

Regulation of the UNC-5 netrin receptor initiates the first reorientation of migrating distal tip cells in *Caenorhabditis elegans*

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SUMMARY

Cell migrations play a critical role in animal development and organogenesis. Here, we describe a mechanism by which the migration behaviour of a particular cell type is regulated temporally and coordinated with over-all development of the organism. The hermaphrodite distal tip cells (DTCs) of *Caenorhabditis elegans* migrate along the body wall in three sequential phases distinguished by the orientation of their movements, which alternate between the anteroposterior and dorsoventral axes. The ventral-to-dorsal second migration phase requires the UNC-6 netrin guidance cue and its receptors UNC-5 and UNC-40, as well as additional, UNC-6-independent guidance systems. We provide evidence that the transcriptional upregulation of *unc-5* in the DTCs is coincident with the initiation of the second migration phase, and that premature UNC-5 expression in these cells induces precocious turning in an UNC-6-dependent manner. The DAF-12 steroid hormone

receptor, which regulates developmental stage transitions in *C. elegans*, is required for initiating the first DTC turn and for coincident *unc-5* upregulation. We also present evidence for the existence of a mechanism that opposes or inhibits UNC-5 function during the longitudinal first migration phase and for a mechanism that facilitates UNC-5 function during turning. The facilitating mechanism presumably does not involve transcriptional regulation of *unc-5* but may represent an inhibition of the phase 1 mechanism that opposes or inhibits UNC-5. These results, therefore, reveal the existence of two mechanisms that regulate the UNC-5 receptor that are critical for responsiveness to the UNC-6 netrin guidance cue and for linking the directional guidance of migrating distal tip cells to developmental stage advancements.

Key words: Netrin, Cell migration, UNC-5, *Caenorhabditis elegans*

INTRODUCTION

Cell migrations are a key component of development and organogenesis, yet the regulatory mechanisms of cell migrations are poorly understood, particularly the events that cause changes in migratory trajectory responsible for shaping organs and organ systems. Directional information for migrating cells comes from extracellular cues that act through cell surface receptors to regulate the cytoskeletal machinery required for cell polarity and motility. Long-range cell and growth cone migrations typically proceed via a series of sequential, shorter migrations. Transitions between these shorter migrations may involve a change in the direction of movement or in the substratum over which migration occurs. For example, the migrating germ cell precursors of *Drosophila* associate with, in order, the posterior midgut primordium, the mesoderm and the somatic gonad primordium in a sequence precisely coordinated with embryonic development (Zhang et

al., 1996). In principle, the transitions between migratory phases might include changes either in the way the cell responds to its environment or in the environment itself, but how these changes are initiated and executed is not completely understood.

The bilobed gonad of *C. elegans* hermaphrodites develops during larval development from a 4-cell primordium positioned in the ventral midbody. The shape of the two gonad arms is determined by the migratory path of a cell called a distal tip cell (DTC) at the leading edge of each arm (Hedgecock et al., 1987). DTC migrations proceed through three sequential linear phases, with each phase occurring along one of the natural body axes and at right angles to the previous phase (Fig. 1A-E). The first migratory phase is a centrifugal migration along the ventral band of body wall muscles away from the midbody. One DTC migrates toward the anterior and the other toward the posterior. The second phase begins with an orthogonal (i.e., 90°) turn of the DTCs and consists of a migration along the inner surface of

the epidermis from the ventral to the dorsal muscle bands. Finally, during the third phase, the DTCs turn 90° and migrate centripetally along the dorsal muscle bands back toward the midbody. This series of linear migrations on the body wall gives rise to two mirror-image C-shaped adult gonad arms (one for each DTC; Figs 1E, 2A; also see Hedgecock et al., 1987). A similar, triphasic C-shaped migratory path is followed by the male linker cell (MLC) that shapes the single (anterior) male gonad arm in *C. elegans* and by many neuronal growth cones. For example, the growth cones of the DD motoneurons initially project from their cell bodies longitudinally within the ventral nerve cord, then make a 90° turn to extend circumferentially in a ventral-to-dorsal direction. Upon reaching the appropriate location of the dorsal nerve cord, they turn once more in a retrograde longitudinal direction, resulting in a C-shaped axonal morphology (White et al., 1986).

Circumferential cell and growth cone migrations in *C. elegans*, including the ventral-to-dorsal second phase of the DTC and MLC migration, are mediated in part by the netrin family member UNC-6 and its receptors UNC-5 and UNC-40 (Hedgecock et al., 1990; Culotti and Merz, 1998). In the DTCs and MLC, UNC-5 and UNC-40 mediate chemorepulsion away from ventrally expressed UNC-6 (Wadsworth et al., 1996). A role for netrins and their receptors in complex cell migrations in mammalian development was demonstrated by the molecular characterization of the murine *rostral cerebellar malformation* (*rcm*) locus, which encodes an UNC-5 homologue (UNC5H3; Ackerman et al., 1997). Mutations at this locus disrupt the migrations of granule cell precursors in the external germinal layer of the developing cerebellum (Ackerman et al., 1997; Przyborski et al., 1998).

The timing of the turns made by the DTCs and the MLC are synchronized with progression through the larval stages of development. The progression from the centrifugal first phase to the circumferential (i.e., ventral-to-dorsal) second phase of migration occurs at a precise time late in the second larval stage (L2) for the MLC and the third larval stage (L3) for the DTCs. In mutants of *unc-5*, *unc-6* and *unc-40*, the second phase of DTC and MLC migration frequently fails, but the first and third phases occur with the proper timing (Hedgecock et al., 1990; see Fig. 2B for resulting gonad morphology). Mutations in two genes, *daf-12* (particularly alleles formerly known as *mig-7*) and *mig-8*, disrupt the navigational program of the DTCs, such that the second and third migratory phases are delayed or absent (Hedgecock et al., 1987; Antebi et al., 1998). These mutants were isolated because they have linear gonad arms that result from the failure of the DTC program to advance from the second to the third larval stage (Hedgecock et al., 1987; see Fig. 2F). *daf-12* encodes a nuclear hormone receptor that is a critical regulator of larval developmental stage progressions for somatic worm tissues (Yeh, 1991). It has been proposed that DAF-12 acts to advance larval stage-specific developmental programs of several different somatic cell types (Antebi et al., 1998). The single existing allele of *mig-8* appears to affect only the progression of the navigational program of the DTCs (Antebi et al., 1998).

A detailed in vivo characterization of the mechanisms by which a specific cell migrates may uncover principles broadly applicable to development and cellular biology. It may also reveal basic mechanisms of axon guidance, as directional cues like UNC-6 are utilized by both migrating cells and neuronal

growth cones. The purpose of the present study was to determine the molecular mechanisms involved in DTC navigation, particularly the regulation of the first change in direction of DTC movement. The phenotypes of several mutants that affect DTC migrations suggest that the initiation of the ventral-to-dorsal (i.e., second) phase of DTC migration involves the activation or disinhibition of the UNC-6-mediated guidance system by MIG-8 and DAF-12. In principle, this could be achieved by regulating any one or more of the components of the UNC-6 system. Here we report that this second phase of DTC migration is initiated largely by the MIG-8 and DAF-12-dependent upregulation of *unc-5* gene expression. A premature high level of expression of UNC-5 using a heterologous promoter induces precocious ventral-to-dorsal DTC migration, indicating that UNC-5 upregulation can be sufficient to drive this migratory phase. However, we also find genetic evidence that UNC-5 function is facilitated at the time of turning, possibly through release from an opposing or inhibiting pathway. Together, these two mechanisms ensure that responsiveness to the UNC-6 directional signal is initiated at the appropriate time in the DTC migration pattern. The regulation by DAF-12 and MIG-8 of the UNC-5 guidance receptor is thus one of the pathways linking this specific cell migration event to the progression of the whole animal through developmental stages.

