonet et al., 1998; Ogawa et al., 1998; Saifee et al., 1998; Maruyama and Brenner 1991; Malsam et al., 2008; Südhof and Rothman, 2009). However, the localization of Venus::UNC-6 was not altered in these mutants (Table 2). Thus, the mechanism by which UNC-18 regulates the UNC-6 localization remains an unsolved question.

**Mutant showing the abnormal localization of Venus::UNC-6 in vulval precursor cells (VPCs).**

We identified one gene required for the VPC localization of Venus::UNC-6. In the mutant gh25, the intensity of Venus::UNC-6 was increased in the VPCs and vulval cells (Fig. 5B, C, Table 1), but its distribution was normal in the other cell types of this mutant (data not shown), suggesting that VPCs have a specific mechanism for secreting UNC-6.

Since the VPCs are the UNC-6 source for HSN axon guidance (Asakura et al., 2007), we analyzed the HSN axon morphology on gh25 mutants. We found that gh25 mutants had HSN
guidance defects (Fig. 5D), suggesting that the VPC localization defects in gh25 mutants result in the impaired UNC-6 secretion and the HSN axon guidance defects.

**Mutants showing a high level of Venus::UNC-6 expression in all the UNC-6-expressing cells.**

We identified one gene required globally for the normal expression level of Venus::UNC-6. In the mutant gh36, the Venus::UNC-6 fluorescence intensity was increased in all of the Venus::UNC-6-expressing cells (Fig. 6B, D, F, G, Table 1). We found that UNC-5::GFP fluorescence intensity was not increased in gh36 (Fig. S2), indicating that gh36 did not affect transgene expression or fluorescence levels in general. Unlike the other mutants, the distribution of the intracellular Venus::UNC-6 distribution was normal for all cell types. Therefore, the responsible gene product in gh36 may negatively regulate unc-6 expression.

**unc-104, unc-18, and unc-68 are required for AVM ventral guidance**

Given that UNC-6 is required for dorso-ventral axon guidance, we predicted that the altered localization of Venus::UNC-6 in these identified mutants probably caused or reflected UNC-6 secretion defects, which should result in dorso-ventral axon guidance defects. In support of this hypothesis, unc-51 and unc-14 mutants show defective dorsally-directed axon pathfinding by DD/VD neurons (McIntire et al., 1992). Furthermore, unc-51 and unc-14 interact genetically with unc-6 to influence DD/VD axon guidance (Ogura and Goshima 2006). To test this hypothesis, we examined ventrally-directed AVM axon guidance in unc-104, unc-18, and unc-68 mutants.

The AVM cell body is located laterally, and its axon grows ventrally to the ventral nerve cords (Fig. 7A, B). The ventrally directed AVM axon guidance is effected by two parallel guidance cues, UNC-6/Netrin and Slt-1/Slit. Ventral UNC-6 attracts and dorsal SLT-1 repels the AVM axon (Fig. 7A; Hao et al., 2001). The AVM axon grows ventrally at L1 stage. We confirmed that unc-104, unc-18 and unc-68 mutants had localization defects of Venus::UNC-6 at the L1 stage as well (Fig. S3).

In unc-104(e1265), unc-18(e234), and unc-68(e540) mutants, minor defects of the AVM axon guidance were observed (Fig. 7G). Although unc-104; unc-6 double mutants exhibited no enhancement in AVM defects compared to unc-6 single mutants, unc-104; slt-1 double mutants
exhibited enhanced defects compared to slt-1 single mutants. These results suggest that unc-104 is in the unc-6-pathway, and is consistent with our hypothesis that the UNC-6-localization defect by unc-104 mutation reflects the axon guidance defect of the AVM neuron.

