Axon Guidance along the Dorsoventral Axis of *Caenorhabditis elegans*

by

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A thesis submitted in conformity with the requirements for the degree of Ph.D.
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University of Toronto

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Axon Guidance along the Dorsoventral Axis of Caenorhabditis elegans

Doctor of Philosophy, 1998
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Abstract

Axon guidance along the dorsoventral axes of animals belonging to diverse phyla involves secreted netrin/UNC-6 laminin-related guidance cues and the UNC-5 and DCC/UNC-40 receptor families. Genetic studies in C. elegans reveal that both UNC-5 and UNC-40 are required to repel motile cells or axons away from UNC-6, while UNC-40 is required to attract motile cells or axons toward ventral sources of UNC-6.

The UNC-5 guidance receptor, in response to the UNC-6/netrin path cue, orients growing axons in a dorsal direction along the epidermis of C. elegans. When ectopically expressed in the touch receptor neurons, which normally extend ventrally or longitudinally, UNC-5 is able to reorient their axons toward the dorsal side in an UNC-6 dependent manner. This property forms the basis of a genetic screen to identify other mutations that, like unc-6 mutations, suppress unc-5-induced growth cone guidance. These mutations may identify new components required for pioneer axon guidance by unc-5. In this study, I describe eight genes that are required for ectopic unc-5-induced growth cone steering. Four are the previously known axon guidance genes unc-6, unc-40, unc-34, and unc-44, and four are the novel genes: unc-129, seu-1, seu-2, and seu-3. I propose that some or all of these genes may function in a developmentally important unc-5 signaling pathway.

Mutations in unc-129 cause dorsal axon guidance defects that are similar to those exhibited by mutations in unc-5, unc-6, and unc-40. I show that unc-129 encodes a novel member of the transforming growth factor-β (TGF-β) family of secreted signaling molecules and is expressed in a restricted pattern that includes motor neurons and dorsal body wall muscle. By further restricting expression to either motor neurons or to muscle, I found that expression in muscle is required for axon guidance. Ectopic expression studies indicate that asymmetric expression of unc-129, with expression in dorsal, but not ventral body wall muscle, is essential for axon guidance. The work discussed here provides the first functional evidence that a TGF-β is involved in a phylogenetically conserved axon guidance mechanism.
Acknowledgements

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<th>Definition</th>
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<tbody>
<tr>
<td>A/P</td>
<td>anteroposterior</td>
</tr>
<tr>
<td>AbA</td>
<td>antibody buffer A</td>
</tr>
<tr>
<td>AbB</td>
<td>antibody buffer B</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>bli</td>
<td>blister</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>bnl</td>
<td>branchless</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>btl</td>
<td>breathless</td>
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<tr>
<td>cam</td>
<td>CAN abnormal migration</td>
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<tr>
<td>cAMP</td>
<td>adenosine-3', 5'-cyclic phosphate</td>
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<tr>
<td>ceh</td>
<td>C. elegans homeobox</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CRMP-62</td>
<td>collapsin response mediator protein</td>
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<tr>
<td>CUB</td>
<td>complement C1r/s homology domain</td>
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<tr>
<td>D/N</td>
<td>dorsoventral</td>
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<td>daf</td>
<td>dauer larva formation abnormal</td>
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<tr>
<td>dbl</td>
<td>dpp-BMP-2,4-like</td>
</tr>
<tr>
<td>dcc</td>
<td>deleted in colorectal cancer</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DiO</td>
<td>3,3'-dioctadecloxacarbocyanine perchlorate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DNC</td>
<td>dorsal nerve cord</td>
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<td>dNTP</td>
<td>deoxyribonucleoside triphosphates</td>
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<td>decapentaplegic</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ELF</td>
<td>EPH ligand family</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscope</td>
</tr>
<tr>
<td>EMS</td>
<td>ethyl methanesulfonate</td>
</tr>
<tr>
<td>enu</td>
<td>enhancer of uncoordinated behaviour</td>
</tr>
<tr>
<td>epi</td>
<td>epithelialization abnormal</td>
</tr>
<tr>
<td>fam</td>
<td>fasciculation and cell migration defective</td>
</tr>
<tr>
<td>fax</td>
<td>defective fasciculation of axons</td>
</tr>
<tr>
<td>fem</td>
<td>feminization</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FNIII</td>
<td>fibronectin type III</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GDF</td>
<td>growth and differentiation factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphatidyl inositol</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>ina</td>
<td>integrin α-subunit</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LG</td>
<td>linkage group</td>
</tr>
<tr>
<td>lin</td>
<td>lineage abnormal</td>
</tr>
<tr>
<td>mau</td>
<td>maternal effect uncoordinated</td>
</tr>
<tr>
<td>mes</td>
<td>maternal effect sterile</td>
</tr>
<tr>
<td>mig</td>
<td>cell migration abnormal</td>
</tr>
<tr>
<td>MIS</td>
<td>Mullerian-inhibiting substance</td>
</tr>
<tr>
<td>mu</td>
<td>map unit</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization sequence</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>pag</td>
<td>pattern of reporter gene expression</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-N,N'-bis[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>RAGS</td>
<td>repulsive axon guidance signal</td>
</tr>
<tr>
<td>rcm</td>
<td>rostral cerebellar malformation</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPTK</td>
<td>receptor protein tyrosine kinase</td>
</tr>
<tr>
<td>sax</td>
<td>sensory axon defects</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>seu</td>
<td>suppressor of ectopic unc-5</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>sma</td>
<td>small</td>
</tr>
<tr>
<td>tax</td>
<td>chemotaxis abnormal</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TSP</td>
<td>thrombospondin</td>
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<tr>
<td>unc</td>
<td>uncoordinated</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>vab</td>
<td>variable abnormal morphology</td>
</tr>
<tr>
<td>VNC</td>
<td>ventral nerve cord</td>
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<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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<tr>
<td>YAC</td>
<td>yeast artificial chromosome</td>
</tr>
<tr>
<td>ZO-1</td>
<td>zona occludens-1</td>
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CHAPTER ONE

General Introduction
A. General Introduction and Outline of Thesis

The human nervous system is a complex structure comprised of more than a trillion neuronal connections. Axon growth cones, the motile tips of growing neurons, migrate over a complex extracellular environment to make stereotypical connections with target cells. What are the factors that guide axons to distant target cells? How are complex nervous systems wired? These are fundamental questions in developmental neurobiology. I have addressed these questions by studying axon guidance in Caenorhabditis elegans, an anatomically simple and genetically tractable model organism. The usefulness of studying processes in simple model organisms has been demonstrated by the identification of components of signaling cascades that have been conserved between invertebrates and vertebrates (Kopan and Turner, 1996; Skaer, 1997).

To study axon guidance mechanisms, I initiated a genetic screen to identify new genes that interact with UNC-5, a transmembrane protein that is required for dorsally directed growth cone and cell migration along the basal surface of the epidermis of C. elegans. UNC-5 is a component of a conserved mechanism involved in axon and cell migration along the dorsoventral (D/V) axes of invertebrates and vertebrates. In Chapter I, I provide a general overview of the nervous system of C. elegans and mechanisms of axon guidance, emphasizing the UNC-6/netrin pathway. In Chapter II, I describe and discuss the results of a genetic screen for suppressors of the axon guidance defects caused by ectopic UNC-5 expression in a subset of sensory neurons. Some of the mutations identified in this screen defined a new axon guidance gene that was designated unc-129. In Chapter III, I present the results of the molecular cloning and analysis of unc-129 and discuss possible implications for axon guidance along the D/V axis of C. elegans. Finally, Chapter IV provides a general discussion and possibilities for future experiments.

B. Caenorhabditis elegans: A Model Organism for Studies on Axon Guidance

Sydney Brenner pioneered the use of Caenorhabditis elegans, a free-living soil nematode, as a model organism for genetic studies of animal development (Brenner, 1974). The short life cycle of approximately three days involves passage through an embryonic stage and
four larval stages before reaching adulthood. *C. elegans* can differentiate as two sexual forms: hermaphrodite or male. However, a wild-type population is primarily composed of self-fertilizing hermaphrodites. The adult hermaphrodite, approximately 1 mm in length, consists of only 959 somatic cells of which 302 are neurons.