MATERIALS AND METHODS

Strains

C. elegans were incubated and handled as previously described (Brenner, 1974). Unless otherwise noted, all cultures were grown at 20°C. Most strains not derived in our laboratories were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). Transgenic lines are described below. *mig-6(e1931)* homozygotes were obtained from the self-fertilization of + *mig-6(e1931)/dpy-11(e224)* + *trans* double heterozygotes. All other strains were maintained as homozygotes. Males were obtained from *him-5* double mutant strains.

Scoring of DTCs

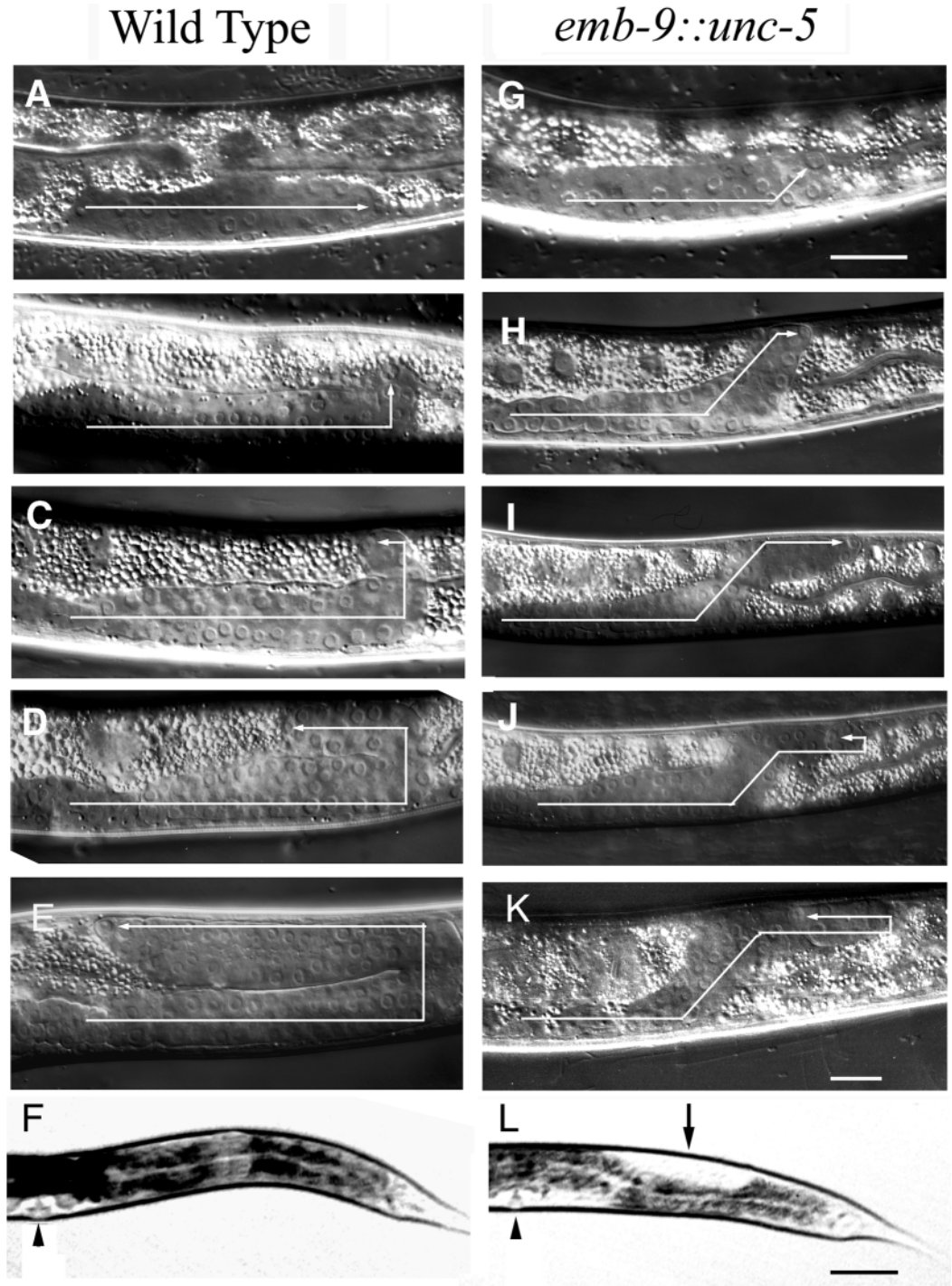
The morphology of somatic gonad arms in wild-type and mutant worms has been described previously (Hedgecock et al., 1987). Adult gonad morphologies reflect the pathway of DTC migrations. The misshapen gonads produced by DTC or MLC migration defects may be scored either using a dissecting microscope as clear patches produced by displacements of the intestine (Hedgecock et al., 1990), or at higher magnification under Differential Interference Contrast (DIC) optics. Standard errors for the penetrance of DTC defects were calculated assuming a binomial distribution of the actual sample size (Hedgecock et al., 1990; Antebi et al., 1998). To assess the timing of migrations, newly hatched larvae were picked and incubated at 20°C for the desired period of time. Worms were then mounted for examination under DIC optics using 1 mM levamisole as anaesthetic. As a control for accuracy of timing, the developmental state of the vulval precursor cells (VPCs) was also determined. The VPCs (e.g. P6,p) divide at 30 hours after hatching, coincident with dorsalward turning of the DTCs (Sulston and Horvitz, 1977). The timing of VPC divisions was normal in the strains for which timing experiments were carried out, including *evls99*, *him-5* and *mig-6*.

Derivation of transgenic lines

Transgenes were introduced into *C. elegans* by germline transformation using the gonad injection method previously described (Mello et al., 1991; Mello and Fire, 1995). The co-injected co-

Fig. 1. DTC migrations in wild-type and in *emb-9::unc-5* transgenic animals that precociously express *unc-5*. In all panels, dorsal is up and anterior is to the left. (A-E) Arrows show the migration pattern of DTCs in the wild type (see Hedgecock et al., 1990); (G-K) arrows show the pattern in the *evIs99* strain, which carries an integrated array of the *emb-9::unc-5* transgene.

Arrowheads point to the DTC. The panels represent a time-lapse series of DTC position and consequent gonad arm morphology. Only the posterior gonad arm is shown for simplicity, but similar (mirror image) trajectories are observed for the anterior DTC. The first phase of DTC migration in wild type (A) and *evIs99* (G) is away from the midbody (centrifugal); however, the DTC in the *evIs99* strain has already begun to move toward the dorsal side at a stage in which the wild-type DTC is still moving centrifugally. In *evIs99* the DTC migrates obliquely with respect to the dorsoventral axis until it reaches the dorsal muscle band (H,I). The transition between the centrifugal and centripetal migratory phases consequently occurs on the dorsal side (J) and the DTCs migrate back towards midbody (K). The resulting shapes of the gonad arms in wild-type and *emb-9::unc-5* animals are diagrammed in Fig. 2A-E, respectively). (A-E) The gonad arms correspond to Fig. 2A. (F,L) The opaque intestine covers the entire dorsoventral axis, except in the forming vulva (arrowhead) in wild-type animals (F), but *emb-9::unc-5* animals with precociously turned gonad arms, push the intestine ventrally leaving a dorsal clear patch (L). Scale bars for A and G (in G), B-E and H-K (in K) are 10 μ m. The scale bar for F and L (in L) is 50 μ m.



transformation marker was either the pRF4 plasmid containing the *rol-6(su1006dm)* gene (Kramer et al., 1990), which creates a dominant rolling (Rol) phenotype, or pMH86 (Clark et al., 1995), which contains the wild-type *dpy-20* gene and rescues a *dpy-20* mutation present in the injected line. Extrachromosomal arrays were integrated by gamma-irradiation (3000 rads) followed by selection for Mendelian inheritance. Integrated transgenic arrays were assigned to linkage groups and were passed into mutant backgrounds using standard genetic methods.