On the other hand, unc-68; unc-6 double mutants exhibited enhanced defects compared to unc-6 single mutants (Fig. 7G). unc-18; slt-1 and unc-68; slt-1 double mutants exhibited suppressed defects compared to slt-1 single mutants (Fig. 7G). The enhancement in unc-68; unc-6 double mutants may result from the accumulation of other axon guidance molecules, such as SLT-1, in unc-68 mutants. The absence of enhancement in unc-18; slt-1 and unc-68; slt-1 double mutants suggest that UNC-6 expressed by the muscle cells do not participate in the AVM ventral guidance. UNC-6 is also required for synaptic development of the DA9 neuron (Poon et al., 2008). We analyzed the synaptic development of the DA9 neuron in unc-18 and unc-68 mutants. However, we did not found their defects and it is reported that synaptic development of the DA9 neuron is normal in unc-18 mutants (data not shown, Zhao and Nonet, 2000), suggesting that UNC-6 expressed by muscle cells does not participate in the synaptic development of the DA9 neuron as well. UNC-6 is also required for dorsal migration of distal tip cells (Hedgecock et al., 1990). However, we did not found dorsal migration defects of the distal tip cells in unc-18 and unc-68 mutants (data not shown). We could not find clear defects on the UNC-6 function in unc-18 and unc-68 mutants.
Discussion

Identification of genes required for proper Venus::UNC-6 localization.

The secretory axon guidance molecule UNC-6/Netrin provides positional information for axon guidance (Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001; Dickson, 2002; Chilton, 2006; Killeen and Sybingco, 2008). The temporal and spatial expression of UNC-6/Netrin has been well documented and plays an important role in neural network formation (Wadsworth et al., 1996; Watanabe et al., 2006; Asakura et al., 2007). A model for the patterning mechanism, in which the Netrin receptor frazzled rearranges secreted Netrin in *D. melanogaster* has been proposed (Hiramoto et al., 2000). However, little is known about the localization/secretion mechanisms of UNC-6/Netrin.

In this study, we identified thirteen genes required for the cellular localization of Venus::UNC-6 in *C. elegans*. The responsible genes include four required only for Venus::UNC-6’s appropriate localization in neurons, seven for only its localization in muscle, one for VPCs only (vulval precursor cells), and one controlling the global expression level of Venus::UNC-6. We think that, in these cells, these genes regulate the processes associated with the secretion of UNC-6, including its expression, maturation, sorting, transport, and exocytosis, and that each of these cell types has a specific mechanism for regulating UNC-6 localization.

Genes required for the UNC-6 localization in neurons.

Venus::UNC-6 is expressed in neurons, including PVT, AVG, RIF, AVA, AVB, PVQ, VA, and VB (Wadsworth et al., 1996; Asakura et al., 2007). In the *unc-51*, *unc-14*, *unc-104*, and gh23 mutants, Venus::UNC-6 accumulated in the neuronal cell bodies, but there was little fluorescence in the axons, suggesting that these genes regulate the transport of UNC-6 from the neuronal cell body to the axon. In *unc-51*, *unc-14*, and *unc-104* mutants, UNC-6/Netrin-mediated dorso-ventral axonal guidance is defective (McIntire et al., 1992; this study). In addition, *unc-51*, *unc-14*, and *unc-104* interact genetically with *unc-6* (Ogura and Goshima, 2006; this study). These findings suggest that the defects in UNC-6 transport in these mutants disrupted the normal secretion of UNC-6 from the neurons.

