The Roles of Multiple UNC-40 (DCC) Receptor-Mediated Signals in Determining Neuronal Asymmetry Induced by the UNC-6 (Netrin) Ligand

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ABSTRACT

The polarization of post-mitotic neurons is poorly understood. Preexisting spatially asymmetric cues, distributed within the neuron or as extracellular gradients, could be required for neurons to polarize. Alternatively, neurons might have the intrinsic ability to polarize without any preestablished asymmetric cues. In Caenorhabditis elegans, the UNC-40 (DCC) receptor mediates responses to the extracellular UNC-6 (netrin) guidance cue. For the HSN neuron, an UNC-6 ventral-dorsal gradient asymmetrically localizes UNC-40 to the ventral HSN surface. There an axon, which is ventrally directed by UNC-6. In the absence of UNC-6, UNC-40 is equally distributed and the HSN axon travels anteriorly in response to other cues. However, we find that a single amino acid change in the UNC-40 ectodomain causes randomly oriented asymmetric UNC-40 localization and a wandering axon phenotype. With UNC-6, there is normal UNC-40 localization and axon migration. A single UNC-6 amino acid substitution enhances the mutant phenotypes, whereas UNC-6 second-site amino acid substitutions suppress the phenotypes. We propose that UNC-40 mediates multiple signals to polarize and orient asymmetry. One signal triggers the intrinsic ability of HSN to polarize and causes randomly oriented asymmetry. Concurrently, another signal biases the orientation of the asymmetry relative to the UNC-6 gradient. The UNC-40 ectodomain mutation activates the polarization signal, whereas different forms of the UNC-6 ligand produce UNC-40 conformational changes that allow or prohibit the orientation signal.

A major challenge for developmental neuroscience has been to understand how axons are able to detect and follow molecular gradients of different extracellular guidance cues. Attractive guidance cues are proposed to stimulate cytoplasmic signaling pathways that promote actin polymerization (Huber et al. 2003). Thus the direction of axon outgrowth is directly linked to the extracellular gradient of the guidance cue; i.e., there is greater extension on the side of the neuron that is closest to the source of the cue. Netrins are bifunctional guidance cues that are attractive to some axons but repulsive to others. Studies have shown that the axon response to netrin is determined by the composition of netrin receptors on the cell surface and the internal state of the growth cone (Round and Stein 2007). The UNC-6 (netrin) guidance cue in Caenorhabditis elegans interacts with the UNC-40 (DCC) receptor to mediate attraction (Hedgecock et al. 1990; Ishii et al. 1992; Chan et al. 1996). The AVM and HSN neurons are useful for studying UNC-40-mediated responses to UNC-6. The cell bodies of these neurons are situated on the lateral body wall and send a single axon ventrally during larval development.

In AVM and HSN, a signaling module comprising UNC-6, UNC-40, phosphoinositide 3-kinase (PI3K), Rac, and MIG-10 (lamellipodin) is thought to transmit the directional information provided by the graded distribution of extracellular guidance cues to the internal cellular machinery that promotes directed outgrowth (Adler et al. 2006; Chang et al. 2006; Quinn et al. 2006, 2008). MIG-10 appears to provide an important link because this family of proteins can interact with proteins that promote actin polymerization, and it is associated with asymmetric concentrations of f-actin and microtubules in turning growth cones (Krause et al. 2004; Quinn et al. 2008). MIG-10 is observed as asymmetrically localized to the ventral site of axon outgrowth in developing HSN neurons. This MIG-10 localization is sensitive to the source of UNC-6. Normally, the source of UNC-6 is ventral; in the absence of UNC-6, there is an equal distribution of MIG-10 along the cell surface, whereas ectopic UNC-6 expression from dorsal muscles causes dorsal MIG-10 localization (Adler et al. 2006). The UNC-40 receptor is also asymmetrically localized in HSN, and this localization is also dependent on UNC-6 (Adler et al. 2006). UNC-40 signaling activates Rac GTPase, and MIG-10 interacts specifically with the activated Rac (Quinn et al. 2008). Therefore, the asym-

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metric activation of Rac through UNC-40 recruits asymmetric MIG-10 localization.

By activating or directing components to the surface nearest the UNC-6 source, the asymmetric distribution of UNC-6 could polarize the neuron. However, an alternative idea is suggested from studies of chemotaxing cells. This model predicts that chemoattractant signaling involves two different elements: one that activates the intrinsic ability of cells to generate asymmetry and another that biases the orientation of the asymmetry (Weidlich-Soldner and Li 2003). The polarization signal does not depend on the spatial information provided by the chemoattractant gradient, whereas the orientation signal does. The asymmetric localization of the UNC-40 and MIG-10 signaling complex is suggestive of the segregation of signaling components into separate “front” and “rear” regions during chemotactic cell migration (Weiner 2002; Mortimer et al. 2008). It is hypothesized that this segregation is accomplished through short-range positive feedback mechanisms that promote the local production or recruitment of signaling molecules. In addition, a long-range inhibition mechanism globally increases the degradation of these molecules. Together such mechanisms could strongly amplify the asymmetric distribution of molecules needed for directed movement. This model has been put forth to explain why chemotactic cells polarize and move in a random direction when encountering a uniform chemoattractant concentration. Although the chemoattractant receptors may be uniformly stimulated across the surface of the cells, randomly oriented asymmetry can be established through these mechanisms.