Several factors establish *C. elegans* as an excellent model system for genetic analysis. The worm's small size, short life cycle and large brood size allow it to be easily maintained and grown in large quantities in the laboratory (Brenner, 1974). Its anatomical simplicity and transparency have allowed the description of the complete somatic cell lineage of the worm (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). In addition, through EM reconstructions, a full catalogue of neuronal connections has been made (White *et al.*, 1976; White *et al.*, 1986).

*C. elegans* has a relatively small genome size for a metazoan. The haploid genome is about 100 Mb compared to 3000 Mb for human (Waterston *et al.*, 1997). Furthermore, the genome is packaged into only six chromosomes, which greatly facilitates genetic analysis. Point mutations and genomic rearrangements can be readily generated using irradiation and chemical mutagens (Johnsen and Baillie, 1997). At present, the genetic map contains more than 1500 mapped genes and more than 500 deficiencies and duplications (Hodgkin *et al.*, 1997).

A number of tools and procedures are available to study development in *C. elegans*. Cells can be selectively killed or ablated by a laser to determine their role in a particular behaviour (Chalfie, 1983). Mosaic analysis can be performed to determine the site of action of any gene that has been disrupted by mutation (Herman, 1989). In addition, germline transformation allows the introduction and stable transmission of exogenous DNA through the germline of *C. elegans* and facilitates cloning of genes identified by mutation (Mello and Fire, 1995).

The *C. elegans* genome will be the first of any metazoan to be completely sequenced. The genome sequence is an invaluable resource that has already expedited genetic and molecular analyses (Wilson *et al.*, 1994). In addition, recently developed techniques have made possible the identification of mutations in any sequenced gene (Zwaal *et al.*, 1993). These techniques, known as reverse genetics, complement the traditional identification of mutations by forward genetic screens.
An Overview of the Nervous System of *C. elegans*

Detailed knowledge of the nervous system of *C. elegans* provides an essential foundation from which to initiate genetic studies of the underlying molecular processes and pathways that are responsible for nematode behaviour. The position and connectivity of all 302 neurons in the adult are known from detailed reconstruction of serial sections of electron micrographs (White et al., 1976, 1986). Neurons and their processes are located between the hypodermis and its underlying basal lamina. Most neurons are structurally simple, extending a single unbranched axon along either a longitudinal or circumferential trajectory on the hypodermis. A variety of different neuronal types exist. Neurons are classified as motor, sensory, or interneurons or combinations of these classes based on their function and their connectivity (Chalfie and White, 1988).

Neurons and their processes are found throughout the body but most are bundled in the nerve ring that encircles the pharynx and in the ventral nerve cord (VNC) that runs longitudinally along the ventral midline from the head to the tail. A subset of neurons contribute axonal processes to lateral nerve bundles that run longitudinally along the body wall. The VNC consists of 57 evenly distributed motoneurons, processes of interneurons that originate in the head and tail, and processes from neurons located at lateral axial positions and from sensory ganglia in the head and tail (White et al., 1976; Hall and Russell, 1991). Some motoneurons in the VNC extend axons as commissures to the dorsal midline. At this point they turn longitudinally to establish the other major nerve tract in the worm, the dorsal nerve cord (DNC). Figure 1.1 displays schematic diagrams of the *C. elegans* nervous system in an L1 larva.

There are 95 body wall muscles organized into four quadrants on the body, with two subdorsal and two subventral rows on each side (Waterston, 1988). Eight classes of motoneurons, DA, DB, DD, AS, VD, VA, VB, and VC, control movement by forming neuromuscular junctions with these muscles. DA, DB, and DD neurons arise during embryogenesis, whereas VA, VB, VC, VD, and AS neurons arise during the first larval stage (White et al., 1976). VA, VB, and VC motor axons project along the VNC to innervate ventral body wall muscles, whereas DA, DB, and AS motor axons project dorsally to innervate dorsal body wall muscles (White et al., 1976). DD and VD motoneurons are located in the VNC and project axons along both the VNC and the DNC. DD motor axons innervate ventral muscle during
Figure 1.1. Schematic diagrams of the *C. elegans* nervous system. (A) The position of neuronal cell bodies in an L1 larva. This figure is a modified version of one appearing in *Developmental Biology* [Sulston et al. (1983)], Copyright (1983) Academic Press and is reprinted with permission. (B) A schematic drawing of a L1 larva cut at the dorsal midline and opened showing the direction of axon outgrowth. This figure is reprinted with permission from *Development* [Hedgecock et al. (1987)] Copyright (1987) The Company of Biologists Limited.
embryonic development but then undergo synaptic rewiring at the end of the first larval stage to innervate dorsal muscles (White et al., 1978). At the same time that DD axons are being rewired, VD motor axons form neural muscular junctions with ventral muscle (White et al., 1978).

Examination of the behavioural consequences of laser ablation of specific motorneurons and inferences made from the pattern of synaptic connections revealed the circuitry that mediates movement (Chalfie et al., 1985; Chalfie and White, 1988). The DB and VB and DA and VA classes of motorneurons are excitatory, and together with their associated interneurons, they stimulate muscle contractions to mediate forward and reverse movement, respectively. The DD and VD classes of motorneurons are inhibitory, and they act to relax the muscles that are on the opposite side of those undergoing contraction. The propagation of reciprocal contraction and relaxation of body wall muscles along the D/V plane results in the normal sinusoidal-like motion of worms.

Visualizing Axons in C. elegans

As C. elegans is a transparent animal, it offers the advantage of directly examining internal structures using light microscopy. Neuronal subsets can be visualized directly by immunostaining with antisera against neurotransmitters such as γ-aminobutyric acid (GABA) (McIntire et al., 1993) or serotonin (Desai et al., 1988) or against microtubules (Siddiqui et al., 1989). Some chemosensory axons, such as the amphids and phasmids, that have ciliated endings exposed to the external environment can be visualized easily because they take up fluorescent dyes such as FITC or DiO from the growth medium (Hedgecock et al., 1985; Starich et al., 1995).

Once a gene is cloned, a transcriptional fusion to sequence encoding β-galactosidase or green fluorescent protein (GFP) can be constructed to examine its expression pattern (Fire et al., 1990; Chalfie et al., 1994). GFP allows the trajectories of axons and motile cells to be followed in living animals as its fluorescence does not require the addition of exogenous cofactors (Chalfie et al., 1994). In contrast, histochemical staining for β-galactosidase requires time-consuming permeabilization and fixation steps.

Given the ease of detecting GFP, the construction of GFP-tagged proteins has become a popular alternative or a first step before generating antibodies to a particular protein (Chan et al.,
1996; Zipkin et al., 1997). A GFP-tagged protein may be used for localization studies if it is first tested for normal function in germline transformation experiments.

**Genetic Screens to Identify Axon Guidance Genes**

Genetic screens in simple organisms like *C. elegans* are powerful tools to identify the components of developmentally important pathways. A genetic pathway can be dissected and studied by inducing mutations that selectively disrupt or perturb normal gene functions. The role of the wild-type gene may then be deduced from the effects of mutations in the gene on the organism.

Many mutants that exhibit axon guidance errors have been identified based on their behavioural defects. For example, genetic screens have been designed to isolate mutants that exhibit uncoordinated movement (Unc) (Brenner, 1974), egg-laying defects (Trent et al., 1983), or are unable to respond to external stimuli such as volatile compounds (Starich et al., 1995). A subset of these behavioural mutants show defects in axon guidance while others are defective in cell fate (Finney et al., 1988), synaptogenesis (Hall and Hedgecock, 1991), neurotransmitter function (McIntire et al., 1993), cytoskeletal components (McKim et al., 1994), or muscle function (Benian et al., 1996).

Recently introduced GFP-based (Chalfie et al., 1994; Cubitt et al., 1995) and histochemical (Xie et al., 1995) techniques that facilitate visualization of axons in either living animals or in gravid hermaphrodites containing viable embryos without killing embryos developing *in utero* have formed the basis of powerful genetic screens. For example, a cell-specific GFP reporter transgene was used in a visual screen to identify mutants with defects in the longitudinal migrations of the canal-associated neurons (Forrester et al., 1998). These types of screens bypass the requirement for behavioural defects altogether and allow direct screening for axon guidance errors.