UNC-104 is a homolog of a kinesin motor protein, KIF1A, which transports the precursors of synaptic vesicles (SVs) and dense core vesicles (DCVs) from neural cell bodies to synapses.
(Otsuka et al., 1991; Hall and Hedgecock, 1991; Yonekawa et al., 1998; Zahn et al., 2004; Hirokawa and Noda, 2008). The accumulation of Venus::UNC-6 in the unc-104 mutant was very similar to the accumulation of SVs and DCVs in unc-104 mutants (Hall and Hedgecock, 1991; Nonet, 1999; Zahn et al., 2004), supporting our hypothesis that UNC-104 transports vesicles containing UNC-6 from the neuronal cell body to the axon. In addition, UNC-6 may be secreted by neurons at the synapse, since UNC-104/KIF1A transports the precursors of synaptic vesicles (Hirokawa and Noda, 2008). The phenotype of the gh23 mutant was very similar to that of unc-104, therefore the responsible gene product in gh23 may be involved in UNC-104 function. In mutants of unc-116 and osm-3, which encode the kinesin motor proteins UNC-116/KIF5 and OSM-3/KIF17, respectively (Patel et al., 1993; Shakir et al., 1993), the localization of Venus::UNC-6 was identical to that in wild-type animals, indicating that these kinesin motor proteins are not involved in the transport of UNC-6. This is consistent with the results of a series of recent studies showing that the kinesin and dynein motor proteins use specific adaptor or scaffold proteins to recognize and bind different cargoes (Hirokawa and Takemura, 2005).

The Venus::UNC-6 accumulation phenotype in the unc-51 and unc-14 mutants was different from that in the unc-104 mutant. In the unc-51 and unc-14 mutants, Venus::UNC-6 accumulated unevenly in the cell body, whereas in the unc-104 mutant, it accumulated evenly throughout the cell body except in the nucleus. The difference may reflect the roles of these gene products in the regulation of UNC-6 localization. UNC-51 is a serine/threonine kinase, which binds UNC-14, a RUN domain protein (Ogura et al., 1994; Ogura et al., 1997). UNC-51 and UNC-14 are predicted to play important roles in vesicle trafficking. UNC-51 can bind VAB-8, a kinesin-like protein, and phosphorylate VAB-8 in vitro (Wolf et al., 1998; Lai and Garriga, 2004). UNC-51 and VAB-8 cooperatively regulate the posterior axonal outgrowth of CAN neurons. UNC-14 can bind UNC-16/JIP, and together they cooperate with a kinesin motor protein UNC-116/KIF5 to transport synaptic vesicles (Sakamoto et al., 2005). The UNC-51 of D. melanogaster phosphorylates UNC-76/FEZ1, a kinesin heavy chain adaptor protein, to regulate axonal transport (Toda et al., 2008). In addition, UNC-51 and UNC-14 regulate the localization (trafficking) of UNC-5, a receptor for UNC-6, in neurons (Ogura and Goshima 2006). Therefore, UNC-51 and UNC-14 may regulate the processing involved in UNC-6’s localization in neuronal cell bodies, including UNC-6’s maturation, selection, or transport (Fig. 8A). UNC-104 may function as a motor protein in concert with the activities of UNC-51 and UNC-14 to transport vesicles containing UNC-6 from cell bodies to axons.

UNC-51 is also required for autophagy in C. elegans (Meléndez et al., 2003). However, the traditional autophagy pathway probably does not participate in the Venus::UNC-6 localization,
since the RNAi of other genes required for autophagy resulted in normal Venus::UNC-6 localization.

**Genes required for appropriate UNC-6 localization in muscle cells.**

We identified seven genes required for the proper localization of Venus::UNC-6 in muscle cells. These were *unc-18, unc-68, syd-1, rpm-1*, and the responsible genes of *gh33* (*gh27, gh38*), and *gh26*. In the *unc-18* mutant, Venus::UNC-6 accumulated in muscle cells. This finding was unexpected, because UNC-18 belongs to the SM (Sec1/Munc18-like) protein family (Gengyo-Ando et al., 1993; Malsam et al., 2008; Südhof and Rothman, 2009), which regulates vesicle exocytosis by interacting with syntaxin, a member of the SNARE proteins in neurons (Sassa et al., 1999; Weimer et al., 2003; Malsam et al., 2008; Südhof and Rothman, 2009). Indeed, UNC-18 is expressed in neurons but not in muscle (Gengyo-Ando et al., 1993). We showed that the muscle-specific expression of *unc-18* did not rescue the Venus::UNC-6 accumulation in *unc-18* mutants, whereas the neuron-specific expression of UNC-18 did. These results suggested that the UNC-18 in neurons regulates the UNC-6 localization in muscle.