***unc-5B* reporter constructs**

unc-5B reporter constructs were made by fusing a 4.6 kb fragment of genomic DNA 5' to the start of *unc-5* exon 2 to the coding sequence

of *lacZ*, *lacZ* with nuclear localization sequence (NLS), or *gfp* reporter genes previously described (Hamelin et al., 1993; Fire et al., 1990; Colavita et al., 1998). pYZ129 (*unc-5B::lacZ*) contains the 4.6 kb promoter fused to *lacZ*, and the *evIs54* transgenic strain contains the array [*unc-5B::lacZ;rol-6(su1006dm)*] integrated on LGII. pIS21 (*unc-5B::lacZ* with NLS) contains the 4.6 kb promoter fused to *lacZ* with an NLS and the *evIs51* line contains the array [*unc-5B::lacZNLS;rol-6(su1006dm)*]. The chromosomal site of integration for this line is unknown. pSU15 (*unc-5B::gfp*) comprises the 4.6 kb promoter fused to the *gfp* gene and the *nuls9* line contains the array [*unc-5B::gfp;myo-3::lacZ;dpy-20(+)*] integrated on LGI in a *dpy-20(e1282)* background.

The *unc-5B::HA*-tagged reporter construct (pU5 derived from pYZ108 described in Hamelin et al., 1993, corrected to a wild-type

coding sequence) was made by fusing a 4.6 kb fragment of genomic DNA 5' to the start of exon 2 to an *unc-5* cDNA with 1 kb of the normal 3' genomic region appended to its 3' end. Three in-frame HA tags were cloned into a *Bgl*III site after codon D889 (30 codons upstream from but still in frame with the normal stop codon) to derive pU5::HA. An *unc-5::gfp*-tagged reporter construct was made by cloning a full-length *gfp* gene (from pD9.75 described in Fire et al., 1990) into a *Bsa*B1 site after the codon F909 in pU5.

Cell identifications

We found that *lacZ* and GFP reporters were distributed throughout cell bodies and neuronal processes (except *unc-5B::NLS-lacZ* (pIS21). This facilitated identification of cells. The *unc-5B::lacZ* reporter with an NLS (pIS21) sometimes expressed in several additional unidentified neurons in the head and tail that were not stained by constructs lacking the NLS. Because the β -gal staining was faint and axons were not visible, these cells were not identified. The GFP reporters were used for experiments that required careful timing and simultaneous DIC observations in live animals.

Construction of the *emb-9::unc-5* transgene

An *Xba*I-*Pvu*I fragment containing 1.65 kb of the *emb-9* promoter just 5' to the initiation codon (from pJJ239) was fused to the wild-type *unc-5* cDNA previously described (Hamelin et al., 1993) to make *emb-9::unc-5* (pSU16). This construct is missing 15 bp immediately 5' to the *emb-9* initiation codon, but contains the entire *unc-5* coding region and 3' UTR. The *emb-9::lacZ* construct (pJJ318) that was co-injected with *emb-9::unc-5* in these experiments was constructed as described (Graham et al., 1997). The *evls99* transgenic line contains a multicopy array of [*emb-9::unc-5*; *emb-9::lacZ*; *dpy-20(+)*] integrated on LG1.

β -gal staining

Staining for β -galactosidase activity was carried out as described by Ruvkun and Giusto (1989) with modifications suggested by M. Finney and G. Ruvkun (Mass. Gen. Hospital) and by E. Aamodt and G. Xie (Louisiana State University). Briefly, animals were washed from NGM plates with water and pelleted in a microcentrifuge tube. The supernatant was removed and 500 μ l of ice-cold MRWB (160 mM KCl, 40 mM NaCl, 20 mM Na₂EGTA, 10 mM spermidine, 30 mM PIPES pH 7.4 and 50% methanol), 100 μ l of 20% paraformaldehyde and 400 μ l of water were added, mixed and incubated for 35 minutes at 4°C with occasional mixing. The worms were washed twice with 1.0 ml TTB (100 mM Tris-HCl pH 7.4, 1% Triton X-100 and 1 mM EDTA) by pelleting. To the pellet was added 960 μ l of TTB, 30 μ l of 20% paraformaldehyde and 10 μ l of 2-mercaptoethanol. The animals were incubated for 15 minutes at room temperature, pelleted by settling (animals are fragile to centrifugation at this stage), then washed once with 1 \times BO₃ and incubated for 15 minutes at room temperature with 200 mM DTT in 1 \times BO₃. The worm pellet was washed twice with β -galactosidase staining mix (370 μ l water, 500 μ l 0.8 M sodium phosphate pH 7.5, 2 μ l 1 M MgCl₂, 4 μ l 1% SDS, 100 μ l Redox Buffer and 50 mM each of potassium

ferrocyanide and potassium ferricyanide), 20 μ l 2.5% X-Gal in DMF and 1.0 μ l 1 mg/ml DAPI, then incubated overnight at room temperature or for 2 hours at 37°C. A similar permeabilization and fixation protocol was used for immunolocalization of HA-tagged UNC-5 protein using anti-HA antibodies (Wadsworth et al., 1996).

RESULTS

DTC migrations in the wild type have been well characterized (Hedgecock et al., 1987; Antebi et al., 1998). The centrifugal first phase of DTC migration is initiated early in L3 about 25 hours after hatching. The anterior and posterior DTCs migrate away from the midbody (i.e., centrifugally) along the ventral muscle bands (Figs 1, 2A). Late in L3, about 30 hours after hatching, the DTCs reorient and migrate circumferentially toward the dorsal muscle band, where at approximately 33 to 34 hours, they turn once more and migrate back toward the midbody (i.e., centripetally). In worms with an extended body length, such as *lon-2*, the first turn of the DTCs occurs at approximately the same distance from the midbody as in the wild type (our unpublished data, also see Morita et al., 1999). This indicates that turning occurs at the normal time in development even though the position of turning relative to many other tissues along the anteroposterior axis may be altered.