**C. General Mechanisms of Axon Guidance**

Growth cones, the motile tip of axons, migrate to target cells by sensing and responding to extracellular guidance information found in their local environment. Growth cone migration requires an environment that is permissive for growth; that is, one that permits transient cell
attachments to the underlying substratum to be established. Components of the extracellular matrix (ECM) such as laminin (Buettner and Pittman, 1991), tenascin (Gotz et al., 1996), and thrombospondin (Osterhout et al., 1992) have been demonstrated to act as substrates that promote axon outgrowth.

In addition to a permissive ECM, guidance information must also be distributed along the migratory path to provide directional cues to motile cells. Four general mechanisms of axon guidance have been identified: target-derived diffusible chemo-attractive and -repulsive cues and contact-dependent attractive and repulsive cues (Tessier-Lavigne and Goodman, 1996). The netrin family provides good examples of secreted proteins that can act as either long-range chemoattractive or chemorepulsive cues. The semaphorin and EPH families of axon guidance molecules are examples of proteins that generate short-range repulsive cues that guide axons by establishing boundaries or regions that inhibit axon migration. These families of axon guidance molecules will be discussed in more detail below. Short-range attractive guidance forces act by providing pre-existing 'highways' that axons follow. These 'highways' may take the form of differentially marked neuroepithelium (Hidalgo et al., 1995) or other axon processes (Wightman et al., 1997). The latter scenario is known as fasciculative axon guidance. The L1/NgCAM subfamily of neuronal cell adhesion molecules has been implicated in guiding axons across the midline of the developing spinal cord by mechanisms involving fasciculation and contact-dependent guidance (Stoeckli et al., 1996; Stoeckli et al., 1997).

D. Axon Guidance in C. elegans

Several mutants in C. elegans that were identified on the basis of aberrant behaviours, such as uncoordination, were found on subsequent inspection to contain neuroanatomical abnormalities such as premature axon termination (outgrowth or elongation defects), loss of cohesion in axonal bundles (fasciculation defects), or inappropriate axonal trajectories (guidance defects) (Hedgecock et al., 1987; Hedgecock et al., 1990; Siddiqui and Culotti 1991; McIntire et al., 1992). Recently, direct visualization of axon morphology using GFP-based reporters, histochemical staining, or immunostaining methods has revealed additional axon pathfinding mutants (Xie et al., 1995; Forrester and Garriga, 1997; Wightman et al., 1997). Table 1.1
Table 1.1. Genes Involved in Axon Guidance in *C. elegans*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Product</th>
<th>Axon Guidance Role</th>
<th>Axon Guidance Defects</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td><strong>unc-5</strong></td>
<td>see Table 1.2</td>
<td>see Table 1.2</td>
<td>see Table 1.2</td>
<td>1McIntire et al., 1992</td>
</tr>
<tr>
<td><strong>unc-6</strong></td>
<td>see Table 1.2</td>
<td>see Table 1.2</td>
<td>see Table 1.2</td>
<td>2Ogura et al., 1997</td>
</tr>
<tr>
<td><strong>unc-14</strong></td>
<td>novel protein(^{2})</td>
<td>General role in axon outgrowth, guidance, and fasciculation. UNc-14 interacts directly with UNC-51(^{2}).</td>
<td>HSN ventral outgrowth and longitudinal elongation defect(^{1}), premature termination of DD axons in DNC(^{3}), premature termination of dorsal outgrowth of DD and VD motor axons(^{4}), fascication defects in VNC and DNC(^{5}), abnormal varicosities along axons(^{6}).</td>
<td>(^{1})McIntire et al., 1992 (^{2})Ogura et al., 1997</td>
</tr>
<tr>
<td><strong>unc-30</strong></td>
<td>homeodomain protein(^{1})</td>
<td>Pioneering of left VNC.</td>
<td>Defect in PVPR pioneering the left VNC bundle(^{3}), PVQL, AVKR, and HSNL axons extend along wrong VNC bundle(^{3}).</td>
<td>(^{1})Jin et al., 1994 (^{2})Wightman et al., 1997</td>
</tr>
<tr>
<td><strong>unc-33</strong></td>
<td>related to collapsin-response mediator protein-CRMP-62 (^{4,5})</td>
<td>General role in axon outgrowth, guidance, and fasciculation.</td>
<td>Amphids exhibit defasciculation and premature termination in nerve ring(^{1}), premature termination of phasmids in VNC(^{1}), PDE axon outgrowth and guidance defects(^{1}), abnormal neuronal microtubules, PHC and PVN axon guidance defects(^{2}), HSN ventral outgrowth and longitudinal elongation defect(^{2}), premature termination of DD axons in DNC(^{3}), fasciculation defects in VNC and DNC(^{5}), mild defects in circumferential elongation of DD and VD motor axons(^{5}).</td>
<td>(^{1})Hedgecock et al., 1985 (^{2})Siddiqui and Culotti, 1991 (^{3})McIntire et al., 1992 (^{4})Li et al., 1992 (^{5})Goshima et al., 1995</td>
</tr>
<tr>
<td><strong>unc-34</strong></td>
<td>?</td>
<td>Longitudinal and circumferential axon guidance. Role in axon outgrowth along other axons. Axon fasciculation.</td>
<td>Premature termination of axons (DD, VD, HSN, phasmids) along VNC(^{1,2}), premature termination of DD axons in DNC(^{1}), fascication defects in VNC and DNC(^{5}), defects in dorsally-directed guidance of DD and VD motor axons(^{5}), commissures sometimes on wrong side, premature termination of posterior directed CAN axon(^{2}), HSN axons extend together in VNC(^{2}), also cell migration defects(^{2}).</td>
<td>(^{1})McIntire et al., 1992 (^{2})Forrester and Garriga, 1997</td>
</tr>
<tr>
<td><strong>unc-40</strong></td>
<td>see Table 1.2</td>
<td>see Table 1.2</td>
<td>see Table 1.2</td>
<td>(^{1})McIntire et al., 1992 (^{2})Ogura et al., 1997</td>
</tr>
<tr>
<td><strong>unc-42</strong></td>
<td>homeodomain protein</td>
<td>Pioneering of left VNC.</td>
<td>Defect in PVPR pioneering the left VNC bundle; PVQL, AVKR, and HSNL extend along wrong VNC bundle.</td>
<td>Wightman et al., 1997</td>
</tr>
<tr>
<td>Gene</td>
<td>Gene Product</td>
<td>Axon Guidance Role</td>
<td>Axon Guidance Defects</td>
<td>References</td>
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<tr>
<td>unc-44</td>
<td>ankyrin-related^4</td>
<td>General role in axon outgrowth, guidance, and fasciulation.</td>
<td>Amphids exhibit defasciculation and premature termination in nerve ring^1^, premature termination of phasmids in VNC^1^, PDE axon outgrowth and guidance defects^1^, PHC and PVN axon guidance defects^2^, HSN ventral outgrowth and longitudinal elongation defect^3^, premature termination of DD axons in DNC^3^, fasciculation defects in VNC and DNC^3^, mild defects in circumferential elongation of DD and VD motor axons^3^</td>
<td>^1Hedgecock et al., 1985^2Siddiqi and Culotti, 1991^3McIntire et al., 1992^4Otsuka et al., 1995</td>
</tr>
<tr>
<td>unc-51</td>
<td>cytoplasmic serine/threonine kinase^4</td>
<td>General role in axon outgrowth, guidance, and fasciulation. UNC-51 interacts directly with UNC-14^2^</td>
<td>Premature termination of phasmids in VNC^1^, PDE axon outgrowth and guidance defects^1^, PHC and PVN axon guidance defects^2^, termination of dorsal outgrowth of DD and VD motor axons^3^, mild defects in ventral outgrowth of HSN axon^3^, fasciculation defects in VNC and DNC^3^, abnormal varicosities along axons^3^</td>
<td>^1Hedgecock et al., 1985^2Siddiqi and Culotti, 1991^3McIntire et al., 1992^4Ogura et al., 1994^5Ogura et al., 1997</td>
</tr>
<tr>
<td>unc-53</td>
<td>novel protein^2</td>
<td>Axon guidance along antero-posterior axis.</td>
<td>PDE axon outgrowth and guidance defects^1^, axon guidance defects along the antero-posterior axis^2^, also cell migration defects^1^</td>
<td>^1Hedgecock et al., 1985^2McIntire et al., 1992^3Maillet and T. Bogaert, personal communication</td>
</tr>
<tr>
<td>unc-71</td>
<td>?</td>
<td>Role in axon outgrowth along other axons.</td>
<td>PHC and PVN axon guidance defects^1^, HSN elongation defect along VNC^3^, fasciculation defects in VNC^3^, also cell migration defects^3^</td>
<td>^1Siddiqi and Culotti, 1991^2McIntire et al., 1992</td>
</tr>
<tr>
<td>unc-73</td>
<td>exchange-factor for Rho-family GTPases^2</td>
<td>General role in axon outgrowth, guidance, and fasciulation.</td>
<td>PHC and PVN axon guidance defects^1^, PDE axon outgrowth and guidance defects^2^, HSN ventral outgrowth and longitudinal elongation defect^3^, defects in circumferential elongation and guidance of DD and VD motor axons^3^, fasciculation defects in VNC and DNC^3^, CAN axon guidance defects^4^, commissures sometimes on wrong side^4^, also cell migration defects^2^</td>
<td>^1Siddiqi and Culotti, 1991^2McIntire et al., 1992^3Hedgecock et al., 1987^4Forrester and Garriga, 1997^5Steven et al., 1998</td>
</tr>
<tr>
<td>unc-76</td>
<td>novel protein^3</td>
<td>Longitudinal axon guidance. Role in axon outgrowth along other axons. Axon fasciulation.</td>
<td>Amphids exhibit defasciculation and premature termination in nerve ring^1^, premature termination of phasmids in VNC^3^, PDE axon outgrowth and guidance defects^1^, HSN elongation defect along VNC^2^, fasciculation defects in VNC and DNC^3^</td>
<td>^1Hedgecock et al., 1985^2McIntire et al., 1992^3Bloom and Horvitz, 1997</td>
</tr>
<tr>
<td>Gene</td>
<td>Gene Product</td>
<td>Axon Guidance Role</td>
<td>Axon Guidance Defects</td>
<td>References</td>
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</table>
| unc-115 | actin-binding         | General role in axon                | AVKR and HSNL axons extend along wrong VNC bundle, defasculation defects in VNC, defects in circumferential elongation of DD and VD motor axons, premature termination of sublateral cord neurons. | 1Wightman et al., 1997  
|         | protein               | outgrowth, guidance, and fasciculation. |                                                                                                         | 2E. Lundquist and C. Bargmann, personal communication                                         |
| unc-119 | novel protein         | Axon fasciculation and              | VNC is highly defasciculated, motor axons exhibit ectopic branching.                                      | Maduro and Pilgrim, 1995                                                                                                                                |
|         |                       | outgrowth.                          |                                                                                                         |                                                                                                 |
| unc-130 | transcription         | Axon guidance along                 | Defects in circumferential guidance of motor axons, AVM, and PVM, also cell migration defects.            | B. Nash and J.G. Culotti, unpublished results                                                  |
|         | factor                | dorsoventral axis.                  |                                                                                                         |                                                                                                 |
| cam-1   | ?                     | CAN axon guidance.                  | Premature termination of posterior-directed CAN axon, cell migration defects.                              | Forrester and Garriga, 1997                                                                    |
| cam-2   | ?                     | CAN axon guidance.                  | Premature termination of posterior-directed CAN axon, HSN axons extend together in VNC, also cell migration defects. | Forrester and Garriga, 1997                                                                    |
| ceh-10  | homeodomain protein   | CAN axon guidance.                  | Premature termination of posterior-directed CAN axon, also cell migration defects.                       | 1Svendsen and McGhee, 1995  
|         |                       |                                    |                                                                                                         | 2Forrester and Garriga, 1997                                                                 |
| enu-1   | ?                     | Axon guidance along VNC.            | AVKR and HSNL axons extend along wrong VNC bundle, enhances vab-8 mutations.                            | Wightman et al., 1997                                                                        |
| epi-1   | laminin A subunit     | General role in axon                | Premature termination of posterior-directed CAN axon, HSN axons extend together in VNC, defects in circumferential elongation and guidance of DD and VD motor axons, commissures sometimes on wrong side, fasciculation defects in VNC. | 1Forrester and Garriga, 1997  
|         | 2                    | outgrowth, guidance, and fasciculation. |                                                                                                         | 2J. Kelichiro and E. Hedgecock, personal communication                                         |
| fam-1   | ?                     | General role in axon                | HSN axons extend together in VNC, mild defects in circumferential elongation and guidance of DD and VD motor axons, commissures sometimes on wrong side, fasciculation defects in VNC. | Forrester and Garriga, 1997                                                                    |
Table 1.1. Genes Involved in Axon Guidance in *C. elegans*.