How does UNC-18 regulate the Venus::UNC-6 localization in muscle? We showed that, except for UNC-18, mutations in the genes encoding SNARE proteins and other proteins that are essential for the exocytosis of neurotransmitters (Malsam et al., 2008; Südhof and Rothman, 2009) did not cause the abnormal localization of Venus::UNC-6 (Table 2), indicating that the traditional machinery for neurotransmitter release is not involved in the UNC-18 function. In addition, there were no defects in the localization of Venus::UNC-6 in the muscle of the *unc-13, unc-25, and unc-31* mutants (Table 2). UNC-13/Munc13, UNC-25/glutamate decarboxylase 1, and UNC-31/CAPS are, respectively, required for acetylcholine (ACh) secretion (Maruyama and Brenner 1991;), GABA synthesis (Jin et al., 1999), and neuropeptide secretion (Berwin et al., 1998; Speese et al., 2007). These findings therefore indicate that traditional neurotransmitters such as ACh, GABA, or neuropeptides are also not involved in the UNC-18 function. The normal Venus::UNC-6 localization observed in the *unc-25* mutant indicated that the muscle homeostasis regulated by GABA (Garcia et al., 2007) is not involved in the UNC-18 function. It is also unlikely that the abnormal Venus::UNC-6 localization in *unc-18* resulted from the severe defect in muscle contraction observed in this mutant, since the *unc-13* mutant, which also displays a paralyzed phenotype, showed normal localization of Venus::UNC-6.

We propose that an unknown signal from neurons, mediated by UNC-18, regulates the UNC-6 localization in muscle (Fig. 8B). The unknown signal is probably secreted at synapses,
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since Venus::UNC-6 also accumulated in the muscles of syd-1; rpm-1 mutants, in which presynaptic components are reduced and disrupted (Nakata et al., 2005). The secretion machinery associated with this unknown signal is probably different from that of traditional neurotransmitters. Although the signal and its secretory machinery remain unidentified, the characterization of gh27, gh33, or gh26 may reveal the mechanisms responsible for the proper localization of UNC-6 in muscle.

Venus::UNC-6 also accumulated in muscle in the unc-68 mutant. UNC-68 is homologous to ryanodine receptors (RyRs), a class of Ca\(^{2+}\) channels (Maryon et al., 1996; Sakube et al., 1997; Zalk et al., 2007). UNC-68 plays important roles in muscle contraction, mediating Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the endoplasmic reticulum (ER). In addition, CICR in neurons is required for regulating both spontaneous and evoked neurotransmitter release (Liu et al., 2005). Therefore, the simplest explanation for the UNC-68 function is that, in the muscle cells and/or neurons, CICR from the ER mediated by UNC-68 is required for the proper localization of UNC-6 in muscle. Unfortunately, however, we could not make an UNC-68 construct that was expressed specifically in the muscle or neurons, because of the large size of the ORF (15.6 kb). It therefore remains unclear where and how UNC-68 regulates the localization of UNC-6 in muscle cells. As discussed above, however, the aberrant accumulation of Venus::UNC-6 in the muscle cells of the unc-18 mutant argues for a neuronal function of UNC-68 in the regulation of UNC-6’s localization in muscle.

We found that unc-18 and unc-68 mutants had localization defects of Venus::UNC-6 in muscle cells, but our results suggest that unc-18 and unc-68 do not appear to participate in known UNC-6 functions including AVM axon guidance, DA9 synaptic development, cell migration of distal tip cells. We think that UNC-6 expressed by muscle cells may have unknown functions, or that functions of UNC-6 expressed by muscle cells may be masked by UNC-6 expressed by neurons. Another possibility is that, although unc-18 and unc-68 have the Venus::UNC-6 localization defects in muscle cells, UNC-6 secretion from the muscle cells could be normal in these mutants.