The ventral-to-dorsal second phase of DTC migration is specifically disrupted by mutations in *unc-6*, *unc-5* or *unc-40*

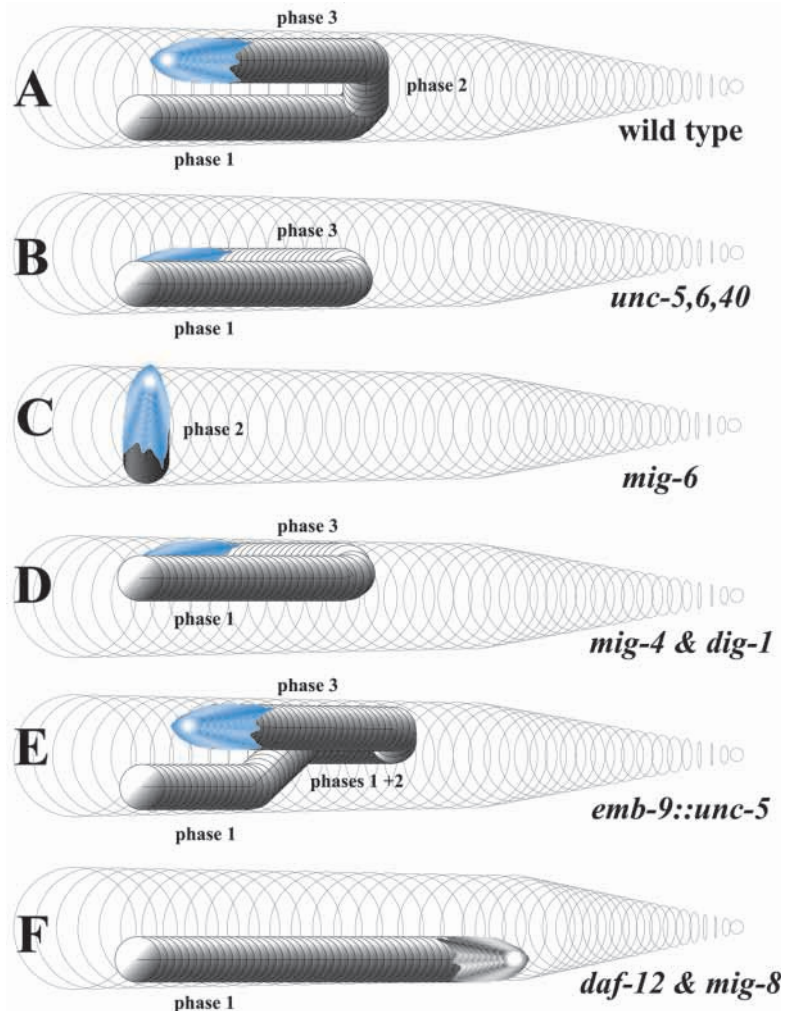
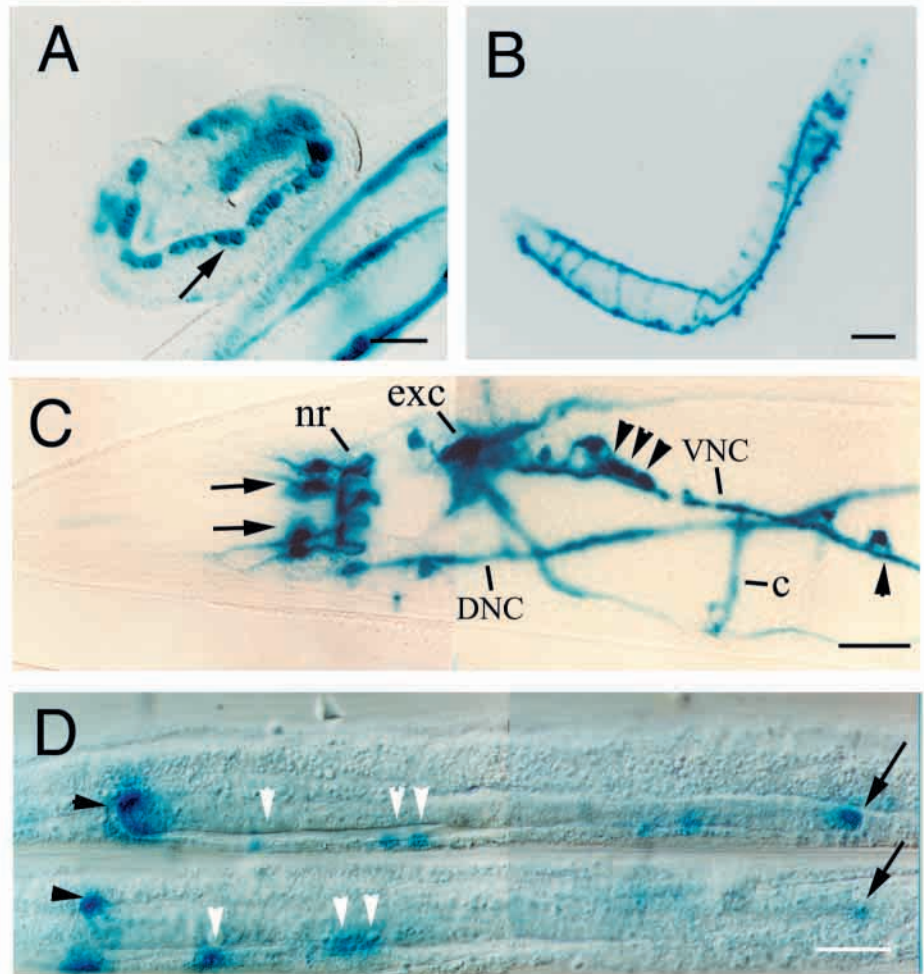


Fig. 2. Shapes of gonad arms in the wild type and mutants with altered DTC migrations. Dorsal is up and anterior is left. Only posterior gonad arms are shown. The shape of each gonad arm is a history of the migratory path of the DTC. DTCs are colored blue if they expressed UNC-5 reporters with normal timing and white if they failed to express the same UNC-5 reporters. The DTCs are depicted as large cells because they form a hood, with veil-like cytoplasmic extensions that cover a large portion of the distal end of each gonad arm.

Fig. 3. *unc-5* expression patterns. Anterior is to the right in A and B, to the left in other panels. (A-D) Animals have a twisted (roller) body wall. (A) *unc-5B::lacZ* shows embryonic expression in neurons, including ventral cord motoneurons (arrow) that will project axons to the dorsal nerve cord. (B) Larvae (shown is an L2 worm) exhibit *unc-5B::lacZ* expression in VD and DD motoneurons and (C) in several classes of sensory neurons in the head (arrows indicate IL2 sensory neurons). The excretory cell (exc), dorsal nerve cord (DNC), ventral nerve cord (VNC) and motoneurons (arrowheads) in the VNC express *unc-5::lacZ* (c is a motoneuron commissure). (D) Two animals are present. Anterior DTCs (arrowheads) and posterior DTCs (arrows) express *unc-5B::lacZ* coincident with the initiation of the second, ventral-to-dorsal migratory phase. The anterior DTCs express *unc-5* reporters and turn approximately 15 minutes before the posterior DTCs. The posterior DTC in the upper animal is poised to turn. The equivalent cell in the lower animal is out of focus, but has begun to migrate from ventral to dorsal and expresses *unc-5::lacZ*. White arrowheads are VNC motoneurons. Scale bars are 10 μ m.



(Hedgecock et al., 1990). In these mutants, the DTCs frequently migrate back toward the midbody along the ventral rather than the dorsal muscle bands and do so with normal timing (i.e., after hesitating a few hours on the ventral side) (Hedgecock et al., 1987; see Fig. 2B for resulting gonad morphology). This indicates that the centripetal third phase of the migratory program can proceed independently of the execution of the second phase and that the third migratory phase can occur on either the dorsal or the ventral side of the animal.

The reported phenotype of *dig-1* and *mig-4* mutants is consistent with this interpretation. In these mutants, the entire gonad primordium is frequently displaced to the dorsal side (Hedgecock et al., 1987; Thomas et al., 1990; see Fig. 2D). Consequently, no ventral-to-dorsal migratory phase occurs, and both centrifugal and centripetal migrations proceed with approximately normal timing along the dorsal muscle bands. These observations suggest that the intrinsic navigational program of the DTCs proceeds independently of the execution of the ventral-to-dorsal second migratory phase and that the centrifugal first phase can occur on the dorsal side as well as on the ventral side.