If the AVM and HSN neurons behave similarly to chemotactic cells, then uniformly stimulating UNC-40 receptors might similarly cause nonspecific asymmetric UNC-40 localization and axon migrations in varying directions. However, this is difficult to test in vivo. Unlike exposing chemotactic cells to a uniform concentration of a chemoattractant in vitro, there is no reliable way to ensure that a neuron in vivo is exposed to a uniform concentration of UNC-6. The pseudocoelomic cavity of C. elegans is fluid filled, and UNC-6 expression patterns are spatially and temporally complex (Wadsworth et al. 1996). How the distribution of UNC-6 is affected by interactions with the extracellular matrix and cell surfaces is unknown.

Using a genetic approach, we have found an UNC-40 mutation that triggers randomly oriented neuronal asymmetry. On the basis of the models proposed for chemotactic cells, we suggest that there is an UNC-6/UNC-40-mediated signal that specifically induces the neuron’s intrinsic ability to polarize. The UNC-40 mutation activates this signal; however, a second signal, which normally would concurrently orient asymmetry relative to the UNC-6 gradient, is not activated. Single amino acid changes within the UNC-6 ligand can enhance or suppress the randomly oriented asymmetry phenotype caused by the UNC-40 mutation. This suggests that specific UNC-40 conformations uncouple the activation of the different signals.

MATERIALS AND METHODS

Strains: A list of strains constructed and used in this study is provided in the supporting information, File S1.

Analysis of phenotypes: For analysis of the AVM migration phenotype, L4 stage larvae were mounted on a 5% agarose pad. The AVM axon was visualized in L4 stage larvae expressing the zds5 transgene, which encodes mec-4::GFP. AVM axon guidance was considered ventral migration defective if the axon traveled laterally and failed to reach the ventral nerve cord posterior to the nerve ring. The AVM axon migration was considered dorsal or posterior if the axon migrated posteriorly for a distance greater than three cell-body lengths from the cell body or if it migrated posteriorly or dorsally during any other phase of its trajectory. The AVM was considered multipolar if more than one process, greater than one cell-body length, was observed. The HSN axon was visualized in L4 stage larvae with the kyIs262 [unc-86::myr-GFP and odr-1::DsRed] transgene. HSN axon guidance was considered ventral migration defective if the axon traveled laterally for a distance greater than two cell bodies without reaching the ventral nerve cord dorsal or posterior if the axon migrated posteriorly for a distance greater that three cell-body lengths or if it migrated posteriorly or dorsally at any other point during the trajectory. The HSN was considered multipolar if more than one process, greater than one cell-body length, was observed. The DA and DB motor neurons were visualized in L4 stage larvae using the edIs82a [unc-129::GFP] transgene. DA and DB dorsal guidance was scored as defective if the axons between the pharynx and vulva failed to reach the dorsal midline region where the dorsal cord forms.

Image analysis: For analysis of UNC-40::GFP in HSN neurons, L2 stage larvae expressing the kyEx1212[unc-86::unc-40::GFP; odr-1::DsRed] transgene were mounted in M9 buffer with 10 mM levamasole. Images were taken using epifluorescent microscopy with a Zeiss 63× water immersion objective. To judge the HSN dorsoventral position and developmental stage, each larva was also imaged by differential interference contrast microscopy. The UNC-40::GFP localization was determined by measuring the average intensity under lines drawn along the dorsal and ventral edges of each HSN cell body (IP lab software).

RESULTS

Selection and characterization of new unc-6 and unc-40 mutations: To explore further the molecular mechanisms that control responses to the UNC-6 guidance cue in C. elegans, we undertook a genetic screen for mutations that could suppress dorsal guidance defects caused by the unc-6(rh46) mutation. The DA and DB ventral cord motor neurons send axons dorsally, traveling circumferentially along the body wall away from ventral sources of the UNC-6 guidance cue (Hedgecock et al. 1990; Wadsworth et al. 1996). The unc-6(rh46) mutation is a partial loss-of-function allele that is temperature sensitive; in mutants, 80% of the DA and DB motor axons fail to reach the dorsal cord at 20°C (Hedgecock et al. 1990; Wadsworth et al. 1996).
Suppressor mutations were selected by improved mobility of the animals and enhanced guidance of motor neuron axons to the dorsal cord (Kulkarni et al. 2008; Li et al. 2008). We reasoned that the unc-6(rh46) mutation, which causes a A157P substitution within the laminin-like domain VI region of the molecule, may result in a protein that is secreted but cannot efficiently stimulate UNC-6 cell-surface receptor signaling at restrictive temperatures. Results of the characterization of two mutations isolated from the screen are consistent with this interpretation. We found that loss of rpm-1 or clec-38 function enhances UNC-6 receptor activity and improves axon migrations in the unc-6(rh46) mutants (Kulkarni et al. 2008; Li et al. 2008). RPM-1 is a member of the conserved Pam/Highwire/RPM-1 protein family, and CLEC-38 has predicted transmembrane and C-type lectin-like domains. These genes function cell autonomously to regulate the receptors. The results indicate that the mutations enhance the ability of UNC-6 A157P to cause a signal within the neurons and that the directional information provided by the distribution of UNC-6 A157P is adequate to guide axon migrations.

