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Genes Required for Cellular UNC-6/Netrin Localization in *Caenorhabditis elegans*

Taro Asakura, Naoko Waga, Ken-ichi Ogura[1] and Yoshio Goshima

Department of Molecular Pharmacology and Neurobiology, Yokohama City University Graduate School of Medicine, Yokohama, 236-0004, Japan

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ABSTRACT

UNC-6/Netrin is an evolutionarily conserved, secretory axon guidance molecule. In *Caenorhabditis 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 13 genes, which are involved in the cellular localization of Venus::UNC-6. For example, in *unc-51*, *unc-14*, and *unc-104* mutants, the neurons showed an abnormal accumulation of Venus::UNC-6 in the cell body and less than normal level of Venus::UNC-6 in the axon. An aberrant accumulation of Venus::UNC-6 in muscle cells was seen in *unc-18* and *unc-68* mutants. *unc-51*, *unc-14*, and *unc-104* mutants also showed defects in the guidance of dorso-ventral axons, suggesting that the abnormal localization of UNC-6 disturbed the positional information it provides. We propose that these genes regulate the process of UNC-6 secretion: expression, maturation, sorting, transport, or exocytosis. Our findings provide novel insight into the localization mechanism of the axon guidance molecule UNC-6/Netrin.

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, providing the positional information to axons, are expressed either on the surface of cells or secreted into the extracellular space. The axons, receiving positional information from the axon guidance molecules, express axon guidance receptors at the growth cone.

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 the ventral cells, including epidermoblasts, glia, neurons, muscle cells, and vulval precursor cells (VPCs) (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 while repelling others to 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 13 genes required for the cellular localization of Venus::UNC-6, including *unc-51*, *unc-14*, *unc-104*, *unc-18*, and *unc-68*. In addition to being involved in the localization of UNC-6, *unc-51*, *unc-14*, and *unc-104* mutants also showed defects in UNC-6-mediated axon guidance, suggesting that the inappropriate UNC-6 localization 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.

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.110.116293/DC1.

[1]*Corresponding author:* Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama, 236-0004, Japan. E-mail: kenogura@med.yokohama-cu.ac.jp

Mutations 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-3*p::*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-3*p::*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 was 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 F_2 generation was screened for animals that exhibited localization defects of Venus::UNC-6. We screened ~3000 haploid genomes. The *gh36* mutation was dominant, and the other mutations were recessive. Single nucleotide polymorphism (SNP) 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 article, the RNAi-hypersensitive double mutant *rrf-3(pk1426); hpl-2(ok917)* was used (Wang *et al.* 2005).

Molecular analysis: We used KOD-Plus (Toyobo, KOD-201) for the PCR experiments. The *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 (*myo-3* promoter::*mCherry*) or pNW19 (*H20* promoter::*mCherry*), resulting in the *myo-3*p::*unc-18*::*mCherry* (pNW7) and *H20*p::*unc-18*::*mCherry* (pNW20) constructs.

Transformation of *C. elegans*: Transformation was performed as described by Mello *et al.* (1991). *myo-2*p::*mRFP* (Campbell *et al.* 2002) (pmy2P-mR) was used as the marker (10 ng/μl). pBluescript SK+ was used to equalize the amount of DNA in the transformations. Mixtures of [pBluescript SK+ (40 ng/μl), pmy2P-mR (10 ng/μl), and pNW7 (50 ng/μl)] or [pBluescript SK+ (40 ng/μl), pmy2P-mR (10 ng/μl), and pNW20 (50 ng/μl)] were injected into the YC81[*unc-18(e234); ghIs9*] adult gonad, resulting in YC84[*unc-18(e234); ghIs9; ghEx20(myo-3p::unc-18::mCherry; myo-2p::mRFP)*] or YC85[*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 (Figure 1). Venus::UNC-6 was detected in dorsal muscle cells in the tail (Figure 1). In male worms, Venus::UNC-6 was expressed in the ray (data not shown). The general distribution pattern of Venus::UNC-6 in the wild-type genetic background was similar to that of 3xHA-tagged UNC-6, reported previously (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 (Figure 1, data not shown). These differences were probably due to the different fixation methods used, because with Bouin's fixative (Nonet *et al.* 1997), HA staining of *urIs1* (3xHA::UNC-6, Wadsworth *et al.* 1996) showed an identical pattern to *ghIs9* (data not shown). In addition, an *unc-6* promoter::mRFP fusion gene also showed the same pattern (data not shown).

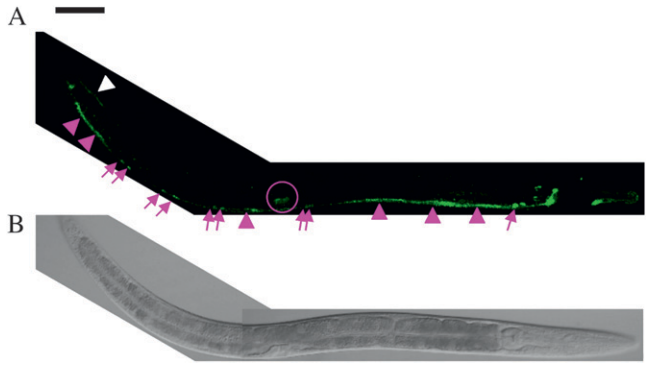

Figure 1.—Expression of Venus::UNC-6 in living *C. elegans*. An L4 worm. Right lateral view, anterior is to the right. (A) Expression of Venus::UNC-6. (B) DIC image of the same worm. Bar, 50 μm. As described previously (Wadsworth *et al.* 1996), ventral neurons expressed Venus::UNC-6 (magenta arrows). 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.