The leader cell in the development of the male gonad is the male linker cell (MLC), which is known to follow a migration pattern that differs from that of the hermaphrodite DTCs both in sequence and in timing (Sulston and Horvitz, 1977; Hedgecock et al., 1987). MLC migrations begin in the L2 stage with a migration along the ventral muscle band from the ventral midbody toward the head. At the end of L2, the MLC turns and migrates from the ventral to the dorsal muscle band one full larval stage earlier than the hermaphrodite DTCs. Subsequently, the MLC turns toward the posterior and migrates toward the tail,

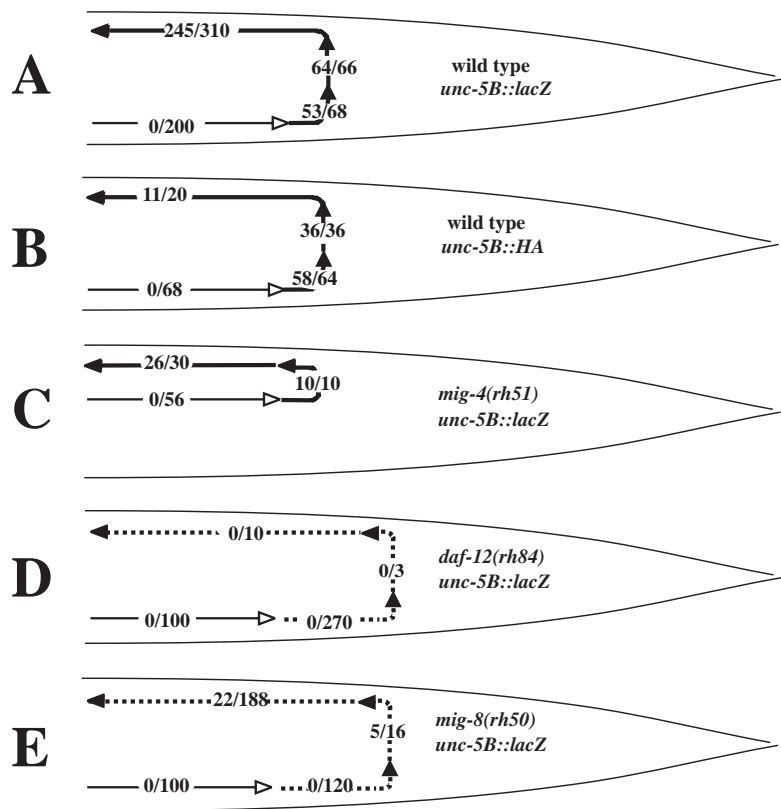
simultaneously descending from the dorsal to the ventral muscle bands. As for the hermaphrodite DTCs, the second, ventral-to-dorsal, migratory phase of the MLC is selectively disrupted by mutations in *unc-5*, *unc-6* or *unc-40* (Hedgecock et al., 1990).

unc-5 expression pattern

The products of the *unc-5*, *unc-6* and *unc-40* genes act together to mediate ventral-to-dorsal DTC migration (Hedgecock et al., 1990; see Fig. 2B). In principle, the transition from the centrifugal first phase of DTC migration to the dorsally oriented second phase could involve changes in the function of any component of the UNC-6 guidance system. UNC-6 is expressed by ventral epidermiblasts beginning in embryonic development and continues to be expressed at the time of the DTC migrations by neurons in the ventral nerve cord (Wadsworth et al., 1996). UNC-40, which functions cell-autonomously in migrating cells and neuronal growth cones, is expressed in the DTCs at a detectable and approximately constant level throughout their migrations (Chan et al., 1996). We examined *unc-5* gene expression using *lacZ* and *gfp* as transcriptional reporters (Fig. 3) and we also tagged UNC-5 with GFP and with an HA epitope. The *unc-5* gene encodes two SL1-spliced transcripts (A and B) which are identical except for an 82 nt first exon present only in the longer *unc-5A* transcript (Leung-Hagesteijn et al., 1992).

unc-5B transcriptional reporters were made by fusing 4.6 kb

Fig. 4. Timing of *unc-5B* expression in the DTCs. (A,B) The position of the DTC at which expression of *unc-5B::HA* (A) or *unc-5B::lacZ* (B) is detected. Denominators indicate the number of DTCs that were observed in a particular range of positions (demarcated by arrowheads), numerators indicate the number that expressed *unc-5B::lacZ*. Expression of the reporter constructs is never observed during the first migratory phase (arrows with open heads), but begins at the time of the initiation of the second phase (arrows with closed heads). Data from the anterior and posterior DTCs are pooled. (C) In *mig-4(rh51)* mutants, the gonad primordium is often displaced to the dorsal side. Although the first migratory phase occurs on the dorsal side, *unc-5B::lacZ* expression is still observed only in subsequent phases. Similar results were obtained for mutants of *dig-1*, which have a similar gonad defect (data not shown). (D,E) In *daf-12(rh84)* (D) and *mig-8(rh50)* (E) mutants, the DTCs often delay (dashed arrow with closed head) or fail (dashed arrows with open heads) to carry out the second and third migratory phases. In these mutants, *unc-5B::lacZ* expression is also delayed or absent.



of genomic DNA upstream of exon 2 to *gfp* or *lacZ* reporter genes. This fragment contains sufficient regulatory sequence to rescue cell migration and axon guidance phenotypes of *unc-5* mutants when fused to an *unc-5* cDNA (Hamelin et al., 1993; M.-W. S. and J.G.C., unpublished data). *unc-5B::HA* and *unc-5B::GFP* translational reporters that rescued the *unc-5* mutant defects were also made. Expression was observed in the hermaphrodite DTCs and MLCs as well as in all five classes of motoneurons that exhibit axon guidance defects in *unc-5* mutants (DA, DB, DD, VD, AS; Fig. 3A,B). In addition, expression was observed in several classes of neurons in the head that are not visibly affected by *unc-5* mutations. These included 12 sensory neurons of the OLQ, OLL and IL2 classes (Fig. 3C), and the interneurons RIA, RIH and ASE.

Expression of *lacZ* or *gfp* transcriptional reporters in the DA, DB and DD embryonic motoneurons was first detected in late stage embryos at about the time when these neurons are known to extend pioneer axons along the epidermis (aka, hypodermis) to the dorsal side (Fig. 3A). Expression in the DA and DB neurons was transient, and only DD staining persisted into larval and adult stages (Fig. 3B). Expression in larval motoneurons was also consistent with the role of UNC-5 in pioneer axon guidance. The VD and AS classes of motoneurons are born at the L1 to L2 molt, and extend axons toward the dorsal side soon thereafter. The VD motoneurons expressed intensely at this and at later times (Fig. 3B), while the AS neurons expressed faintly and sporadically (not shown). The expression of *unc-5B* reporters in cells known to require *unc-5* for guided migrations is entirely consistent with previous mosaic analysis, which showed that UNC-5 acts cell-autonomously in the DTCs and motoneurons (Leung-Hagesteijn et al., 1992).