**Genes required for the UNC-6 localization in vulval precursor cells.**

UNC-6 expressed by the VPCs (vulval precursor cells) is essential for proper HSN axon guidance (Asakura et al., 2007). Venus::UNC-6 was accumulated in the VPCs in the gh25 mutant. In addition, HSN axon guidance defects were observed in the gh25 mutants. Taken together, these findings suggest that the responsible gene of gh25 plays an important role in the
UNC-6 secretion by the VPCs.

The Venus::UNC-6 accumulation in the gh25 mutant resembled the EGL-17/FGF accumulation in EGL-17-secretion defective mutants (Kamikura and Cooper, 2003; Kamikura and Cooper, 2006). EGL-17 is a secreted protein that is expressed in the VPCs and attracts sex myoblasts (Burdine et al., 1998). The UNC-6 secretion by the VPCs may be mediated by a mechanism similar to that for EGL-17 secretion.

Genes required for UNC-6 expression.

In mice, the transient expression of Netrin 1 at the dorsal spinal cord is required for the accurate axon guidance of primary sensory axons (Watanabe et al., 2006), and transcription factors such as Runx3 are also involved in the axon guidance of primary sensory axons (Inoue et al., 2002). In C. elegans, the upregulation of UNC-6 in the vulval precursor cells is essential for the complex axon guidance of the HSN neurons (Asakura et al., 2007). These observations suggest that accurate construction of the nervous system requires axon guidance molecules to be expressed at the proper time, place, and concentration.

In the mutant gh36, the Venus::UNC-6 fluorescence intensity was increased in all the cells that expressed Venus::UNC-6, without any alteration in its intracellular distribution. Therefore, the responsible gene of this mutant may negatively regulate unc-6 expression. The analysis of the gh36 mutant may provide information about how UNC-6 expression is regulated.

Other mechanisms of Netrin localization.

In D. melanogaster, a model for Netrin’s patterning mechanism has been proposed, in which Netrin’s localization is regulated by interaction with its receptor, Frazzled/UNC-40 (Hiramoto et al., 2000). In C. elegans, we could not find any alteration in the localization of Venus::UNC-6 in unc-40 mutants (data not shown). Since the nervous system of C. elegans is extremely simple compared to that of D. melanogaster, such a re-distribution mechanism of UNC-6 might not be required for it to form. Determining whether such a re-distribution mechanism for Netrin is conserved in mammalian species is an important issue to be addressed in the future.

Finally, we used strong loss-of-function alleles or RNAi to examine the localization of Venus::UNC-6 on the known mutants or genes. Since all of them are not null alleles, it remains possible that the genes listed in the Table 2 could be involved in regulating the localization of
UNC-6. In addition, all the new mutants except for gh36 were recessive alleles, therefore, we think that these mutants except for gh36 are loss-of-function alleles. However, they could be gain-of-function alleles, since we did not identify the responsible genes.
Acknowledgments

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Figure legends

**Fig. 1. Expression of Venus::UNC-6 in living *C. elegans***.
An L4 worm. Right lateral view, anterior is to the right. (A) Expression of Venus::UNC-6. (B) DIC image of the same worm. Bar represents 50 μm. As described previously (Wadsworth et al., 1996), ventral neurons expressed Venus::UNC-6 (magenta arrows). In addition, ventral muscle (magenta arrowheads), dorsal muscle (white arrowheads), and vulval cells (magenta circle) expressed Venus::UNC-6. Venus::UNC-6 expressed by the ventral muscle in the central part of the worm is not visible, because the intensity of the Venus::UNC-6 in these cells was very low.