From the screen, four intragenic mutations were also identified after mapping each to the X linkage group at the unc-6 position and sequencing the unc-6 gene in the mutants. Each of the four mutations was independently isolated from different mutagenesis experiments; previously, a suppressor had been isolated as a spontaneous revertant (Wadsworth et al. 1996). Two of the mutations, ev436 and ur301, are missense mutations altering A214 to T214 or V214, respectively, and three of the mutations, ur282, ur296, and ur300, are missense mutations altering P216 to S216 (Figure 1A). The second-site mutations are located near the A157P change caused by the rh46 mutation. The rh46 mutation (A157P), as well as the second-site mutations, is within the domain VI sequence. This domain is required for all UNC-6 guidance functions, although mutations within the domain can selectively affect attractive and repulsive guidance and the responses from cells and growth cones (Lim and Wadsworth 2002).

The intragenic suppressors restore unc-6 guidance functions (Figure S1). The loss of unc-6 function disrupts the dorsal and ventral guidance of cells and axons (Hedgecock et al. 1990), and we observe that both the unc-6(rh46ur282) and the unc-6(rh46ur301) alleles improve guidance functions relative to unc-6(rh46), with unc-6(rh46ur282) being the strongest suppressor. The expression pattern of UNC-6 is dynamic; however, for the phenotypes that we examined the dorsal migrations correspond to repulsion from UNC-6-secreting cells, whereas ventral migrations correspond to attraction (Wadsworth et al. 1996). The second-site mutations restore both the attraction and the repulsion responses.

We also recovered a new unc-40 allele. The ur304 mutation was characterized because animals of a mutagenized strain showed unique movements during the initial screening process for suppressor mutations of the dorsal guidance defects caused by unc-6(rh46). However, this strain proved to have multiple phenotypes that could not be mapped to single loci. We found, however, that the strain had a GCA → CCA transversion causing an A157P amino acid substitution in the unc-6 (rh46) allele. Intragenic suppressor mutations of unc-6 (rh46) were identified: ur301 is a GCG → GTG transition causing an A214V substitution; ev436 is a GCG → ACG transition causing an A214T substitution; and the three mutations ur282, ur296, and ur300 each have a CCT → TCT transition causing an P216S substitution. The unc-40 protein consists of four Ig domains; six fibronectin type III domains; a transmembrane domain; and the three cytoplasmic domains P1, P2, and P3 (Chan et al. 1996). The unc-40(ur304) mutation results in an alanine-to-valine amino acid substitution at position 1056 within the juxtamembrane extracellular region.

**Figure 1.**—Schematic of the UNC-6 and UNC-40 protein and location of mutations in this study. (A) UNC-6/netrin-1 family members are composed of domains VI, V, and C (Ishii et al. 1992). The GCA → CCA transversion causes an A157P amino acid substitution in the unc-6 (rh46) allele. Intragenic suppressor mutations of unc-6 (rh46) were identified: ur301 is a GCG → GTG transition causing an A214V substitution; ev436 is a GCG → ACG transition causing an A214T substitution; and the three mutations ur282, ur296, and ur300 each have a CCT → TCT transition causing an P216S substitution. (B) The UNC-40 protein consists of four Ig domains; six fibronectin type III domains; a transmembrane domain; and the three cytoplasmic domains P1, P2, and P3 (Chan et al. 1996). The unc-40(ur304) mutation results in an alanine-to-valine amino acid substitution at position 1056 within the juxtamembrane extracellular region.

We examined whether the unc-40(ur304) mutation alone causes cell or axon migration defects such as those caused by other unc-40 alleles. We find that there are no differences from wild type. We also tested double mutants with alleles of unc-5,slt-1, and sax-3, which encode products that might affect unc-40 function, but found no evidence for genetic interactions. We next created double mutants with unc-40(ur304) and different unc-6 alleles. The phenotypes of the dorsal migrations of DA and DB motor neuron axons, which express UNC-40 but primarily utilize the UNC-5 receptor for a repulsive response away from the ventral UNC-6 sources, are not significantly different from the defects caused by the unc-6 mutations themselves (Table S1).