Since UNC-6 is a secreted protein, we expected that some Venus::UNC-6 would be detected outside of the 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 so in this article, the "localization" of Venus::UNC-6 refers to not the extracellular but the cellular localization of Venus::UNC-6. We believe that the observed cellular Venus::UNC-6 localization largely reflects the 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

TABLE 1

Summary of mutants displaying Venus::UNC-6 localization defects

Gene	Allele	LG	Mammalian homolog	Reference or source
		Accumulated unevenly in the cell body of neurons		
unc-14	*e57, gh34*	I	—	Ogura *et al.* (1997)
unc-51	e369	V	ULKI	Ogura *et al.* (1994)
		Accumulated evenly in the cell body of neurons		
unc-104	*e1265*	II	KIFIA	Otsuka *et al.* (1991)
ND	*gh23*	II	ND	
		Accumulated in muscle cells		
ND	*gh33*	I	ND	
unc-68	*e540, gh21, gh22, gh28, gh29, gh32, gh37, r1162*	V	Ryanodine receptor	Maryon *et al.* (1996); Sakube *et al.* (1997)
ND	*gh27, gh38*	V	ND	
ND	*gh26*	X	ND	
unc-18	*e234*	X	Sec1/Munc18	Gengyo-Ando *et al.* (1993)
syd-1; rpm-1	*ju82; ju44*	II/V	SYDEI; Pam/Highwire	Nakata *et al.* (2005)
		Accumulated in vulval precursor cells and vulval cells		
ND	*gh25*	*IV*	ND	
		Strongly expressed in *unc-6*-expressing cells		
ND	*gh36*	*IV*	ND	

ND, not determined.

secretion. From these experiments, we isolated or identified 13 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 (Figure 2A). We identified four genes required for this localization of Venus::UNC-6 within the neurons (Figure 2, B–E; Table 1). In these mutant worms, Venus::UNC-6 was expressed abnormally in the neurons, but the expression in other cell types was similar to that in the wild type (data not shown), suggesting that UNC-6 localization in the neurons is regulated by a unique mechanism. In *unc-51(e369)* and *unc-14(e57)* mutants, the Venus::UNC-6 was accumulated unevenly in the neuronal cell body, and little Venus::UNC-6 was present in the axon (Figure 2, B and 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, a RUN domain protein, is the binding partner of UNC-51 (Ogura *et al.* 1997). Although the precise molecular functions are unknown, UNC-51 and UNC-14 have been implicated in membrane trafficking and localization of UNC-5, which is the receptor for UNC-6 (Ogura and Goshima 2006).

UNC-51 has been reported to be involved in the autophagy in *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 the RNAis of other genes required for autophagy (*bec-1/atg-6*, *atg-7*, *lgg-1/atg-8*, and *atg-18*) in an RNAi-hypersensitive mutant strain showed the normal Venus::UNC-6 localization (Table 2). In *unc-104(e1265)* mutants, the Venus::UNC-6 was accumulated evenly throughout the neuronal cell body, and little Venus ::UNC-6 was present in the axon (Figure 2, D and F). *unc-104* encodes a kinesin motor protein, which is homologous to KIF1A in vertebrate (Otsuka *et al.* 1991; Hirokawa and Noda 2008). These findings therefore suggest that UNC-6 might be transported by the motor protein UNC-104 from the neuronal cell body to the axon. In *gh23* mutants, Venus::UNC-6 in neurons also accumulated evenly in the cell body, with very little appearing in the axon (Figure 2, E and F), which was similar to that in *unc-104(e1265)* mutants (Figure 2D). Complementation analysis revealed that *gh23* was not an allele of *unc-104*. The similar expression patterns indicated that the responsible gene product of *gh23* might be involved in the molecular function of UNC-104 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 (Figure 3A). We identified seven genes required for this Venus::UNC-6 localization within muscle cells (Table 1). In these mutant worms, Venus::UNC-6 was accumulated abnormally in the muscle cells (Figure 3, B–D), but a normal distribution pattern in other cell types was observed (data not shown), suggesting that, like

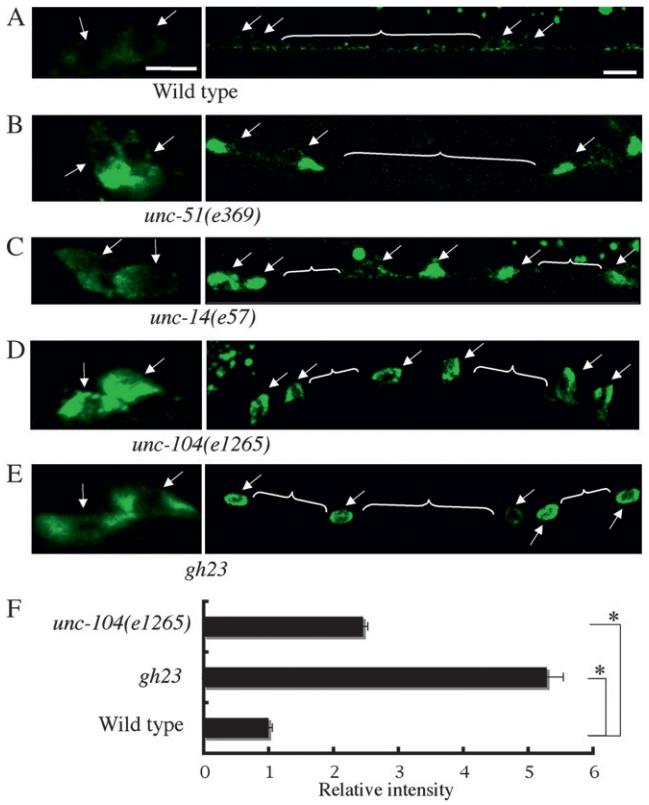

Figure 2.—Mutants that exhibit abnormal Venus::UNC-6 localization in neurons. (A) Wild type. (B) *unc-51(e369)* mutant. (C) *unc-14(e57)* mutant. (D) *unc-104(e1265)* mutant. (E) *gh23* mutant. Arrows indicate cell bodies. White lines indicate axons. Anterior is to the right, lateral view. Bars, 5 μm. 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 could not compare the fluorescence intensity between these mutants.