<table>
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<th>Gene Product</th>
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<th>Axon Guidance Defects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>fam-2</td>
<td>nuclear hormone receptor-transcription factor&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Axon Fasciculation.</td>
<td>PVQL, AVKR, and HSNL axons extend along wrong VNC bundle&lt;sup&gt;1&lt;/sup&gt;, fasciculation defects in VNC&lt;sup&gt;2&lt;/sup&gt;.</td>
<td>&lt;sup&gt;1&lt;/sup&gt;Wightman et al., 1997  &lt;sup&gt;2&lt;/sup&gt;B. Wightman and G. Garriga, personal communication</td>
</tr>
<tr>
<td>fax-1</td>
<td>α integrin subunit&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Axon fasciculation.</td>
<td>HSN axons extend together in VNC&lt;sup&gt;1&lt;/sup&gt;, commissures sometimes on wrong side&lt;sup&gt;1&lt;/sup&gt;, fasciculation defects in VNC&lt;sup&gt;2&lt;/sup&gt;, also cell migration defects&lt;sup&gt;1,2&lt;/sup&gt;.</td>
<td>&lt;sup&gt;1&lt;/sup&gt;Forrester and Garriga, 1997  &lt;sup&gt;2&lt;/sup&gt;Baum and Garriga, 1997</td>
</tr>
<tr>
<td>ina-1</td>
<td>?</td>
<td>Axon guidance along both antero-posterior and dorso-ventral axes.</td>
<td>Maternal effect axon guidance defect; CAN, AVM, and PLM axons misrouted, also cell migration defects.</td>
<td>Takagi et al., 1997</td>
</tr>
<tr>
<td>mau-2</td>
<td>?</td>
<td>Axon guidance along both antero-posterior and dorso-ventral axes.</td>
<td>HSN elongation defect along VNC, HSN axons extend together in VNC, mild defects in circumferential elongation and guidance of DD and VD motor axons, commissures sometimes on wrong side, fasciculation defects in VNC.</td>
<td>Forrester and Garriga, 1997</td>
</tr>
<tr>
<td>mig-2</td>
<td>Rho GTPase&lt;sup&gt;2&lt;/sup&gt;</td>
<td>HSN axon guidance. Note: only activating mutations in <em>mig-2</em> cause axon guidance defects&lt;sup&gt;1&lt;/sup&gt;.</td>
<td>HSN ventral outgrowth and longitudinal elongation defect&lt;sup&gt;1,2&lt;/sup&gt;, HSN axons extend together in VNC&lt;sup&gt;1&lt;/sup&gt;, commissures sometimes on wrong side&lt;sup&gt;1&lt;/sup&gt;, also cell migration defects&lt;sup&gt;1,2&lt;/sup&gt;.</td>
<td>&lt;sup&gt;1&lt;/sup&gt;Forrester and Garriga, 1997  &lt;sup&gt;2&lt;/sup&gt;Zipkin et al., 1997</td>
</tr>
<tr>
<td>tax-2&lt;sup&gt;1&lt;/sup&gt;; tax-4&lt;sup&gt;2&lt;/sup&gt;</td>
<td>cyclic nucleotid-gated channel subunits</td>
<td>Amphid axon guidance.</td>
<td>Amphid axons extend past normal termination sites.</td>
<td>&lt;sup&gt;1&lt;/sup&gt;Coburn and Bargmann, 1996  &lt;sup&gt;2&lt;/sup&gt;Komatsu et al., 1996</td>
</tr>
<tr>
<td>sax-3</td>
<td>transmembrane protein</td>
<td>General role in axon guidance.</td>
<td>Nerve ring axons misrouted, PVQ axon repeatedly crosses the midline, head interneurons and HSN motor axons misrouted, also cell migration defects.</td>
<td>J. Zallen and C. Bargmann, personal communication</td>
</tr>
<tr>
<td>vab-8</td>
<td>kinesin-related protein&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Axon outgrowth along antero-posterior axis. Required for posterior-directed axon outgrowth.</td>
<td>Premature termination of posterior-directed axons of AVA, AVB, AVD, PDE and CP in the VNC&lt;sup&gt;1&lt;/sup&gt;; premature termination of posterior-directed axons of ALA and CAN neurons in lateral nerve bundle&lt;sup&gt;1&lt;/sup&gt;, also cell migration defects&lt;sup&gt;1&lt;/sup&gt;.</td>
<td>&lt;sup&gt;1&lt;/sup&gt;Wightman et al., 1996  &lt;sup&gt;2&lt;/sup&gt;F. Wolf and G. Garriga, personal communication</td>
</tr>
</tbody>
</table>
provides a comprehensive list of genes that are involved in axon outgrowth and guidance in *C. elegans*.