**Mutations that induce new axon migration patterns:** The unc-40(ur304) mutation causes dorsal and posterior migrations in unc-6 mutant backgrounds. In double
mutants with unc-6(ev400) or unc-6(e78) alleles, some AVM and HSN axons migrate dorsally and posteriorly instead of migrating ventrally as in wild type or mainly anteriorly at various dorsoventral positions as in unc-6 or unc-40 loss-of-function mutants (Figure 2; Tables 1 and 2). The unc-6(ev400) mutation is a predicted null allele, whereas unc-6(e78) is a partial loss-of-function allele (Hedgecock et al. 1990; Wadsworth et al. 1996). The penetrance of this phenotype is higher than in the unc-40(e1430) mutants, where UNC-40 is predicted to be absent. The penetrance of each phenotype varies with the AVM and HSN axon. It is possible that, in the absence of all guidance cues, the axons would have a more wandering phenotype; however, in the absence or with a reduction in the guidance cues directing ventral migration, the effects of cues that anteriorly direct the axons are revealed. These cues may have a stronger influence on AVM than on HSN so that the HSN axon can migrate dorsally or posteriorly in unc-6 or unc-40 loss-of-function mutants, whereas the AVM axons are guided only anteriorly.

The phenotypes resulting from the combinations of the unc-40 and unc-6 alleles suggest that conformational changes resulting from the interactions of UNC-6 and UNC-40 are important. The unc-6(rh46) allele is a temperature-sensitive mutation, and we scored the AVM axon migration in the double mutant at the high and low temperatures (Table 1) in the unc-40(ur304) background. The difference

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**Figure 2.**—unc-6 mutations and an unc-40 ectodomain mutation act together to induce new axon migration patterns. (A) Schematic of the ventral axon migrations of AVM and HSN neurons. The axons migrate toward ventral UNC-6 sources. (B–G) Photomicrographs of L4 stage animals showing AVM axon migration. Ventral is down and anterior is to the left. Bar, 20 μm. In the wild-type pattern, the AVM axon migrates ventrally and then turns anteriorly to migrate along the ventral nerve cord (B). Loss of unc-6 function causes the axon to migrate anteriorly at abnormal dorsoventral positions (C). In unc-40(ur304);unc-6(rh46) mutants, AVM axons frequently migrate dorsally (D), posteriorly (E), or in trajectories that have both dorsal and posterior components (F). In addition, the AVM neurons sometimes have extra extensions (G). (H–M) Photomicrographs of L4 stage animals showing HSN axon migrations. Ventral is down and anterior is to the left. Arrow indicates the PLM axon. Bar, 10 μm. In the wild-type pattern, the HSN axon extends ventrally and then anteriorly, defasciculating from the ventral nerve cord to make synapses at the vulva (H). Loss of unc-6 function causes the axon to migrate anteriorly at abnormal dorsoventral positions (I). In unc-40(ur304);unc-6(rh46) mutants, HSN axons frequently migrate dorsally (J), posteriorly (K), or in trajectories that have both dorsal and posterior components (L). In addition, the HSN neurons sometimes have extra extensions (M).
in penetrance of the phenotypes is consistent with the enhanced dorsal and posterior migrations being caused by the A157P change of the unc-6(rh46) mutation. Since studies indicate that netrin induces DCC homodimerization (Stein et al. 2001), we also tested whether unc-40(ur304) might act as a dominant gain-of-function allele in the unc-6(rh46) background. Consistent with this, we find no difference in the penetrance of the AVM

| Strain | Temperature | Wild type* | Anterior migration* | Dorsal or posterior migration* | Multipolar outgrowth |
|--------|-------------|------------|---------------------|-------------------------------|---------------------|
| Wild type | 20° | 100 | 0 | 0 | 0 | 200 |
| unc-40(ur304) | 20° | 100 | 0 | 0 | 0 | 263 |
| unc-40(ur304) | 15° | 100 | 0 | 0 | 0 | 242 |
| unc-6(rh46) | 20° | 72±3 | 28±3 | 0 | 0 | 235 |
| unc-6(rh46) | 15° | 82±3 | 18±3 | 0 | 0 | 207 |
| unc-6(ev400) | 20° | 80±1 | 20±1 | 0 | 0 | 231 |
| unc-6(e78) | 20° | 91±2 | 9±2 | 0 | 0 | 228 |
| unc-6(e1430) | 20° | 76±1 | 24±1 | 0 | 0 | 257 |
| unc-6(rh46ur282) | 20° | 58±2 | 14±1 | 22±2 | 6 | 664 |
| unc-6(rh46) | 15° | 72±1 | 17±1 | 8±1 (P = 0.006) | 3 (P = 0.004) | 501 |
| unc-6(ev400) | 20° | 73±1 | 19±1 | 5±1 (P = 0.002) | 3 (P = 0.006) | 463 |
| unc-6(e78) | 15° | 70±2 | 24±2 | 5±1 (P = 0.003) | 1 (P = 0.002) | 432 |
| unc-6(e78) | 20° | 89±2 | 9±2 | 2 (P = 0.001) | 0 | 588 |
| unc-6(e78) | 15° | 86±2 | 13±2 | 1±1 (P = 0.001) | 0 | 444 |
| unc-6(e1430) | 20° | 73±4 | 27±4 | 0 | 0 | 224 |
| unc-6(e1430) | 20° | 70±2 | 30±2 | 0 | 0 | 174 |
| unc-6(e1430) | 20° | 100 | 0 | 0 | 0 | 200 |
| unc-6(rh46ur282) | 20° | 100 | 0 | 0 | 0 | 247 |
| unc-6(rh46ur301) | 20° | 96±1 | 4±1 | 0 | 0 | 256 |
| unc-6(rh46ur301) | 20° | 98±1 | 2±1 | 0 | 0 | 190 |