neurons, a unique mechanism for controlling UNC-6 localization exists in muscle cells. 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 (Figure 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 release of Ca^{2+} 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. Since *gh27* and *gh38* are mapped to LGV, in which *unc-68* is located (supporting information, Figure S1), we performed complementation analysis with *unc-68(e540)*. Venus::UNC-6 accumulated in the muscle cells of the *trans*-heterozygous strain *e540/gh27*, but the level was clearly lower than that of the homozygous 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), interallelic 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, but *gh27* and *gh38* did not. 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 was also accumulated in the muscle cells of the *unc-18(e234)* mutants (Figure 3C). UNC-18, which is homologous to the SM (Sec1/Munc18-like) proteins, regulates a multistep 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 regulates 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 gene, 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 (Figure 4A), but its neuron-specific expression did (Figure 4B). These results suggested that neuronal UNC-18 cell nonautonomously 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 (Figure 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.

Since *unc-18* and *unc-68* are known to regulate the exocytosis of synaptic vesicles in neurons, we exam-

TABLE 2

Summary of mutants displaying normal Venus::UNC-6 localization

	Gene	Allele	Mammalian homolog	Reference or source
Molecular motors				
	unc-116	*e2310, rh24*	KIF5	Patel *et al.* (1993)
	osm-3	*p802*	KIF17	Shakir *et al.* (1993)
Exocytosis-related proteins				
	snt-1	*md290*	Synaptotagnmin	Nonet *et al.* (1993)
	snb-1	*md247*	Synaptobrevin	Nonet *et al.* (1998)
	unc-64	*e246*	Syntaxin	Ogawa *et al.* (1998); Saifee *et al.* (1998)
	unc-31	*e169, e928*	CAPS	Speese *et al.* (2007)
	unc-13	*e51*	Munc13	Maruyama and Brenner (1991)
	egl-19	*n582*	L-type Ca^{2+} channel	Lee *et al.* (1997)
	nrx-1	*ds1*	Neurexin	Shen *et al.* (2007)
	unc-10	*e102*	RIM	Koushika *et al.* (2001)
	rab-3	*y250*	Rab3A	Nonet *et al.* (1997)
UNC-6/netrin receptors				
	unc-5	*e53*	UNC5H	Leung-Hagesteijn *et al.* (1992)
	unc-40	*n324*	DCC	Chan *et al.* (1996)
Autophagy-related proteins				
	bec-l/atg-6	*RNAi*	Atg6/beclin1	Meléndez *et al.* (2003)
	atg-7	*RNAi*	Atg7	Meléndez *et al.* (2003)
	lgg-1/atg-8	*RNAi*	Atg8/LC3	Meléndez *et al.* (2003)
	atg-18	*RNAi*	Atg18/WIPI1	Meléndez *et al.* (2003)
Others				
	unc-25	*e156*	Glutamate decarboxylase 1	Jin *et al.* (1999)
	unc-33	*e204, mn407*	CRMP2	Li *et al.* (1992)
	unc-44	*e362*	Ankyrin	Otsuka *et al.* (1995)
	unc-16	*jul46*	JIP3	Byrd *et al.* (2001)
	unc-101	*ml*	AP-1	Lee *et al.* (1994)
	unc-53	*e234*	NAV	Stringham *et al.* (2002)

ined 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* (Maruyama and Brenner 1991; Nonet *et al.* 1993; Nonet *et al.* 1998; Ogawa *et al.* 1998; Saifee *et al.* 1998; 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.

Mutants showing the abnormal localization of Venus::UNC-6 in VPCs: We identified one gene required for the localization of Venus::UNC-6 in VPCs. In *gh25* mutants, the intensity of Venus::UNC-6 was increased in the VPCs and vulval cells (Figure 5, B and C; Table 1), but its distribution was normal in the other cell types (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 in *gh25* mutants. We found that *gh25* mutants had HSN guidance defects (Figure 5D), suggesting that the abnormal Venus::UNC-6 expression 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 *gh36* mutants, Venus::UNC-6 fluorescence intensity was increased in all of the Venus::UNC-6-expressing cells (Figure 6, B, D, F, G; Table 1). However, we found that UNC-5::GFP (Killeen *et al.* 2002) fluorescence intensity was not increased in *gh36* mutants (Figure 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 expression was normal for all cell types. Therefore, the responsible gene product of *gh36* may negatively regulate *unc-6* expression.