Axons in *C. elegans* grow either longitudinally along the anteroposterior (A/P) axis, usually in one of the major longitudinal axon bundles such as the VNC, or circumferentially along the D/V axis (White *et al.*, 1986). At present, the genes that direct circumferential migrations of axons are better understood than those that direct longitudinal migrations. For example, genetic studies have identified three well characterized genes, *unc-5, unc-6,* and *unc-40,* that are primarily involved in circumferential axon and cell migrations on the hypodermis of *C. elegans* (Hedgecock *et al.*, 1990; Culotti, 1994). The products of these genes belong to families of conserved axon guidance molecules that are involved in directing axon guidance along the D/V axes of animals of diverse phyla (Tessier-Lavigne and Goodman, 1996). A similar set of genes acting as part of a common mechanism to direct longitudinal migrations has not been identified.

Several axon guidance mutants share a similar array of axon and cell migration defects, suggesting that the affected genes likely act in the same guidance pathway (McIntire *et al.*, 1992; Forrester and Garriga, 1997; Wightman *et al.*, 1997). For example, mutations in *unc-14* and *unc-51* lead to a similar range of morphological and guidance defects such as atypical varicosities along axonal processes and defects in axon elongation (McIntire *et al.*, 1992). Molecular analysis revealed that *unc-14* encodes a novel protein and *unc-51* encodes a cytoplasmic serine/threonine protein kinase (Ogura *et al.*, 1994, 1997). The discovery of a direct physical association between these two proteins strongly supports their proposed involvement in a common axon guidance mechanism (Ogura *et al.*, 1997).

Genetic studies of axon guidance in *C. elegans* have provided insights on guidance mechanisms common to other invertebrate or vertebrate model systems. For example, the Rho family of small GTP-binding proteins has been implicated in the regulation of the cytoskeleton in cells and axonal growth cones (Ridley and Hall, 1992; Ridley *et al.*, 1992; Nobes and Hall, 1995). Cytoskeletal rearrangements are ultimately responsible for axon and cell migrations and therefore the genes that regulate this process are likely to be targets or downstream components of cell-surface receptors that interpret extracellular guidance cues (Mitchison and Cramer, 1996). Recently, *mig-2* and *unc-73* were shown to encode respectively a new member of the Rho family of GTPases and a guanine nucleotide exchange factor for Rho family GTPases (Zipkin *et al.*, 1997).
1997; Steven et al., 1998). As mutations in these genes cause axon guidance and cell migration defects, these results provide evidence that small GTP-binding proteins and the factors that regulate their activities are involved in normal axon and cell migrations in vivo.

E. The Netrin/UNC-6 Pathway: A Phylogenetically Conserved Mechanism for Circumferential Axon and Cell Guidance

Netrin/UNC-6 family members are components of a phylogenetically conserved pathfinding mechanism that is important for cell and axon migrations in nematodes, insects, and vertebrates. They function as diffusible or substrate-associated guidance cues that are either attractive or repulsive for different populations of neurons and migrating cells. The unc-6 gene was initially implicated in growth cone and cell migration along the D/V axis of *C. elegans* (Hedgecock et al., 1990; Ishii et al., 1992). Subsequently, members of the netrin family were independently identified as factors that can direct the growth of axons in vertebrates (Serafini et al., 1994) and *Drosophila* (Mitchell et al., 1996; Harris et al., 1996). Netrin/UNC-6 signaling pathways are poorly understood, but are known to involve the UNC-5 and DCC/UNC-40 families of transmembrane receptors (Leung-Hagesteijn et al., 1992; Chan et al., 1996). This section will review the present state of knowledge regarding this pathway in *C. elegans* and vertebrates.

The Netrin/UNC-6 Pathway in *C. elegans*

Detailed examination of the circumferential and longitudinal projections of axons and migrating cells in unc-5, unc-6, and unc-40 mutants revealed significant migration defects along the D/V axis (Hedgecock et al., 1990; McIntire et al., 1992). These mutants share several of the same axon and cell migration defects. Their uncoordinated behaviour or Unc phenotype is attributable to qualitatively similar defects in the dorsal migrations of motor axons. Five classes of motorneurons, DA, DB, DD, VD, and AS, extend axons, usually as commissures, from cell bodies in the VNC to innervate muscle targets located at the dorsal midline (White et al., 1986). In unc-5, unc-6, and to a lesser extent, unc-40 mutants, a significant fraction of these axons fail to reach their normal dorsal targets and instead are misrouted along longitudinal trajectories at lateral axial levels (Hedgecock et al., 1990; McIntire et al., 1992).
Another highly visible phenotype expressed by all three mutants is the frequent failure of the dorsal component of the migration of the hermaphrodite distal tip cell (DTC) and male linker cell. The migration of these mesodermal cells along a trajectory that involves both longitudinal and circumferential migrations shapes the elongating gonads. Abnormalities in the dorsally directed migration of the mesodermal head cell and the initial dorsal projection of the ectodermal excretory canals are the only other defects that appear similar in all three mutants. However, like the motor axon migrations, these defects are far more penetrant in unc-5 and unc-6 than in unc-40 mutants.

unc-6 and unc-40 mutants also display disruptions in ventral guidance. A subset of neurons with cell bodies located at lateral positions extend axons ventrally to enter the VNC. In unc-6 and unc-40, but not in unc-5 mutants, these axons are often misrouted along longitudinal trajectories. The guidance defects affect ventral axonal projections of the AVM, PVM, and PDE mechanosensory neurons, PHA and PHB sensory neurons (Hedgecock et al., 1990) and HSN motorneurons (Desai et al., 1988). unc-40 but not unc-6 mutants are also defective in the final phase of the ventrally directed migrations of a subset of ventral epidermoblasts (Hedgecock et al., 1990).

In all cases, apparent null mutations do not result in complete penetrance for any one particular defect, suggesting some redundancy in axon and cell guidance mechanisms (Hedgecock et al., 1990; Chan et al., 1996). The observed penetrance of specific guidance defects also appears to vary depending on cell type or position. For example, approximately 30% of PHA or PHB axons are misguided in unc-6 mutants whereas 40% of AVM or PVM axons are misguided. Similarly, the migration defect displayed by the posterior (left) DTC is far more penetrant than that displayed by the anterior (right) DTC (Hedgecock et al., 1990). Furthermore, pathways in the head appear unperturbed. For example, the amphid commissure pathway, which is a bundle of axons containing chemosensory neurons that first extends ventrally to the VNC then dorsally in the nerve ring, appears normal in unc-5, unc-6, and unc-40 mutants (Hedgecock et al., 1985; Hedgecock et al., 1990).

All double mutant combinations of unc-5, unc-6, and unc-40 were generated to verify the existence of another guidance mechanism. Examination of circumferential migrations revealed that the guidance defects are not significantly more severe in the double mutants than in the single mutants and are therefore non-additive (Hedgecock et al., 1990). This finding, combined
with incomplete penetrance of each defect in null mutants, suggests the involvement of another pathway in circumferential migrations that is, at least partially, independent of *unc-5*, *unc-6*, and *unc-40*.