P-values (two-tailed Student’s t-test) compare differences when the unc-40(ur304); unc-6(rh46) mutants are grown at 20°.

| Strain | Wild type* | Anterior migration* | Dorsal or posterior migration* | Multipolar outgrowth* |
|--------|------------|---------------------|-------------------------------|---------------------|
| Wild type | 100 | 0 | 0 | 0 | 192 |
| unc-40(ur304) | 100 | 0 | 0 | 0 | 205 |
| unc-6(rh46) | 5±2 | 90±3 | 3 (P = 6.6E-06) | 2 (P = 0.0001) | 230 |
| unc-6(ev400) | 4±2 | 90±3 | 4 (P = 8.3E-06) | 2 (P = 0.0002) | 220 |
| unc-6(e78) | 15±2 | 82±3 | 3 (P = 7.2E-06) | 0 | 244 |
| unc-6(e78) | 3±1 | 45±1 | 36±1 | 16±1 | 189 |
| unc-6(e78) | 4±1 | 66±2 | 22±2 (P = 0.002) | 8±1 (P = 0.002) | 204 |
| unc-6(e78) | 39±2 | 51±3 | 4±1 (P = 1.6E-05) | 6±2 (P = 0.02) | 199 |
| unc-6(e1430) | 5±3 | 74±4 | 14±2 (P = 0.0008) | 7±2 (P = 0.02) | 222 |
| unc-6(e1430) | 91±4 | 9±5 | 0 | 0 | 182 |
| unc-6(rh46ur282) | 88±4 | 12±5 | 0 | 0 | 179 |
| unc-6(rh46ur301) | 28±4 | 59±5 | 7±3 (P = 0.0003) | 6±1 (P = 0.003) | 204 |
| unc-6(rh46ur301) | 31±3 | 53±3 | 10±2 (P = 0.0008) | 6±1 (P = 0.002) | 215 |
| cdc-38(ur280) | 100 | 0 | 0 | 0 | 284 |
| cdc-38(ur280); unc-6(rh46) | 16±4 | 80±4 | 4±1 (P = 1.9E-05) | 0 | 400 |
| cdc-38(ur280); unc-6(ev400) | 4±2 | 92±3 | 3 (P = 4.9E-06) | 1 (P = 0.0001) | 398 |

P-values (two-tailed Student’s t-test) compared differences between the unc-40(ur304); unc-6(rh46) double mutants.

*Numbers represent percentage value ± SEM. Schematics depict AVM axon migration patterns. Growth condition was at 20°.
dorsal and posterior migration phenotype among unc-40(ur304)/unc-40(ur304), unc-40(ur304)/unc-40(+), and unc-40(ur304)/unc-40(uf) mutants (Table S2).

Wild-type UNC-6 and the UNC-6 second-site mutations show that single amino acid changes in UNC-6 influence the axon migration pattern in the unc-40(ur304) background. The dorsal and posterior axon migration phenotype of unc-40(ur304); unc-6(rh46) mutants is not observed with wild-type UNC-6 in the unc-40(ur304) mutant background. Compared to unc-40(ur304); unc-6(rh46) mutants, the unc-40(ur304);unc-6(168) and the unc-40(ur304);unc-6(301) mutants have a significantly lower penetrance of dorsal and posterior axon migrations and instead show the wild-type migration pattern (Figure 2 and Tables 1 and Tables 2). These results indicate that the single amino acid differences in the UNC-6 A157P A214V, UNC-6 A157P P216S, or wild-type UNC-6 ligands are able to suppress the response caused by UNC-6 A157P in the UNC-40 A1056V background.