***unc-104*, *unc-18*, and *unc-68* are required for anterior ventral microtubule cell 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). In 2006, we reported that *unc-51* and *unc-14* interact genetically with *unc-6* to influence

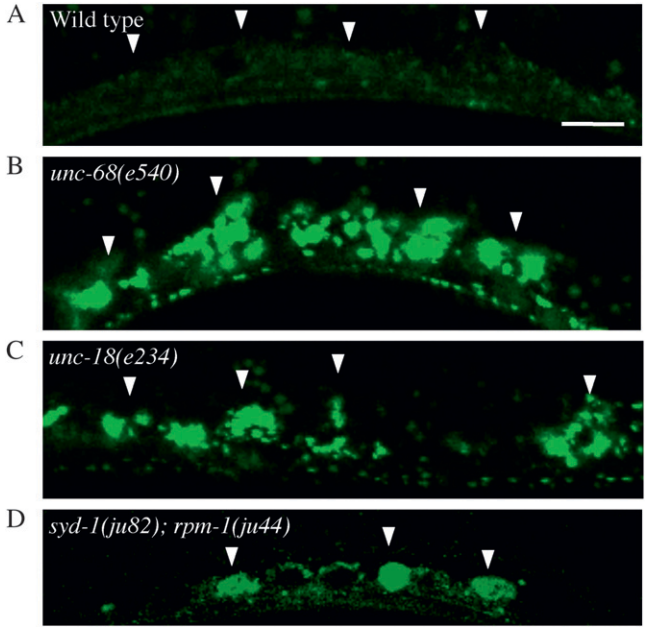

FIGURE 3.—Mutants that exhibit abnormal Venus::UNC-6 localization in muscle cells. Ventral muscle. (A) Wild type. (B) *unc-68(e540)* mutant. (C) *unc-18(n234)* mutant. (D) *syd-1(ju82); rpm-1(js410)* double mutant. Arrowheads indicate ventral muscle cells. Anterior is to the right, lateral view. Bar, 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.

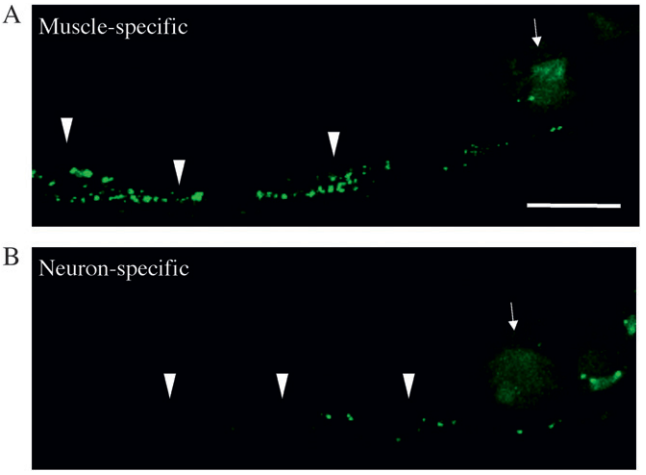

FIGURE 4.—UNC-18 functions cell nonautonomously in neurons to regulate the Venus::UNC-6 localization in muscle. Venus::UNC-6 in a worm expressing muscle-specific UNC-18 (A) or neuron-specific UNC-18 (B). YC84[*unc-18(e234); ghIs9 (Venus::unc-6); ghEx20(myo-3p::unc-18::mCherry; myo-2p::mRFP)*] worms were 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, 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).

DD/VD axon guidance (OGURA and GOSHIMA 2006). To verify this hypothesis further, we examined ventrally directed anterior ventral microtubule cell (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 (Figure 7, A and B). The pathfinding of the AVM axon is affected by two parallel guidance cues, UNC-6/Netrin and Slt-1/Slit. Ventral UNC-6 attracts and dorsal SLT-1 repels the AVM axon (Figure 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 (Figure S3).

In *unc-104(e1265)*, *unc-18(e234)*, and *unc-68(e540)* mutants, minor defects of the AVM axon guidance were observed (Figure 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* has a role in the *unc-6*-pathway and is consistent with our hypothesis that the UNC-6 localization defect reflects the axon guidance defect of the AVM neuron.

Interestingly, *unc-68; unc-6* double mutants exhibited enhanced defects compared to *unc-6* single mutants (Figure 7G). *unc-18; slt-1* and *unc-68; slt-1* double mutants exhibited suppressed defects compared to *slt-1* single mutants (Figure 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 suggests 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 find the defects. It is also reported that synaptic development of the DA9 neuron is normal in *unc-18* mutants (data not shown; ZHAO and NONET 2000). These suggest that UNC-6 expressed by muscle cells does not participate in the synaptic development of the DA9 neuron. UNC-6 is also required for dorsal migration of distal tip cells (HEDGECOCK *et al.* 1990). However, we did not find the dorsal migration defects of the distal tip cells in *unc-18* and *unc-68* mutants as well (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 mol-