Other axon and cell migrations are also affected in these mutants but not as extensively as circumferential migrations. For example, the longitudinal migrations of coelomocyte precursor cells are sometimes perturbed in *unc-5*, *unc-6* and more mildly in *unc-40* mutants (Hedgecock *et al.*, 1990). Moreover, the posterior directed longitudinal migration of the left Q neuroblast is often curtailed prematurely in *unc-40*, but not in *unc-5* or *unc-6* mutants, suggesting that *unc-40* may act independently in longitudinal migrations (Hedgecock *et al.*, 1990). *unc-6* and *unc-40* mutants also show defects in the decussation of the PVP axons at the ventral midline (Wadsworth *et al.*, 1996). Finally, *unc-6* is implicated in maintaining cohesion between axons in the VNC and *unc-5* and *unc-6* mutants display minor perturbations in the position of muscle cells (Hedgecock *et al.*, 1990).

In short, *unc-5*, *unc-6*, and *unc-40* affect circumferential migrations along the entire length of the worm with only mild effects on longitudinal migrations. *unc-5* mutants are defective in dorsally directed migrations, *unc-40* mutants are primarily defective in ventrally directed migrations, and *unc-6* mutants display defects in both dorsal and ventral migrations. A summary of the axon and cell migration defects exhibited by *unc-5*, *unc-6*, and *unc-40* mutants is provided in Table 1.2.

The genes *unc-5*, *unc-6*, and *unc-40* have been recently cloned (Figure 1.2). *unc-5* encodes a transmembrane protein of 919 amino acids that includes two extracellular immunoglobulin (Ig) and two thrombospondin (TSP) type I domains. The intracellular region is largely novel except for a SH3-like motif and a C-terminal death domain motif (Leung-Hagesteijn *et al.*, 1992; Hofmann and Tschopp, 1995). Ig domains are found in many proteins including neuronal cell adhesion molecules and are known to mediate protein-to-protein interactions (Sonderegger and Rathjen, 1992). TSP type I domains were initially identified in the extracellular matrix protein thrombospondin and are known to promote neurite outgrowth *in vitro* (Adams and Lawler, 1993). SH3 motifs are domains that bind proline-rich domains of other proteins and couple them to signaling pathways or to the cytoskeleton (Pawson and Gish, 1992). However, the SH3-like motif found in UNC-5 has only weak similarity and may therefore not be a *bona fide* SH3 domain. Death domains are found in several proteins that
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Product</th>
<th>Expression Pattern</th>
<th>Axon Guidance Role</th>
<th>Mutant Phenotype</th>
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<tbody>
<tr>
<td><em>unc-5</em></td>
<td>transmembrane protein: extracellular Ig and TSP domains, intracellular death domain</td>
<td>All cells that exhibit guidance defects in <em>unc-5</em> mutants. Expression in subset of sensory axon in head (which do not exhibit defects in mutants).</td>
<td>Dorsal guidance of axon and cell migrations.</td>
<td>Uncoordinated, general failure of dorsally-directed axon and cell migrations. Defects in dorsal guidance of commissure motor axons (DA, DB, DD, VD), frequent failure of dorsal migration of DTC and linker cell, failure of initial dorsal migration of excretory canals and head mesodermal cell, mild muscle positioning defects.</td>
</tr>
<tr>
<td><em>unc-40</em></td>
<td>transmembrane protein: extracellular Ig and FNIII domains</td>
<td>Expression in all cells at the onset of gastrulation but at later stages detected in cells that exhibit guidance defects in <em>unc-40</em> mutants</td>
<td>Ventral guidance of axon and cell migrations. Minor role in dorsal guidance of axon and cell migrations.</td>
<td>Uncoordinated, egg laying defective, mild dumpy, general failure of ventrally-directed axon and cell migrations, milder failure of dorsally-directed axon and cell migrations. Ventral guidance of HSN, PHA, PHB, PDE, AVM, and PVM axons perturbed, mild defects in dorsal guidance of commissures, mild defects in dorsal migration of DTC and head mesodermal cell, failure of ventral migration of linker cell. QL posterior migration reversed, failure of ventral P cell intercalations, defects in PVP axon decussation.</td>
</tr>
<tr>
<td><em>unc-6</em></td>
<td>secreted laminin-related protein</td>
<td>Embryonic: ventral epidermalblasts P1/2-P11/12, subset of neurons in head and tail including PVT, sheaths in head. Post embryonic: VNC neurons VA2-VA12, VB3- VB11</td>
<td>Dorsal and ventral guidance of axon and cell migrations.</td>
<td>Uncoordinated, egg laying defective, general failure of dorsal and ventral axon and cell migrations. Ventral guidance of HSN, PHA, PHB, PDE, AVM, and PVM axons perturbed, defects in dorsal guidance of commissure motor axons (DA, DB, DD, VD), frequent failure of dorsal migration of DTC and linker cell and ventral migration of linker cell, failure of initial dorsal migration of excretory canals and head mesodermal cell, defects in PVP axon decussation, mild muscle positioning defects.</td>
</tr>
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Abbreviations: Ig, immunoglobulin; TSP, thrombospondin type I; FNIII, fibronectin type III; DTC, distal tip cell.
Figure 1.2. Domain organization of UNC-5, UNC-6, and UNC-40. UNC-5 contains two immunoglobulin and two thrombospondin type I domains, a transmembrane region, a region that has similarity with a unique region in zona occludens-1 (ZO-1), and a death domain. UNC-40 contains four immunoglobulin domains, six fibronectin type III domains, a transmembrane region, and a novel intracellular region. UNC-6 is a laminin-related molecule that contains a region with similarity to the amino-terminal domain VI of the laminin B subunit, three EGF-like repeats similar to those of domain V of the laminin B subunit, and a unique carboxy-terminal region.
Laminin domain VI
EGF-like repeat
Netrin C-terminus
Immunoglobulin domain (lg)
Fibronectin Type III domain (FNIII)
Thrombospondin Type I domain
Death domain
ZO-like homology
mediate apoptosis such as the TNF receptor and are involved in mediating protein-protein interactions (Hofmann and Tschopp, 1995).

unc-6 encodes a secreted, laminin-related protein of 591 amino acids (Ishii et al., 1992). Laminins, composed of laminin subunits A, B1, and B2, are major components of the extracellular matrix and are known to function in neuron-substrate interactions, in mediating adhesion, and in promoting axon outgrowth (Beck et al., 1990). UNC-6 contains the amino-terminal motifs VI, V-1, V-2, and V-3 that are similar to those found in laminin subunits B1 and B2, and a unique, cysteine-rich, C-terminal region. Laminin domain VI is known to mediate polymerization to form extensive laminin networks while domain V motifs contain EGF-like homologies and are believed to mediate protein-protein interaction (Hedgecock and Norris, 1997). The C-terminal domain of netrin-1, a vertebrate homolog of UNC-6, functions as a binding site for the glycosaminoglycan heparin, a common component of the extracellular matrix in tissues (Serafini et al., 1994).

unc-40 encodes the C. elegans homologue of the vertebrate Deleted in Colorectal Cancer (DCC) and neogenin proteins and Frazzled in Drosophila (Chan et al., 1996; Kolodziej et al., 1996; Keino-Masu et al., 1996). UNC-40 is a transmembrane protein of 1415 amino acids with an extracellular domain composed of four Ig-like and six fibronectin type III (FNIII) repeats and a novel cytoplasmic domain. Ig and FNIII domains are often found together in members of the Ig superfamily, which includes neuronal cell adhesion molecules such as NCAM and L1 (Sonderegger and Rathjen, 1992). The FNIII motifs were initially identified in the extracellular matrix protein fibronectin and function as sites for protein interaction (Adams and Lawler, 1993; Bennett et al., 1997).

The molecular identities of UNC-5, UNC-6, and UNC-40 suggested possible mechanisms for their roles in circumferential migrations. As transmembrane proteins, UNC-5 and UNC-40 could be present on the surface of motile cells or growth cones and respond to guidance information encoded by extracellular guidance cues. Genetic studies indicate that UNC-5 is responsible for dorsal guidance and UNC-40 is primarily involved in ventral guidance but also has some dorsal guidance functions. The molecular identity of UNC-6 as a secreted laminin-related molecule, suggested that its distribution along the D/V axis might form, or be a component of, the extracellular path cues providing D/V polarity information to cells via
interactions with UNC-5 and UNC-40. Expression studies and mosaic analysis supported this initial hypothesis.