Mutations that alter the asymmetric localization of UNC-40: Studies suggest that UNC-6 and UNC-40 function during HSN axon formation to initiate, maintain, and orient asymmetric neuronal growth (Alder et al. 2006). In response to UNC-6, UNC-40::GFP becomes localized to the ventral side of HSN at the early L2 stage of larval growth, accompanying the formation of a leading edge from the point at which the axon will emerge (Alder et al. 2006). We examined whether the leading edge and localization of UNC-40::GFP in HSN might be affected by the mutations (Figure 3). In particular, we predicted that UNC-6 triggers the upregulation of UNC-40 and the asymmetric localization of the receptor to cell surface membranes where axon formation occurs (Kulkarni et al. 2008). We reasoned that, in the unc-40(ur304) strains that have a higher penetrance of dorsal and posterior axon migrations, the average ratio of dorsal-to-ventral UNC-40::GFP intensity might be greater, provided that UNC-40 A1056V is able to direct the UNC-40::GFP. Using image analysis, the average dorsal and average ventral UNC-40::GFP intensity was measured. The ratio of dorsal-to-ventral intensity is greatest in the unc-40(ur304);unc-6(e1430) and unc-40(ur304);unc-6(rh46) mutants (Figure 3), the strains that also have the highest penetrance of dorsal and posterior axon migrations (Table 2). We also observed that when the dorsal-to-ventral intensity is greater, there is a bias for the intensity to be greatest at the anterior, medial, or posterior third of the dorsal surface (48% of unc-

Figure 3.—The unc-6 and unc-40(ur304) mutations affect UNC-40::GFP localization. (A–H) Photomicrographs of the localization of UNC-40::GFP in the HSN neuron of L2 stage larvae. Ventral is down and anterior is to the left. Bar in C, 5 μm. UNC-40::GFP is ventrally localized in the unc-40(ur304) mutant or in unc-40(+) animals (A), but is more evenly distributed in unc-6(rh46) mutants (B). In unc-40(ur304); unc-6(rh46) mutants, UNC-40::GFP localization is shifted dorsally (C). UNC-6 second-site mutations restore UNC-40::GFP ventral localization (D and E). Expression of myr-GFP is evenly distributed in HSN membranes in the wild-type background and was used as a control (F). In clec-38(ur280) mutants, there is an increase in UNC-40::GFP expression, but there is still ventral localization (G). In clec-38(ur280); unc-6(rh46) mutants, UNC-40::GFP distribution resembles the localization in unc-6(rh46) mutants (H). (I) Graph indicating the average ratio of dorsal-to-ventral intensity from line-scan intensity plots of the GFP signal around the periphery of the cell. Values at top indicate the number of samples. The phenotypes of unc-40(e1430) mutants can be rescued by the unc-40::gfp transgene. (*) Statistically different from the myr-GFP control strain (P < 0.05, one-tailed Student’s t-test). Error bars indicate SEM.
Because the UNC-6 A157P A214V or UNC-6 A157P P216S second-site mutations cause wild-type axon migration patterns in the unc-40(ur304) background, we examined whether the average ratio of dorsal-to-ventral UNC-40::GFP intensity was affected in the unc-40(ur304); unc-6(rh46) and unc-40(ur304); unc-6(rh46 ur282) mutants. In both cases, the ratio is lower (Figure 3). These results indicate that signal amino acid changes within UNC-6 are sufficient to alter UNC-40 A1056V-mediated cytoplasmic signaling events regulating directional responses.

The UNC-40 A1056V phenotypes are not similar to those caused by UNC-40 overexpression: We compared the phenotypes and localization of UNC-40 in unc-40(ur304); unc-6(ev400) and unc-40(ur304); unc-6(rh46) mutants to those observed in strains where UNC-40 has increased activity. For example, in contrast to the unc-40(ur304) phenotypes, the expression of a constitutively activated UNC-40 protein, MYR::UNC-40, causes enlarged and deformed cell bodies and additional axons and branches, as well as misguided axons (Gittai et al. 2003). Previously, we showed that loss of clec-38 enhances UNC-40 activity and that, in clec-38 loss-of-function mutants that express the unc-40::gfp transgene, the HSN develops severe morphological defects (see Figure 7 in Kulkarni et al. 2008). Moreover, we observed that UNC-40 at the early L2 stage is ventrally asymmetrically localized in these mutants (Figure 3). We conclude that, while these mutants have enhanced UNC-40 activity that can lead to severe morphological defects, the ability to orient the asymmetric UNC-40 localization is not impaired. Signaling by UNC-40 A1056V in the absence of UNC-6 or by UNC-40 A1056V/UNC-6 A157P is unique in that it produces phenotypes that are not similar to the phenotypes caused by either the loss or the overactivity of UNC-40.