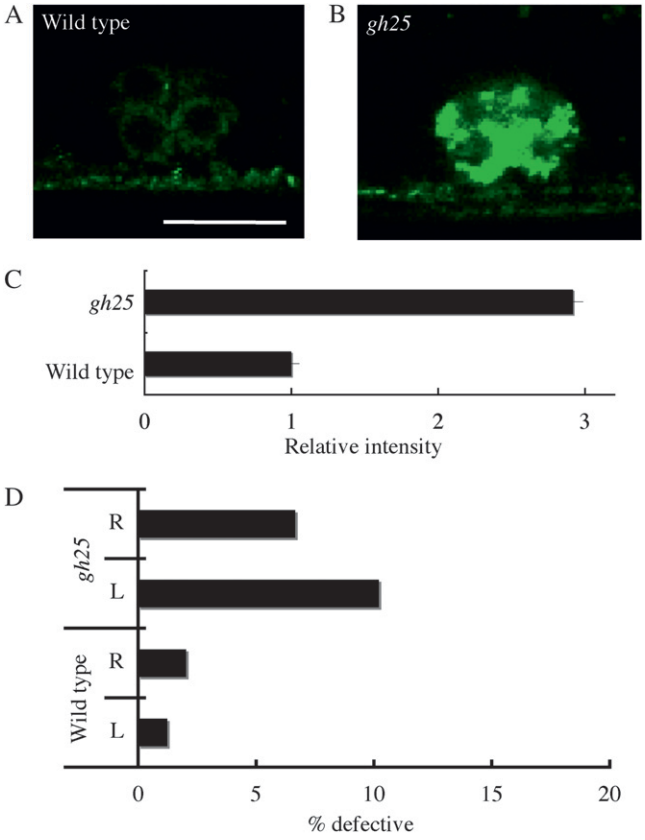

FIGURE 5.—A mutant that exhibits abnormal Venus::UNC-6 localization in the vulval precursor cells (VPCs). P6.p descendants (VPCs) at the eight-cell stage. (A) Wild type. (B) *gh25* mutant. Anterior is to the right, lateral view. Bar, 10 μm. In the *gh25* mutant, Venus::UNC-6 accumulated abnormally in the VPCs. (C) Relative fluorescence intensities of 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.

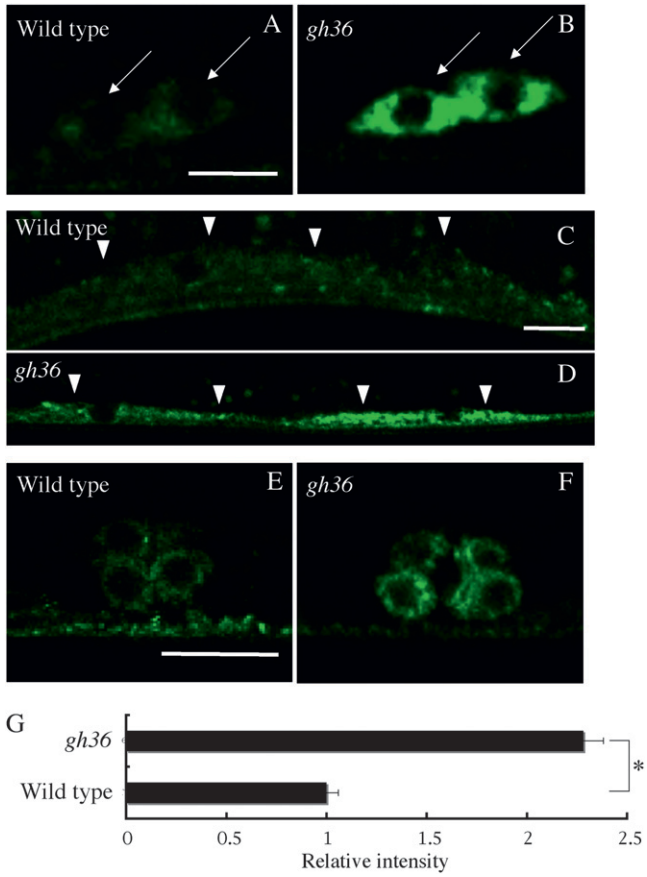

FIGURE 6.—A mutant that exhibits a high level of Venus::UNC-6 expression in all the cells that express it. (A and B) Cell bodies of ventral neurons. (C and D) Ventral muscle cells. (E and F) P6.p descendants (VPCs) at the eight-cell stage. (A, C, and E) Wild type. (B, D, and F) *gh36* mutants. Arrows indicate neuronal cell bodies. Arrowheads indicate muscle cells. Anterior is to the right, lateral view. Bars, 5 μm (A and B); 10 μm (C–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).

ecule 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 *Drosophila 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 13 genes required for the cellular localization of Venus::UNC-6 in *C. elegans*. Four genes were required specifically for the proper localization of Venus::UNC-6 in neurons, 7 for its localization in muscle, 1 for its localization in VPCs, and 1 for controlling the global expression level of Venus::UNC-6. We propose that, in these cells, these 13 genes regulate the processes associated with the secretion of UNC-6: 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 *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 neuro-