***unc-5B* is expressed in DTCs and MLCs at the time of the initiation of dorsalward turning**

Reporter gene expression in the DTCs was never detected during the centrifugal first phase of migration ($n=644$; Fig. 4A,B). *unc-5B::lacZ* and *unc-5B::unc-5HA* expression were first detectable in DTCs at the positions where they normally

begin to reorient in order to migrate toward the dorsal side (Figs 3D, 4A,B). Similar results were obtained in *mig-4(rh51)* mutants in which the gonad primordium was displaced dorsally (Fig. 2D, 4C). For the anterior DTC, this turning position is, with some variability, adjacent to the cell body of the VD4 motoneuron. Occasionally, DTC staining could be observed at positions posterior to VD4, but at no greater frequency than that with which the anterior DTC makes its turn at a position posterior to VD4 (data not shown). Staining was relatively intense in DTCs migrating from ventral-to-dorsal muscle bands and decreased thereafter during the centripetal third phase of migration. In the MLCs, β -gal staining was similarly detected beginning at the position at which the first 90° turn is initiated (data not shown). Importantly, both the HA-tagged and GFP-tagged *unc-5B::unc-5* constructs could rescue nearly completely the DTC migration defects of an *unc-5* null mutant (M. T. K. and J. G. C., unpublished data).

To assess the timing of *unc-5* expression in living animals, *unc-5B* transcriptional and translational GFP reporters were used. As with *lacZ* and HA-tagged reporter constructs, expression was never observed in the DTCs during the centrifugal first migratory phase. In contrast, however, expression of GFP was detectable only after the DTCs had begun their second (ventral-to-dorsal) phase of migration, then persisted until the end of the L4 stage. The delayed initial expression of the GFP reporters relative to the *lacZ* reporter gene may reflect the time required for the activation of the GFP fluorophore.

***unc-5* expression is sufficient to induce dorsalward turning**

The expression of *unc-5B::lacZ* reporter constructs at the time

Table 1. Suppression of precocious DTC migrations by mutations in *unc-6* or *unc-5*

Strain	Wild type ¹		Precocious ²		No dorsal ³		<i>n</i>
	ANT ⁴	POST ⁵	ANT	POST	ANT	POST	
N2	100%	100%	0%	0%	0%	0%	100
<i>evIs99</i>	34±5%	25±4%	66±3%	75±4%	0%	0%	110
<i>evIs99/+</i>	91±2%	91±2%	9±2%	9±2%	0%	0%	160
<i>unc-6(ev400)</i>	56±4%	14±3%	0%	0%	44±4%	86±3%	157
<i>evIs99;unc-6(ev400)</i>	53±5%	11±3%	4±2%	0%	43±5%	89±3%	100
<i>unc-5(e53)</i>	69±4%	27±4%	0%	0%	31±4%	73±4%	110
<i>evIs99;unc-5(e53)</i>	88±3%	59±5%	6±2%	26±4%	6±2%	15±4%	100

The percentages of misreflexed gonad arms is shown ± the standard error of a binomial distribution of the same sample size and observed mean.

¹As in Fig. 2A

²As in Fig. 2E

³As in Fig. 2B

⁴ANT is the anterior DTC

⁵POST is the posterior DTC

of the initiation of the second phase of DTC migration, and the ability of tagged *unc-5B::unc-5* transgenes (which express at the time of the turn) to rescue *unc-5* mutant DTC defects, suggest that *unc-5* gene expression may initiate dorsalward turning. If this were the case, expressing the *unc-5* cDNA in the DTCs at earlier times could induce precocious turning toward the dorsal side. The *unc-5* cDNA was placed under the control of the 5' regulatory sequence of the *emb-9* gene. This promoter drives expression in the DTCs and other somatic cells throughout L3 and L4 (Graham et al., 1997). Most animals that carry the *emb-9::unc-5* transgene in an extrachromosomal or an integrated multicopy array (*evIs99*) exhibit precocious DTC turning (Fig. 1G-K; summarized in Fig. 2E). Consistent with a precocious ventral-to-dorsal migration superimposed upon the normal first-phase navigational program, DTCs that turn precociously follow an oblique angle (relative to the dorsoventral axis) to reach the dorsal side. They then continue to migrate centrifugally along the dorsal muscle band slightly variable distances, but usually distal to the normal position of the second turn, before initiating the centripetal third migratory phase (Figs 1G-K, 2E). The highest penetrance of this defect was observed in the *evIs99* integrated line (Table 1).

In timed experiments (see Methods), it was found that, in *evIs99(emb-9::unc-5)* hermaphrodites, 13 of 30 DTCs had turned obliquely by 28-29 hours after hatching. In contrast, in strains not carrying the *emb-9::unc-5* transgene, no DTCs turned prior to 29 hours after hatching (*n*=16), and ventral-to-dorsal migration was always immediately followed by a centripetal turn. The DTC phenotype in the *evIs99* line was largely copy number dependent, as animals with one copy of the integrated array exhibited few precocious DTC migrations (Table 1). This indicates a strong sensitivity of this phenotype to the dose of prematurely expressed UNC-5. Precocious DTC migration was the only visible phenotype caused by the *emb-9::unc-5* transgene in hermaphrodites. In males, precocious MLC migrations were also induced by *emb-9::unc-5*, but for reasons that are not understood, male gonad arms always failed to reflex toward the posterior and instead extended toward or into the head of the worm, resulting in sterility. This phenotype was also largely dependent on the copy number of the *evIs99* array (data not shown).

Precocious migrations are suppressed by *unc-6* and *unc-5* mutations

Genetic, molecular and biochemical results suggest that UNC-6 is the ligand for the UNC-5 receptor, which is required for ventral-to-dorsal migrations on the epidermis (Hedgecock et al., 1990; Hamelin et al., 1993; Leonardo et al., 1997). The effects of *unc-5* and *unc-6* null mutations on the *emb-9::unc-5*-induced precocious DTC migrations were examined. The precocious DTC migration phenotype in the *evIs99* line was almost completely suppressed by a null mutation in *unc-6* (Table 1). The *evIs99; unc-6(ev400)* double mutant was indistinguishable from *unc-6(ev400)* alone, with the centripetal third migratory phase frequently occurring ventrally. Marginally precocious migrations occurred in only 4 of 200 DTCs examined. The precocious ventral-to-dorsal DTC migrations are therefore dependent upon directional information provided by UNC-6.

Surprisingly, a null mutation in *unc-5* also reduced the frequency of precocious migrations (Table 1). Whereas in the *evIs99* line, 71% (156 of 220) of gonad arms made a precocious dorsalward turn, only 16% (32 of 200) of *evIs99; unc-5(e53)* gonad arms exhibited this phenotype. These results indicate that, although *unc-5* reporter constructs are not expressed in the DTCs during the first migration phase, there is some basal level of expression undetectable with the reporter constructs. This phase one expression, though insufficient to drive ventral-to-dorsal migration, contributes to the precocious migrations in the *emb-9::unc-5* transgenic background. The sensitivity of the precocious DTC migrations in the *emb-9::unc-5* lines to the amount of UNC-5 expressed is also evidenced by the dependence of the phenotype on the copy number of the *evIs99* integrated array (Table 1).