Mosaic analysis revealed that normal guidance requires that unc-5 function be present in migrating cells and neurons: it is not required in path or target cells (Leung-Hagesteijn et al., 1992). For example, loss of wild-type unc-5 function in DA, DB, DD, and VD motoneurons resulted in worms exhibiting uncoordinated movement, presumably due to failure of these axons to form synapses along the dorsal midline. Similarly, only DTCs that were unc-5 (-) in an otherwise unc-5 (+) background were observed to exhibit dorsal migration defects. Subsequent expression studies using an unc-5 promoter fused to lacZ indicated that the unc-5 promoter is active in all of the cells that display axon or cell guidance defects in unc-5 mutants (M.W. Su, Y. Zhou, and J.G. Culotti, unpublished results).

If UNC-5 acts cell-autonomously to mediate dorsal guidance, then misexpression using a heterologous promoter might cause predictable dorsal misrouting if other components of the pathway are present. This experiment was performed by ectopically expressing unc-5 coding sequence in a subset of sensory neurons that do not normally grow dorsally. The touch receptor neurons are mechanosensory neurons that normally extend axons ventrally or longitudinally along the hypodermis. Ectopic expression of the UNC-5 receptor specifically in the touch neurons was sufficient to redirect their axons dorsally (Hamelin et al., 1993). This dorsal steering depends on guidance information provided by UNC-6, as growth cone reorientation does not occur in an unc-6 mutant background. Similarly, premature expression of UNC-5 during the initial longitudinal migrations of DTCs induces their precocious dorsal migration in an UNC-6 dependent manner (M.W. Su, Y. Zhou, and J.G. Culotti, unpublished results). These results demonstrate that UNC-5 acts as part of an instructive mechanism to guide growth cone and cell migrations.

A localization study performed with a functional UNC-40/GFP fusion protein revealed that UNC-40 is present in motile cells and neurons at developmental stages when their migrations occur (Chan et al., 1996). However, expression of unc-40 initially occurs in all cells during gastrulation, and only later becomes restricted to a subset of cells. unc-40 expression is observed in all neurons that display ventral guidance defects in unc-40 mutants, including the AVM, PVM, and PDE mechanosensory neurons, the PHA and PHB sensory neurons, and HSN motorneurons. unc-40 appears to act cell-autonomously in these cells, as reintroduction of
normal copies of \textit{unc-40} in misrouted AVM and PVM axons in an \textit{unc-40} null background is able to restore normal guidance (Chan \textit{et al.}, 1996). \textit{unc-40} is also expressed in the ventral epidermblasts and the Q cells and their descendents; migrations of these cells are perturbed in \textit{unc-40} mutants.

Perhaps most intriguingly, \textit{unc-5} and \textit{unc-40} appear to be co-expressed in all the motile cells and axons that show dorsal guidance defects in mutants. These include the DA, DB, DD, and VD class of motorneurons, DTCs, and the excretory cell (Chan \textit{et al.}, 1996). This result is consistent with the observation that \textit{unc-5} and \textit{unc-40} mutants share qualitatively similar dorsal guidance defects, although these defects appear less severe in \textit{unc-40} mutants. One possibility is that UNC-5 and UNC-40 are components of a receptor complex that guide cells and growth cones away from ventral UNC-6 sources. However, the observation that mutation of \textit{unc-40} has a less severe effect on dorsally directed guidance than does mutation of \textit{unc-5} suggests that UNC-40 may act as an accessory factor that modulates UNC-5’s role in dorsal guidance. It is also possible that other accessory factors may exist that function like UNC-40, but act in parallel with it.

\textit{unc-6} is expressed in a restricted spatial and temporal pattern during embryogenesis and larval stages that is consistent with a role for UNC-6 as a guidance cue for both dorsally and ventrally directed cell and growth cone migrations (Wadsworth \textit{et al.}, 1996). Earliest expression is seen during the late gastrula stage to the early neurula stage in the twelve ventral epidermblasts P1/2 – P11/12 as they migrate ventrally to form a row at the ventral midline. At later embryonic stages, expression is seen in a subset of cells in the head and tail and in axons that pioneer the ventral nerve tracts. Some of this early ventral expression is maintained and augmented by postembryonic expression in most of the VA and VB class of motorneurons that are located in the VNC. \textit{unc-6} expression; however, is not detected in the motile cells or axons that undergo circumferential migrations.

A simple model is that UNC-6 is secreted from the ventral epidermblasts as they migrate ventrally and later by cells and axons in the VNC, forming a D/V gradient with highest levels at the ventral midline (Wadsworth \textit{et al.}, 1996). This gradient can provide polarity information along the D/V axis and would be consistent with a bifunctional role for UNC-6 as both a repulsive and attractive guidance cue for different classes of axons or motile cells. In this way, ventral \textit{unc-6} expression can guide the dorsal projections of motor axons, DTCs, and other
mesodermal or ectodermal cell migrations and the ventral projections of sensory and interneurons affected in *unc-6* mutants. *unc-6* expression along the ventral midline is also consistent with genetic studies that demonstrate a role for *unc-6* in fasciculation of axons in the VNC and the decussation of the PVP axons at the ventral midline (Wadsworth *et al.*, 1996). The latter finding, combined with the fact that *unc-40* mutants display the same defects in fasciculation and PVP decussation as *unc-6* mutants, and the observation that *unc-40* is expressed in PVP neurons, implicates *unc-6* and *unc-40* in mediating local axon guidance decisions at the midline. This is significant because the crossing or decussation of axons at the midline of the *Drosophila* CNS (Kid *et al.*, 1998) or vertebrate spinal cord (Stoeckli *et al.*, 1997) is a common phenomenon during axon guidance.

Wadsworth *et al.* (1996) speculate that a stable D/V gradient may be formed by diffusion of UNC-6 away from ventral sources and its copolymerization with laminin in the basement membrane. Also, UNC-6 protein levels are predicted to be higher in the right than in the left tract of the VNC, suggesting an additional left-right asymmetry of unknown functional significance. However, because it has not yet been possible to detect secreted epitope-tagged UNC-6, there is no direct evidence that UNC-6 is distributed in a gradient in *C. elegans*.

UNC-6 demonstrates properties of being both an attractive and repulsive guidance cue molecule for different classes of neurons and migrating cells. Mutations in *unc-6* exist that selectively disrupt dorsal versus ventral migrations or ectodermal versus mesodermal migrations, indicating that these functions are genetically separable (Hedgecock *et al.*, 1990). Analysis of molecular lesions in *unc-6* mutants implicates the EGF-like module V-2 and domain VI as functionally important sites in UNC-6 that may mediate these differences. A precise deletion of EGF-like module V-2 selectively disrupts dorsal but not ventral migrations, and various mutations within domain VI are able to selectively disrupt either ventral migrations or mesodermal cell migrations (Wadsworth *et al.*, 1996; Antebi *et al.*, 1997).

Wadsworth *et al.* (1996) propose that mutations in *unc-6* that selectively disrupt dorsal guidance, such as loss of the V-2 EGF-like module, are in putative UNC-5 binding sites, as both proteins are believed to interact in the same pathway and *unc-5* and *unc-6* mutants display qualitatively similar guidance defects. However, a direct interaction between UNC-5 and UNC-6 has not been demonstrated. These authors further speculate that mesoderm-specific mutations in domain VI of *unc-6*, known to mediate self-polymerization in laminin, may perturb the
tethering of UNC-6 to the basement membrane such that it is selectively displayed to ectodermal but not mesodermal cells.

The Netrin/UNC-6 Pathway in Vertebrates

In the rat, commissural neurons differentiate in the dorsal region of the spinal cord adjacent to the roof plate around embryonic day 11 and send axon processes along a ventral trajectory to the floor plate (Tessier-Lavigne et al., 1988). The floor plate, a region located at the ventral midline, secretes a chemoattractant that can both induce and reorient the growth of commissural axons originating from rat dorsal spinal cord explants (Tessier-Lavigne et al., 1988). Two factors that mimic the diffusible growth-promoting and chemoattractant activity of rat floor plate were purified from chick brain and found to be homologues of UNC-6. These factors were designated netrin-1 and netrin-2 (Serafini et al., 1994; Kennedy et al., 1994). Either protein expressed from aggregates of heterologous cells functions as a diffusible chemoattractant for commissural axons in vitro (Kennedy et al., 1994). Furthermore, recombinant netrin-1 can act as a chemoattractant for commissural axons derived from different axial levels of the spinal cord (Shirasaki et al., 1995, 1996) and for specific axon populations in the brain (Richards et al., 1997; Metin et al., 1997). These observations suggest that netrins act as globally distributed chemotropic guidance cues.