**Fig. 2. Mutants that exhibit abnormal Venus::UNC-6 localization in neurons.**
(A) Wild type. (B) *unc-51(e369)* mutant. (C) *unc-14(e57)* mutant. (D) *unc-104(e1265)* mutant. (E) *gh23* mutant. Arrows indicate cell bodies. White lines indicate axons. Anterior is to the right, lateral view. Bars represent 5 μm. In the wild-type background, Venus::UNC-6 showed a punctate distribution throughout the cell body and axon, except for the nucleus. In the *unc-51(e369)*, *unc-14(e57)*, *unc-104(e1265)*, and *gh23* mutants, Venus::UNC-6 was accumulated in the neural cell bodies, and little Venus::UNC-6 was in the axons. (F) Relative fluorescence intensities of the Venus::UNC-6 in neuronal cell of *unc-104(e1265)* and *gh23* mutants to wild type worms. In each case, 20 neuronal cell bodies were examined and the results were averaged. Error bars show the standard error. *p < 0.01 (Student’s t-test). In *unc-51(e369)* mutants and *unc-14(e57)* mutants, Venus::UNC-6 is accumulated in some part of the neuronal cell body. Therefore, we couldn’t compare the fluorescence intensity between these mutants.

**Fig. 3. Mutants that exhibit abnormal Venus::UNC-6 localization in muscle cells.**
Ventral muscle. (A) Wild type. (B) *unc-68(e540)* mutant. (C) *unc-18(n234)* mutant. (D) *syd-1(ju82); rpm-1(js410)* double mutant. Arrowheads indicate ventral muscle cells. Anterior is to the right, lateral view. Bar represents 10 μm. Within the muscle cells, Venus::UNC-6 showed a punctate distribution throughout the cell body except for the nucleus. In *unc-68(e540)*, *unc-18(e234)* and *syd-1(ju82); rpm-1(js410)*, Venus::UNC-6 accumulated as fluorescent clusters in the muscle cells.

**Fig. 4. UNC-18 functions cell-non-autonomously in neurons to regulate the Venus::UNC-6 localization in muscle.**
Venus::UNC-6 in a worm expressing muscle-specific UNC-18 (A) or neuron-specific UNC-18
(B). YC84[unc-18(e234); ghIs9(Venus::unc-6); ghEx20(myo-3p::unc-18::mCherry; myo-2p::mRFP)] worms were used for the muscle-specific expression, and YC85[unc-18(e234); ghIs9(Venus::unc-6); ghEx21(H20p::unc-18::mCherry; myo-2p::mRFP)] worms were used for the neuron-specific expression. Arrowheads indicate the accumulated Venus::UNC-6 in muscle cells. Arrows indicate the myo-2p::mRFP fluorescence used as an expression marker. Anterior is to the right. Bar represents 20 µm. The accumulation of Venus::UNC-6 was observed in worms expressing muscle-specific UNC-18 (A), but little accumulation of Venus::UNC-6 was observed with the neuron-specific expression (B).

Fig. 5. A mutant that exhibits abnormal Venus::UNC-6 localization in the vulval precursor cells (VPCs).
P6.p descendants (VPCs) at the eight-cell stage. (A) Wild type. (B) gh25 mutant. Anterior is to the right, lateral view. Bar represents 10 µm. In the gh25 mutant, Venus::UNC-6 accumulated abnormally in the VPCs. (C) Relative fluorescence intensities of the Venus::UNC-6 in the gh25 mutants to wild type worms. In each case, 10 VPCs were examined and the results were averaged. Error bars show the standard error. * p < 0.01 (Student’s t-test). (D) The percentage of HSN axons showing guidance defects. R represents HSN R. L represents HSN L. tph-1p::gfp (Sze et al., 2000) was used to visualize the HSN neuron. In gh25 mutants, HSN axon guidance defects were observed.