DISCUSSION

Our results show that the A1056V amino acid substitution at the juxtamembrane extracellular region of the UNC-40 (DCC) protein allows the random asymmetric localization of UNC-40 in neurons and causes axon migrations to wander. UNC-40 A1056V appears to cause these phenotypes independently of the UNC-6 gradient since they occur in the unc-6 loss-of-function background and also in the presence of UNC-6 A157P, which we have shown can properly orient axon migrations. We propose that UNC-40 signaling comprises multiple signals, one that causes the random asymmetric localization of UNC-40 and another that enables the neuron to interpret the UNC-6 gradient (Figure 5). UNC-40 A1056V allows the polarization signal but prevents the orientation signal. Thus, UNC-40 A1056V is asymmetrically localized within the neurons, but the localization is not properly oriented with respect to the

| E | Strain | anterior | central | posterior | uniform | n |
|---|--------|----------|---------|-----------|---------|---|
| A | unc-40(ur304); unc-6(rh46) | 18 | 15 | 15 | 52 | 35 |
| B | unc-40(ur304); unc-6(ev400) | 16 | 12 | 12 | 60 | 25 |

Figure 4.—UNC-40::GFP is localized to different surfaces (anterior, medial, or posterior) when dorsally localized in mutants. (A–D) Photomicrographs of the localization of UNC-40::GFP in the HSN neuron of L2 stage larvae. Ventral is down and anterior is to the left. Bar, 5 μm. Line-scan intensity plots of the GFP signal across the dorsal periphery of the cell were taken, the dorsal surface was geometrically divided into three equal segments, and the average intensity of each was recorded. The intensity was considered localized if the average intensity value of one segment was greater than the sum of the other two segments. UNC-40::GFP can be localized to the dorsal anterior region (A), the medial region (B), and the posterior region (C) or can be more uniformly distributed across the dorsal surface (D). (E) Table describing the average UNC-40::GFP distribution to the different segments along the dorsal surface.
The UNC-40 A1056V amino acid substitution may cause a conformational change that affects the ability of the receptor to transmit different signals (Figure 5). That unc-40(ur304) acts as a dominant gain-of-function allele and that the UNC-40 A1056V product is capable of asymmetrically localizing the UNC-40::GFP molecule is consistent with other studies indicating that netrin induces DCC homodimerization (Stein et al. 2001). For type I transmembrane receptors, such as the toll-like receptor, epidermal growth factor receptor, and erythropoietin receptor, ligand-induced dimerization or oligomerization promotes conformational changes in the receptor ectodomains to induce stable protein–protein interactions between the receptor chains (Gay et al. 2006; Hubbard and Miller 2007). The ligand-binding region of the ectodomain may function as an autoinhibitor, causing structural hindrance that prevents unregulated dimerization. With the binding of the
ligand, the repression is relieved and the reorientation of the ectodomain and juxtamembrane region positions the transmembrane helices so that the cytoplasmic domains assume new conformations. This allows the recruitment of adaptor molecules that can regulate intracellular signaling pathways. Signaling by UNC-40 A1056V could involve a juxtamembrane extracellular conformational change that allows the cytoplasmic domains to stimulate the intracellular signaling pathways that control UNC-40 receptor asymmetric localization while preventing the stimulation of pathways that control the response to the UNC-6 gradient. The interaction with UNC-6 A157P could further stabilize this conformation, whereas the interaction with wild-type UNC-6, UNC-6 A157P A214V, or UNC-6 A157P P216S may direct the conformation toward the normal UNC-40 conformation, allowing simulation of the pathways that respond to the UNC-6 gradient.

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The Roles of Multiple UNC-40 (DCC) Receptor-Mediated Signals in Determining Neuronal Asymmetry Induced by the UNC-6 (Netrin) Ligand

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Figure S1.— Quantification of dorsal and ventral migration phenotypes in unc-6(rh46) intragenic suppressors. The intragenic suppressors restore unc-6 guidance functions. The phenotypes and assays are as described in Hedgecock, E. M., Culotti, J. G., and Hall, D. H. (1990). DTC ant: anterior distal tip cell. DTC post: posterior distal tip cell. egl: egg laying. The DA/DB and distal tip cell migration are dorsal cell and axon migration, respectively. These migrations are away from the UNC-6 ventral source. The AVM, PVM, and egg laying phenotypes measure ventral axon and cell migrations, which are towards UNC-6 ventral source. Asterisks indicate statistically difference (*$P<0.01$, **$P<0.001$, two-tailed Student’s t test). Error bars indicate s.e.m. Values at top indicate the number of samples.
| Strain                               | Dorsal migration defect | $P$ value* | n  |
|--------------------------------------|-------------------------|------------|----|
| $unc-6(ea400)$                       | 96 ± 1                  | 0.52       | 244|
| $unc-40(ur304);unc-6(ea400)$         | 99 ± 1                  |            | 351|
| $unc-6(rh46)$                        | 82 ± 1                  | 0.71       | 248|
| $unc-40(ur304);unc-6(rh46)$          | 83 ± 1                  |            | 328|
| $unc-6(e78)$                         | 32 ± 2                  | 0.91       | 262|
| $unc-40(ur304);unc-6(e78)$           | 32 ± 1                  |            | 338|
| $unc-40(ur304)$                      | 0                      |            | 200|