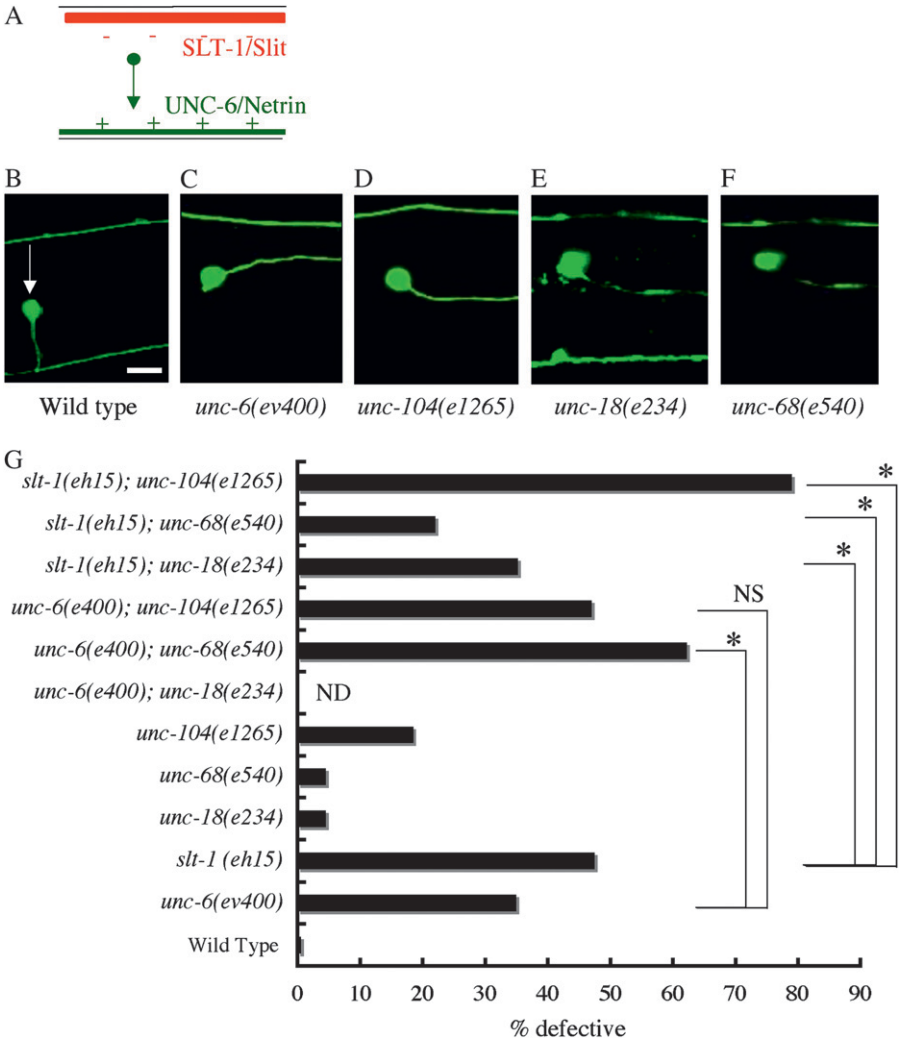

FIGURE 7.—AVM axon guidance defects in UNC-6/Netrin-localization mutants. (A) Schematic drawing of the ventral guidance signals for the AVM (HAO *et al.* 2001). The AVM axon grows ventrally, attracted by ventral UNC-6/Netrin (green) and repelled by dorsal SLT-1/Slit (red). (B–F) The AVM morphology. (B) 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, 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). NS, 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)*.

nal 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 the kinesin motor protein, KIF1A, which transports the precursors of synaptic vesicles (SVs) and dense core vesicles (DCVs) from neuronal cell bodies to synapses (HALL and HEDGECOCK 1991; OTSUKA *et al.* 1991; YONEKAWA *et al.* 1998; ZAHN *et al.* 2004; HIROKAWA and NODA 2008). The pattern of accumulation of Venus::UNC-6 was very similar to that 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 of *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 *unc-51* and *unc-14* mutants was different from that in *unc-104* mutant. In *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

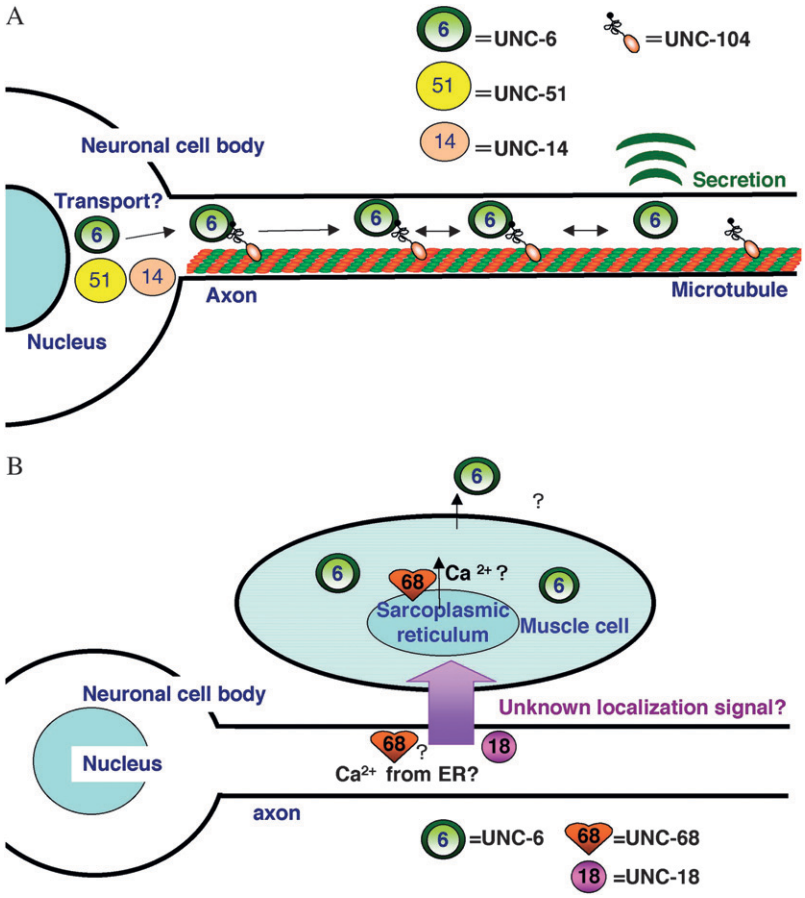

FIGURE 8.—Models of the UNC-6/Netrin localization. (A) Model for the localization of UNC-6/Netrin in neurons: UNC-104/KIF1A transports UNC-6/Netrin-containing vesicles along the axon. UNC-51 and its binding partner UNC-14 are required for the maturation, selection, or transport of UNC-6/Netrin. (B) Model 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.

kinase, which binds UNC-14, a RUN domain protein (OGURA *et al.* 1994, 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 (Figure 8A). Furthermore, 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 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 displayed 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 (Figure 8B). The unknown signal is probably secreted at synapses, 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, we could not make an UNC-68 construct that 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.

We found that *unc-18* and *unc-68* mutants had localization defects of Venus::UNC-6 in muscle cells. However, 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, and 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* mutants showed 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 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, 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 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 redistribution mechanism of UNC-6 might not be required for it to

form. Determining whether such a redistribution 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 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.