The *evIs99; unc-5(e53)* mutants also exhibited a partial suppression of the ventral misreflexion DTC defects of *unc-5*. In *unc-5(e53)* worms, 31% (34 of 110) anterior and 73% (80 of 110) posterior DTCs failed entirely to migrate from ventral to dorsal. In *evIs99; unc-5(e53)*, only 6% (6 of 94) anterior and 20% (15 of 74) posterior DTCs that did not make a precocious dorsalward turn failed entirely to migrate to the dorsal side, indicating that the premature UNC-5 expression that was unable to initiate precocious ventral-to-dorsal migration could nevertheless drive the same migration at the appropriate time in development. This suggests the existence of a facilitating

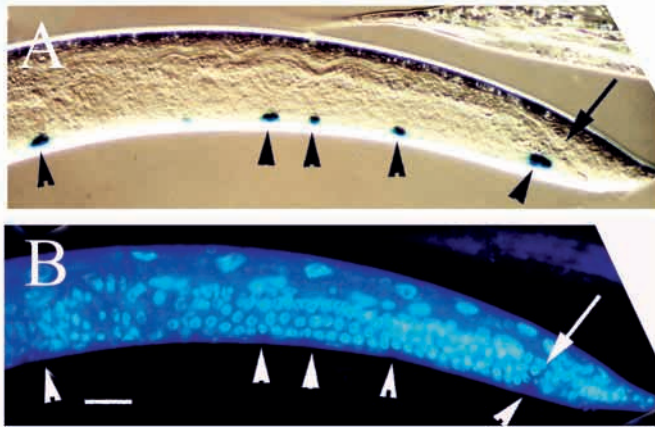


Fig. 5. *unc-5* expression is usually absent or delayed in mutants of *daf-12* and *mig-8*. (A,B) β-gal (A) and DAPI (B) staining of a *daf-12(rh84)* mutant animal in which the posterior DTC (arrow) has migrated into the tail region, fails to execute the second and third migratory phases and fails to express *UNC-5::LacZ*. VNC motorneurons (arrowheads), however, stain with normal intensity. (B) The gonadal germline nuclei just above the motorneurons are arranged in rows that extend the length of the gonad arm. Scale bar is 10 μm.

mechanism that acts with *UNC-5* at the appropriate time to induce reorientations of DTCs.

unc-5B expression in mutants affecting DTC migrations

The expression of the *unc-5B::lacZ* or *unc-5B::GFP* reporter gene was examined in various mutant strains in which DTC migrations are abnormal. Mutations that disrupt only the execution of the second phase of migration would not be expected to affect the expression of these reporter genes. In contrast, mutations that disrupt the developmental program of the DTCs might exhibit defects in *unc-5B* reporter gene expression. In mutants of *unc-130* (E. B. Nash and J. G. C., unpublished data) *unc-5*, *unc-6* or *unc-40* (Hedgecock et al., 1990), the second migratory phase of the DTC frequently fails to occur (Fig. 2B). First and third phase migrations, which occur along the anteroposterior axis, however, are normal in timing and extent. The expression of *unc-5B::lacZ* appeared normal in timing and intensity in these mutants, even though the second migratory phase failed to occur (data not shown). As described above, in *dig-1* or *mig-4* mutants, the entire gonad primordium is often displaced dorsally and the entire DTC migration program is carried out on the dorsal muscle bands. In these mutant backgrounds as well, *unc-5B::lacZ* expression appeared normal (Figs 2D, 4C).

In *mig-6(e1931)* hermaphrodites, the gonad arms do not elongate normally (Hedgecock et al., 1987). There is still, however, ventral-to-dorsal DTC migration in many mutant animals. In 74% (34 of 46) late L4 and young adult hermaphrodites, dorsally positioned DTCs were observed. As in wild-type worms, dorsalward reorientation of the DTCs was never observed prior to Pn.p cell divisions ($n=30$), indicating that the timing of the ventral-to-dorsal phase of DTC migration was approximately normal. In addition, *unc-5B::gfp* expression was frequently observed in these DTCs (Fig. 2C, data not shown). These observations suggest, in this mutant, the DTC

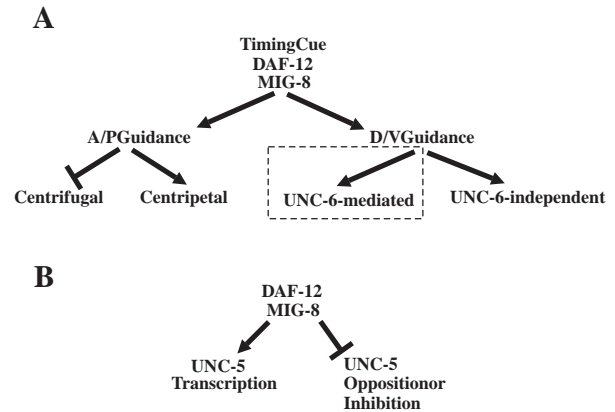


Fig. 6. A model for the regulation of DTC guidance during the *DAF-12/MIG-8*-dependent DTC re-orientation late in L3. (A) At this time, the DTCs, which have been migrating centrifugally along the ventral muscle bands, begin to respond to both *UNC-6*-dependent (dashed box) and *UNC-6*-independent dorsoventral guidance systems. In addition, we propose that the DTCs stop responding to centrifugal guidance cues and begin to respond to centripetal cues. These changes together drive the cells to reflex back toward the midbody along the dorsal muscle bands. (B) The initiation of responsiveness to the *UNC-6* guidance cue involves the transcriptional upregulation of the *unc-5* gene and the facilitation of *UNC-5* function, possibly through the downregulation of an opposing or inhibiting pathway.

developmental program occurs with normal timing even in the absence of DTC migrations along the anteroposterior axis.

In contrast to the above mutants, *daf-12* and *mig-8* mutants are thought to disrupt DTC migrations by blocking progression between larval stage-specific developmental programs (Antebi et al., 1998). Specifically, entry into the L3 or L4 programs is delayed or blocked. In *daf-12(rh84)* animals transgenic for the *unc-5B::lacZ* reporter construct, β-gal staining was never observed in the DTCs, although neuronal staining appeared normal (Figs 4D, 5; summarized in Fig. 2F). This is consistent with the phenotype of *daf-12(rh84)*, as normal gonad morphologies were observed in only 10/304 (3%) of gonad arms. In the remaining animals, the DTCs either did not turn or did so only at the extreme ends of the animal. It is possible that some low level of *unc-5* expression, undetectable with these methods, underlies these infrequent, usually delayed ventral-to-dorsal migrations.

In *mig-8(rh50)* worms, only 13% (52 of 384) of DTCs migrated from ventral to dorsal at the normal time, as judged by gonad morphologies (data not shown). Of these 52 DTCs, 14 expressed *unc-5B::lacZ*. As for *daf-12(rh84)*, most DTCs failed to turn dorsalward at the appropriate time and instead continued toward the head or tail. Some of these (179/334=53%) eventually moved dorsalward at an abnormal anteroposterior position. However, only 18 of these 179 exhibited β-gal staining (Fig. 4E). None of those DTCs that failed to migrate toward the dorsal side exhibited staining. Thus, only *daf-12* and *mig-8* mutations, which are the only mutations known to block or delay the advancement of stage-specific programs affecting the DTCs, are defective in *unc-5* expression. These results are consistent with the hypothesis that *daf-12* and *mig-8* are components of a developmental timing mechanism, which among other things, regulates expression of *UNC-5* in the DTCs to make them

responsive to UNC-6 and change their trajectory. Mutations in other genes that affect only one stage of DTC migration (e.g., *unc-5*, *unc-6*, *unc-40*, *unc-130*, *dig-1* and *mig-4*) all express UNC-5 with normal timing, consistent with the idea that they affect the execution of specific migratory phases, but do not affect the timing program that regulates DTC turning.