† Numbers represent percentage values ± s.e.m.
* Each $unc-6$ mutant was compared to the double mutant with $unc-40(ur304)$. $P$ values are calculated by using a two-tailed Student’s t test.
**TABLE S2**

unc-40(ur304) acts dominantly with unc-6(rh46) (20 °C)

| Strain                          | Dorsal or posterior migration (%) | Multipolar outgrowth (%) | n  |
|--------------------------------|----------------------------------|--------------------------|----|
| unc-6(rh46)                     | 0                                | 0                        | 95 |
| unc-40(ur304); unc-6(rh46)      | 22                               | 6                        | 72 |
| unc-40(ur304); unc-40(+); unc-6(rh46) | 20                               | 6                        | 85 |
| unc-40(ur304); unc-40(e1430); unc-6(rh46) | 18                               | 7                        | 76 |
**FILE S1**

**Strains.** All strains were constructed with the N2 Bristol genetic background. Animals were maintained on NGM plates seeded with OP50 bacteria.

IM983: unc-40(ur304) I, IM989: unc-6(rh46, ur282) X; evIs82a IV, IM990: unc-6(rh46, ur282) X; zdIs5 I, IM991: unc-6(rh46, ur282) X, IM992: unc-6(rh46, ur301) X; evIs82a IV, IM993: unc-6(rh46, ur301) X; zdIs5 I, IM994: unc-6(rh46, ur301) X, IM648: unc-40(e1430) I, zdIs5 I, IM998: unc-40(ur304) I; unc-6(e78) X; evIs82a IV, IM999: unc-6(e78) X; zdIs5 I, IM1000: unc-40(ur304) I; zdIs5 I, IM1001: unc-40(ur304) I; unc-6(rh46, ur282) X; zdIs5 I, IM1002: unc-40(ur304) I; unc-6(rh46, ur301) X; zdIs5 I, IM650: unc-6(e400) X; zdIs5 I, IM838: unc-6(rh46) X; zdIs5 I, IM1003: unc-40(ur304) I; unc-6(rh46) X; evIs82a IV, IM1004: unc-40(ur304) I; unc-6(e400) X; evIs82a IV, IM1005: unc-40(ur304) I; unc-6(rh46) X; zdIs5 I, IM1006: unc-40(ur304) I; unc-6(e400) X; zdIs5 I, IM1007: unc-40(e1430) I; unc-6(e78) X; zdIs5 I, unc-40(e1430) I; unc-6(rh46) X; zdIs5 I, IM1011: unc-40(ur304) I; unc-6(e78) X; zdIs5 I, IM1012: unc-40(e1430) I; unc-6(e400) X; zdIs5 I, IM1072: unc-40(ur304) I; unc-6(rh46) X; kyIs262 IV, IM1073: unc-40(ur304) I; unc-6(e400) X; kyIs262 IV, IM1074: unc-40(ur304) I; unc-6(e78) X; kyIs262 IV, IM1075: unc-40(ur304) I; kyIs262 IV, IM1076: unc-6(rh46) X; kyIs262 IV, IM1077: unc-6(e400) X; kyIs262 IV, IM1078: unc-6(e78) X; kyIs262 IV, IM1079: unc-40(e1430) I; kyIs262 IV, IM1080: unc-40(ur304) I; unc-6(rh46, ur282) X; kyIs262 IV, IM1081: unc-6(rh46, ur282) X; kyIs262 IV, IM1082: unc-40(ur304) I; unc-6(rh46, ur301) X; kyIs262 IV, IM1083: unc-6(rh46, ur301) X; kyIs262 IV, IM1084: unc-40(ur304) I; unc-6(rh46) X; kyEx1212, IM1085: unc-40(ur304) I; unc-6(e400) X; kyEx1212, IM1086: unc-40(ur304) I; unc-6(e78) X; kyEx1212, IM1087: unc-40(ur304) I; kyEx1212, IM1088: unc-6(rh46) X; kyEx1212, IM1089: unc-6(e400) X; kyEx1212, IM1090: unc-6(e78) X; kyEx1212, IM1091: unc-40(e1430) I; kyEx1212, IM1092: unc-40(ur304) I; unc-6(rh46, ur282) X; kyEx1212, IM1093: unc-6(rh46, ur301) X; kyEx1212, IM1095: unc-6(rh46, ur301) X; kyEx1212.

Transgenes not derived in the Wadsworth laboratory were kindly provided by Scott Clark [zdIs5(mec-4::GFP)], Joe Culotti [evIs82a(unc-129::GFP)], Cori Bargmann [kyEx1212(unc-86::unc-40::GFP)] and [kyIs262(unc-86::myr-GFP)].

**Sequence analysis.** The unc-6 and unc-40 coding region and intron-exon boundaries were PCR-amplified from the mutants' genomic DNA using Expand High Fidelity PCR kits (Roche Applied Science, Indianapolis, IN). PCR products were purified according to the protocol of QiaTaq (QIAGEN, Valencia, CA), and then were submitted to DNA Sequencing using the ABI PRISM® 3130xl Genetic Analyzer at UMDNJ-RWJMS DNA Core Facility.