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GENETICS

Supporting Information
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Genes Required for Cellular UNC-6/Netrin Localization in *Caenorhabditis elegans*

Taro Asakura, Naoko Waga, Ken-ichi Ogura and Yoshio Goshima

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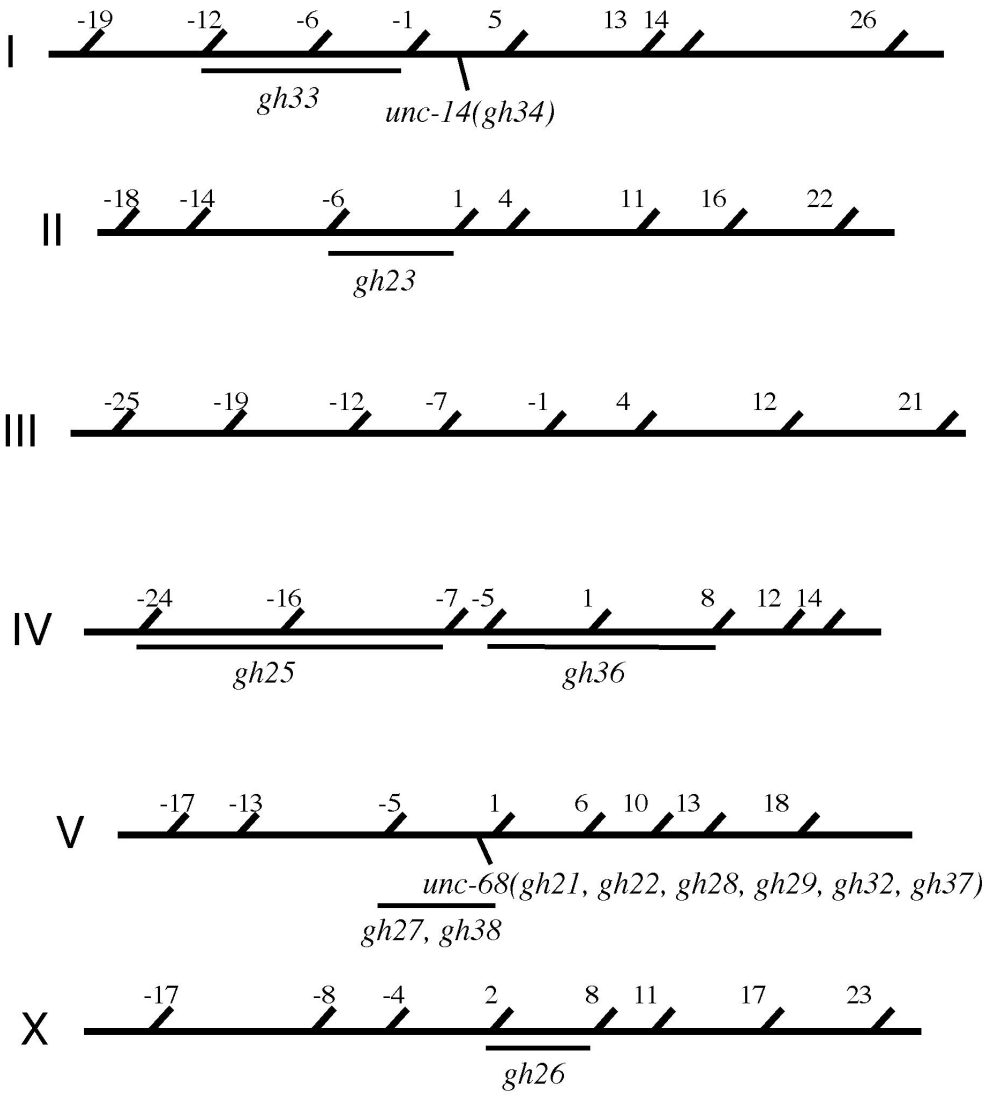

FIGURE S1.—Genetic map positions of the new genes. Each linkage group is described in a horizontal line. Genetic position of SNPs used for mapping is described above each chromosome (Davis et al., 2005). The genes identified in this screen are shown under each chromosome.