DISCUSSION

Previous work has demonstrated that changes in the expression of extracellular cues or of cell surface receptors can initiate cell migrations or cell shape changes. For example, temporal changes in the spatial expression of the *branchless* FGF homologue or the Robo receptor in *Drosophila* direct tracheal and axon extensions, respectively (Sutherland et al., 1996; Seeger et al., 1993; Kidd et al., 1998a,b). Alternatively, *Drosophila* border cell migrations require the transcription factor encoded by the *slow border cells* locus (*slbo*; Montell et al., 1992) to activate directly the expression of the *breathless* FGF receptor and thereby initiate migration (*btl*; Murphy et al., 1995). Post-transcriptional mechanisms have been proposed to mediate changes in the repertoire of cell-surface integrins in response to changes in ligand concentrations (Condic and Letourneau, 1997). Finally, ectopic expression of the UNC-5 netrin receptor in neurons that do not normally express that receptor makes the growth cones of these neurons responsive to UNC-6 (Hamelin et al., 1993). We have extended these findings to demonstrate that the UNC-5 receptor is a critical target for regulation of responsiveness to UNC-6 during the first turn of the DTCs. An understanding of the regulatory pathways directing DTC migrations may shed light on complex cell migrations in other systems, including the developing mammalian cerebellum, where granule cell precursors require the Unc5H3 netrin receptor for correct positioning (Ackerman et al., 1997). In addition, the UNC-6 netrin pathway is utilized in the complex migrations of many neuronal growth cones, and principles of guidance may be shared between cells and growth cones.

The triphasic pattern of DTC migrations is tightly synchronized with the progression of larval development (Antebi et al., 1998). The dorsalward turning of the DTCs occurs about 30 hours after hatching, coincident with other developmental events, such as the cell divisions of the Pn.p vulval precursor cells. The coordinated regulation of the development of gonadal and non-gonadal tissues is evidenced by the arrest of DTC migrations upon entry of the worm into the dauer larval stage. Antebi et al. (1998) have proposed that the steroid hormone receptor DAF-12 is part of a mechanism that advances developmental programs in all somatic tissues of *C. elegans*, including the gonad. Although the identity and source of the DAF-12 ligand are unknown, a diffusible steroid hormone could act simultaneously on many cells to synchronize cell-intrinsic larval stage advancement mechanisms. Mutations in *daf-12* or in *mig-8* can result in the failure of the DTCs to initiate phases 2 or 3 of migration. This is interpreted as the absence or the delay of entry into the normal L4 developmental program (Antebi et al., 1998). Consistent with this, we have found that, in these mutants, but not in mutants affecting other aspects of DTC migrations, transcriptional upregulation of *unc-5* at the appropriate time fails to occur.

Navigational programs of migrating cells may simultaneously regulate more than one guidance mechanism. For example,

transgenic expression of *btl* in *Drosophila* border cells under a conditional promoter rescues the *slbo* mutant phenotype (Murphy et al., 1995); however, the rescue is incomplete, suggesting that *btl* is probably not the sole target of regulation by the *slbo* protein. Similarly, the advancement in developmental stage mediated by *daf-12* and *mig-8* does not affect the DTC migrations exclusively through *unc-5*. The ventral-to-dorsal migration defects in null mutants of *daf-12* or *mig-8* are of higher penetrance than those of null mutants of *unc-5* or *unc-6* (Hedgecock et al., 1990; Antebi et al., 1998). Thus, *unc-5*- and *unc-6*-independent mechanisms of guidance along the dorsoventral axis must also be affected. In addition, as noted above, *unc-5* and *unc-6* mutations, unlike those in *daf-12* and *mig-8*, do not disrupt the third, centripetal phase of migration. We propose that, at the time of DTC re-orientation, guidance systems acting in both the longitudinal and the dorsoventral axes must be turned on or off. As summarized in Fig. 6A, the DAF-12/MIG-8-dependent turning involves the activation of both UNC-6-dependent and UNC-6-independent dorsoventral guidance systems. In addition, DTCs alter their responses to longitudinal cues and re-orient from centrifugal to centripetal migration. These other guidance mechanisms in DTC migrations are not yet understood and their regulation need not occur in the same manner as the UNC-6 guidance system. For example, some heterochronic mutants that, unlike *daf-12*, affect larval stage advancements only of extragonadal tissues, do not themselves cause DTC migration defects, but act as strong enhancers of the DTC migration defects caused by *unc-5* null mutations (D. C. M., unpublished data). In contrast to the DTC-intrinsic mechanism by which DAF-12 affects the UNC-6 guidance system, temporal regulation of UNC-6-independent dorsoventral guidance may involve changes in some DTC-extrinsic guidance factor, possibly a directional cue.

Mosaic analysis has previously demonstrated that UNC-5 is required cell autonomously by cells and neurons that are repelled by UNC-6, including the DTCs (Leung-Hagesteijn et al., 1992). The shorter transcript of the *unc-5* gene begins with exon 2, and this transcript (*unc-5B*), along with its upstream regulatory sequence, is able to rescue completely the known cell migration and axon guidance defects of *unc-5* mutants (Hamelin et al., 1993). Analysis of the timing of reporter construct expression suggests that expression of the *unc-5B* transcript by the DTCs begins as the DTCs reorient from a longitudinal to a dorsally oriented trajectory. However, genetic evidence indicates that some low level of UNC-5 is actually present in the DTCs prior to the initiation of turning, and that endogenous UNC-5 contributes to the precocious migrations caused by premature (*emb-9::unc-5*) transgenic UNC-5 expression. We propose that the *unc-5B* reporter constructs contain an enhancer element that is, directly or indirectly, responsive to a signal from DAF-12. The phase one expression of *unc-5* that we detect genetically may not be visible using our reporters since the reporter constructs, which are based on 5' regulatory sequence, are lacking large intronic fragments that could contain additional regulatory elements. Thus, either *unc-5* transcript (A or B) could normally be expressed in the DTCs prior to their ventral-to-dorsal (second) phase of migration. We interpret this as evidence that inhibiting or competing pathways must act during the first phase of migration to prevent precocious responses to UNC-6 through UNC-5 and UNC-40. This is consistent with the observation that premature transgenic expression of UNC-5 in

the DTCs causes precocious dorsalward migrations in an UNC-5 dosage-sensitive manner. The presence of high levels of UNC-5 is required to initiate turning, while low levels are insufficient to cause precocious turning.

Constitutively expressed transgenic UNC-5 largely rescued the DTC defects of a null *unc-5* mutant, although the frequency of precocious migrations was reduced. Therefore, UNC-5 expression that was insufficient to initiate precocious dorsalward turning during the first migration phase is able to do so at the normal time of reorientation. Therefore, as summarized in Fig. 6B, in addition to transcriptional regulation of *unc-5*, DAF-12 activity may also induce a mechanism that facilitates UNC-5 function during the initiation of turning. The nature of this mechanism is unknown. It presumably is not involved in the transcriptional regulation of *unc-5* but it could affect UNC-5 post-transcriptionally. For example, it could be a mechanism that inhibits the UNC-5 inhibitory (or opposing) pathway that operates during phase I as proposed above. Alternatively, it may be a novel guidance mechanism that acts in parallel with UNC-5 and UNC-6.

These studies have thus far focussed only on the transition between the first and second phases of DTC migrations. The initiation of motility in the DTCs, the transitions between the second and third migratory phases and the cessation of motility should also be accessible to genetic analysis. The combined analyses of cell migration mutant phenotypes and reporter construct expression may be used to distinguish mutations affecting the migration program (or its advancement) from those affecting the execution of the program. It will thus be possible to test the model for the regulation of the UNC-6/netrin system proposed above for its applicability to other transitions in DTC migrations. If such a scheme is generally applicable to cell and growth cone migrations, it may shed light on the mechanisms of cell migration in development and organogenesis in general.

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