Fig. 6. A mutant that exhibits a high level of Venus::UNC-6 expression in all the cells that express it.
(A, B) Cell bodies of ventral neurons. (C, D) Ventral muscle cells. (E, F) P6.p descendants (VPCs) at the eight-cell stage. (A, C, E) Wild type. (B, D, F) gh36 mutants. Arrows indicate neuronal cell bodies. Arrowheads indicate muscle cells. Anterior is to the right, lateral view. Bars represent 5 µm (A, B), 10 µm (C, D, E, F). In the gh36 mutant, the Venus::UNC-6 level was increased in all of the cells that normally express it. (G) Relative fluorescence intensities of the Venus::UNC-6 in the neuronal cell bodies of gh36 mutants to wild type worms. In each case, 20 neuronal cell bodies were examined and the results were averaged. Error bars show the standard error. * p < 0.01 (Student’s t-test).

Fig. 7. AVM axon guidance defects in UNC-6/Netrin-localization mutants.
(A) Schematic drawing of the ventral guidance signals for the AVM (Hao et al., 2001). The AVM axon grows ventrally, attracted by ventral UNC-6/Netrin (green) and repelled by dorsal
SLT-1/Slit (red).

(B-F) The AVM morphology. (B) Wild type. (C) unc-6(ev400). (D) unc-104(1265). (E) unc-18(e234). (F) unc-68 (e540). zdIs5(mec-4::gfp) was used to visualize the AVM neuron (Clark and Chiu, 2003). An arrow indicates the AVM cell body. Right lateral view, anterior is to the right. Bar represents 10 μm. In the wild-type worm, the AVM neuron extended its axon ventrally and then anteriorly. In the unc-6(ev400), unc-104(1265), unc-18(e234), and unc-68 (e540) mutants shown, the AVM neuron extended anteriorly without navigating ventrally.

(G) The AVM axon guidance defects in unc-6(ev400), slt-1(eh15), unc-18(e234), unc-68(e540) and unc-104(e1265) and their double mutants. The genetic distance between unc-6 and unc-18 is too small to make double mutants. n=200-967. * p < 0.01 (Student’s t test). N.S., not significant. unc-104(e1265) did not enhance unc-6(ev400), but it strongly enhanced slt-1(eh15). unc-68(e540) enhanced unc-6(ev400). unc-68(e540) and unc-18(e234) suppressed slt-1(eh15).

Fig. 8. Models of the UNC-6/Netrin localization.

(A) Model for the localization of UNC-6/Netrin in neurons: UNC-104/KIF1A transports UNC-6/Netrin-containing vesicles along the axon. UNC-51 and its binding partner UNC-14 are required for the maturation, selection, or transport of UNC-6/Netrin.

(B) Model for the localization of UNC-6/Netrin localization in muscle: UNC-6/Netrin secretion requires an unknown UNC-18/Sec1-mediated signal from neurons. The UNC-6/Netrin secretion also requires an UNC-68/RyR-mediated process, which may involve calcium release through UNC-68/RyR from the ER in neurons and/or muscle cells.

Fig. S1. Genetic map positions of the new genes.

Each linkage group is described in a horizontal line. Genetic position of SNPs used for mapping is described above each chromosome (Davis et al., 2005). The genes identified in this screen are shown under each chromosome.

Fig. S2. In gh36 mutants, the expression of UNC-5::GFP is not increased.

(A, B) Z-stack images of UNC-5::GFP (Killeen et al., 2002) in the distal tip cell (DTC). (A) wild type. (B) gh36 mutant. A bar represents 10 μm. (C) Relative fluorescence intensities of the UNC-5::GFP in the DTC of gh36 mutants to wild type worms. In each case, the 20 DTCs were examined and the results were averaged. Error bars show the standard error. * p < 0.01 (Student’s t-test).

Fig. S3. Venus::UNC-6 localization at L1 atage.
(A-D) Neural cell bodies. (A) Wild type. (B) unc-51(e369) mutant. (C) unc-14(e57) mutant. (D) unc-104(e1265) mutant. Bars represent 2 μm.