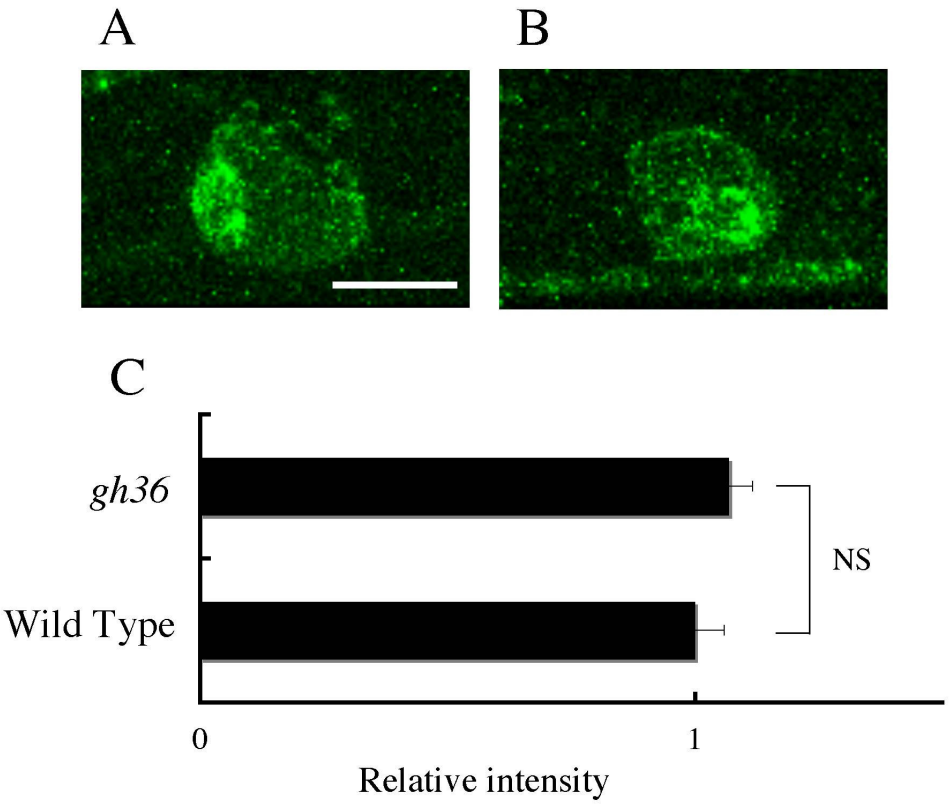

FIGURE S2.—In gh36 mutants, the expression of UNC-5::GFP is not increased. (A, B) Z-stack images of UNC-5::GFP (Killeen et al., 2002) in the distal tip cell (DTC). (A) wild type. (B) gh36 mutant. A bar represents 10 μm. (C) Relative fluorescence intensities of the UNC-5::GFP in the DTC of gh36 mutants 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).

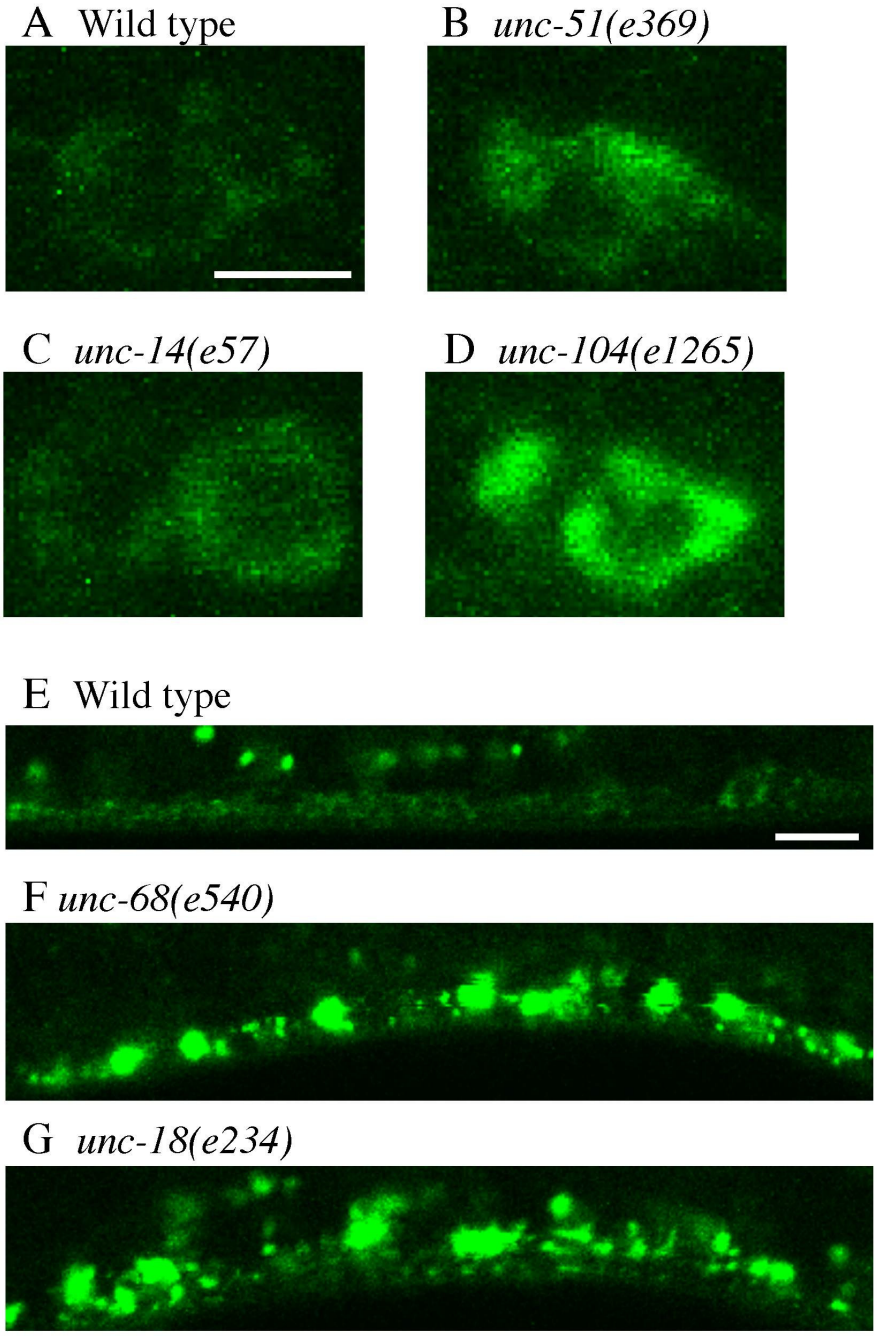

FIGURE S3.—Venus::UNC-6 localization at L1 atage. (A-D) Neural cell bodies. (A) Wild type. (B) unc-51(e369) mutant. (C) unc-14(e57) mutant. (D) unc-104(e1265) mutant. Bars represent 2 μm. (E-G) Muscle cells. (E) Wild type. (F) unc-68(e540) mutant. (G) unc-18(e234) mutant. Bar represents 5 μm.