(E-G) Muscle cells. (E) Wild type. (F) unc-68(e540) mutant. (G) unc-18(e234) mutant. Bar represents 5 μm.
T. Asakura Fig. 2

A. Wild type

B. unc-51(e369)

C. unc-14(e57)

D. unc-104(e1265)

E. gh23

F. Relative intensity

unc-104(e1265)  gh23  Wild type

*
T. Asakura Fig. 3

A

Wild type

B

unc-68(e540)

C

unc-18(e234)

D

syd-1(ju82); rpm-1(ju44)
Neuron-specific

T. Asakura Fig. 4

Muscle-specific

Neuron-specific
Wild type

Relative intensity

% defective

T. Asakura Fig. 5
## Table 1  Summary of mutants displaying Venus::UNC-6 localization defects

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<th>gene</th>
<th>allele</th>
<th>LG</th>
<th>Mammalian homolog</th>
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<tr>
<td>Accumulated unevenly in the cell body of neurons.</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>unc-14</em> <em>1</em></td>
<td>e57, gh34</td>
<td>I</td>
<td>-</td>
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<td>e369</td>
<td>V</td>
<td>ULK1</td>
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<td>Accumulated evenly in the cell body of neurons.</td>
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<td></td>
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<tr>
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<td>e1265</td>
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<tr>
<td>ND</td>
<td>gh23</td>
<td>II</td>
<td>ND</td>
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<tr>
<td>Accumulated in muscle cells.</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>gh33</td>
<td>I</td>
<td>ND</td>
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<tr>
<td><em>unc-68</em> <em>4</em></td>
<td>e540, gh21, gh22, gh28, gh29, gh32, gh37, r1162</td>
<td>V</td>
<td>ryanodine receptor</td>
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<td>gh27, gh38</td>
<td>V</td>
<td>ND</td>
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<tr>
<td>ND</td>
<td>gh26</td>
<td>X</td>
<td>ND</td>
</tr>
<tr>
<td><em>unc-18</em> <em>5</em></td>
<td>e234</td>
<td>X</td>
<td>Sec1/Munc18</td>
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<td><em>syd-1; rpm-1</em> <em>6</em></td>
<td>ju82; ju44</td>
<td>II/V</td>
<td>SYDE1; Pam/Highwire</td>
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<td>Accumulated in vulval precursor cells and vulval cells.</td>
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<td>ND</td>
<td>gh25</td>
<td>IV</td>
<td>ND</td>
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<tr>
<td>Strongly expressed in <em>unc-6</em> expressing cells.</td>
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<tr>
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<td>IV</td>
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Table 2 Summary of mutants displaying normal Venus::UNC-6 localization

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<td>CAPS</td>
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<td>Rab3A</td>
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<td>Atg8/LC3</td>
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<td>NAV</td>
</tr>
</tbody>
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A

---

SLT-1/Slit

- -

UNC-6/Netrin

+++

B

C

D

E

F

Wild type

unc-6(ev400)

unc-104(e1265)

unc-18(e234)

unc-68(e540)

G

slt-1(eh15); unc-104(e1265)

slt-1(eh15); unc-68(e540)

slt-1(eh15); unc-18(e234)

unc-6(e400); unc-104(e1265)

unc-6(e400); unc-68(e540)

unc-6(e400); unc-18(e234)

unc-104(e1265)

unc-68(e540)

unc-18(e234)

slt-1 (eh15)

unc-6(ev400)

Wild Type

% defective

0 10 20 30 40 50 60 70 80 90

* NS ND
Fig. S2

A

B

C

\( gh36 \)

Wild Type

Relative intensity

0

1

NS
Fig. S3

A  Wild type
B  unc-51(e369)
C  unc-14(e57)
D  unc-104(e1265)
E  Wild type
F  unc-68(e540)
G  unc-18(e234)