The netrin genes are expressed before and during the period of axon outgrowth in the developing spinal cord and parts of the midbrain and hindbrain. As well, expression is observed in non-neuronal tissues in the embryo and at later stages of development (Kennedy et al., 1994, Serafini et al., 1996). In chicken, netrin-1 transcripts are present at high levels in the floor plate while netrin-2 transcripts are present at lower levels in the ventral two-thirds of the spinal cord, but not in the floor plate (Kennedy et al., 1994). In mouse, netrin-1 is expressed in the ventral two-thirds of the spinal cord including floor plate cells (Serafini et al., 1996). However, the netrin-2 gene in mouse is not expressed in the spinal cord during periods of axon outgrowth (Serafini et al., 1996).

Netrin-1 has also been shown to function as a chemorepellent in vitro. It directs motor axons away from floor plate explants (Colamarino and Tessier-Lavigne, 1995). The trochlear motor neurons in rat are located adjacent to the floor plate near the midbrain/hindbrain junction and extend axons dorsally to the dorsal midline of the hindbrain and then to their final muscle
targets (Colamarino and Tessier-Lavigne, 1995). Trochlear motor axons were shown to grow away from floor plate cells of the ventral hindbrain/midbrain junction explants in vitro. This chemorepellent activity was identified as netrin-1. A cell line expressing recombinant netrin-1 mimics the diffusible chemorepellent activity of floor plate cells when positioned at a distance from ventral midbrain/hindbrain junction explants (Colamarino and Tessier-Lavigne, 1995). These experiments demonstrate that, like UNC-6 in C. elegans, vertebrate netrins are bifunctional molecules that act as both chemoattractants and chemorepellents.

Kennedy et al. (1994) propose that netrins are distributed as a chemotropic gradient that is highest at the ventral midline of the spinal cord. This gradient would provide a long range directional cue to guide axons to their targets. This view is supported by two observations: first, the ability of recombinant netrin-1 or netrin-2 proteins secreted from a point source to act at a distance to promote and orient the growth of axons from spinal cord explants, and second, the observation that the distribution of netrin-1 and netrin-2 transcripts is graded. Netrin mRNA, which is highest in floor plate cells with lower levels in the ventral two-thirds of the spinal cord, may contribute to the establishment of a gradient of netrin protein. Motor axons may thus be exposed to increasing levels of netrin activity as they project ventrally toward the floor plate and vice versa for dorsally projecting axons.

To determine if netrin acts as an axon guidance molecule in vivo, a mouse line bearing a strong loss-of-function mutation in the netrin-l gene was examined for axon pathfinding errors (Skarnes et al., 1995; Serafini et al., 1996). In homozygous netrin-l deficient animals, many commissural axons are foreshortened and display perturbations from their normal ventrally directed trajectories towards the floor plate (Serafini et al., 1996). This defect results in a reduction of the number of commissural axons that cross the floor plate. In contrast to defects in commissural axon trajectories, the dorsally directed trajectories of the trochlear motor axons appear largely normal (see below) (Serafini et al., 1996).

Striking defects in the formation of commissures in the developing brain were also observed in mice with the netrin-l mutation. The corpus callosum is a commissure in the brain that joins the left and right cerebral cortices. Similarly, the hippocampal commissure joins the left and right hippocampi. The anterior commissure is another major commissure in the brain and is located at the ventral midline. In netrin-l deficient animals, these commissures are completely absent or in the case of the anterior commissure severely reduced (Serafini et al.,
1996). *In situ* hybridization indicates that netrin-1 transcripts are present along the pathway of these commissures before and during commissure formation (Serafini et al., 1996). Together, these defects provide strong evidence that at least some of the growth-promoting and chemotropic activities of netrin-1 seen *in vitro* are physiologically important *in vivo*.

The observations that some commissural axons reach the ventral midline of the spinal cord normally and that there are no defects in trochlear axon guidance in netrin-1 mutants suggest redundancy underlying axon guidance mechanisms in the mouse. The existence of netrin-1-independent guidance mechanisms is supported by the ability of floor plate explants from netrin-1 mutant embryos to chemoattract commissural axons and chemorepel trocheolar motor axons *in vitro* (Serafini et al., 1996). In addition, a floor plate-derived chemorepellent activity that is distinct from netrin has been shown to guide the dorsalward projection of axons in the posterior commissure *in vitro* (Shirasaki et al., 1996). The posterior commissure is located in the mesencephalic region of the brain and is normal in netrin-1 mutants (Serafini et al., 1996). A floor plate derived chemorepellent distinct from netrin-1 has also been shown to repel olfactory interneuron precursors. This provides further evidence that netrins are not the only repulsive guidance cues present in the floor plate (Hu and Rutishauser, 1996). The chemorepulsive factor semaphorin III/collapsin is also known to act as a guidance cue in the developing spinal cord and may mediate some of the netrin-independent axon guidance functions (Messersmith et al., 1995; Behar et al., 1996; Varela-Echavarria et al., 1997). These findings are consistent with the putative existence of redundant mechanisms operating on circumferential migrations in the worm.

The identification of vertebrate netrins as structurally and functionally homologous to UNC-6 in *C. elegans* suggested that further insights into the mechanisms of axon guidance in *C. elegans* would come by also studying the vertebrate homologues of the putative guidance receptors UNC-40 and UNC-5. As stated above, *unc-5* and *unc-40* in *C. elegans* appear to act cell-autonomously within motile cells and growth cones to interpret path cue information encoded by *unc-6* (Leung-Hagesteijn, 1992; Chan et al., 1996). *unc-40* homologues have been identified in vertebrates as DCC and neogenin (Chan et al., 1996).

*Dcc* was initially identified as a putative tumor suppressor gene expressed in gastrointestinal tissue. Loss of heterozygosity for a region encompassing the *Dcc* locus is observed in greater than 70% of colorectal cancers (Fearon et al., 1990; Hedrick et al., 1994).
However, on the basis of recent experiments (see below), the role of DCC in cancer progression is uncertain (Fearon, 1996). Neogenin was initially identified in chicken as a cell surface protein expressed on terminally differentiated neurons before and during axon outgrowth (Vielmetter et al., 1994). Early evidence suggested a role for these genes in cell differentiation and proliferation. For example, DCC expression on the surface of fibroblast cells co-cultured with neuronal cells can induce terminal differentiation, including neurite outgrowth, in the neuronal cell line (Pierceall et al., 1994).

Antibody-blocking experiments using function-blocking anti-DCC antibodies demonstrated that DCC is required for netrin-1 dependent axon outgrowth in spinal cord explant assays (Keino-Masu et al., 1996). In contrast, anti-DCC antibodies did not block the ability of floor plate or netrin-1 to reorient the growth of commissural axons (Keino-Masu et al., 1996). A possible trivial explanation for this result is that anti-DCC antibodies did not penetrate the explant tissue sufficiently. Interestingly, although anti-DCC antibody prevents the response of axons to netrin, the antibodies do not disrupt the interaction between DCC and netrin-1. This suggests that other factors may be involved in DCC/netrin responses (Keino-Masu et al., 1996). However, another study also implicated DCC in the chemotropic response elicited by netrin-1. In this study anti-DCC antibodies were found to abolish the growth cone turning behaviour that is normally displayed by retinal neurites in response to a gradient of netrin-1 in vitro (de la Torre et al., 1997).

DCC and netrins are expressed in distinct regions of the developing spinal cord. DCC transcripts are expressed widely in neuronal and non-neuronal tissue but are highest in the brain and other regions of the CNS, especially during embryonic stages (Fearon et al., 1990). Examination of DCC mRNA and protein distribution in the developing rat spinal cord revealed that DCC is present in neurons and not in the neuroepithelial cells that underlie their path (Keino-Masu et al., 1996). At embryonic day 11 (E11) DCC transcripts are detected strongly in ventrally located motor neurons and are detected less intensely in dorsally located commissural neurons. In contrast, netrin-1 is expressed in neuroepithelial and floor plate cells in the ventral two-thirds of the spinal cord. At E13, DCC transcript levels appear to be higher in commissural neurons than in motor neurons. Immunostaining shows DCC protein on the surface of axons and, at least in the case of commissural neurons, on their growth cones (Keino-Masu et al., 1996). DCC expression is also observed on distinct neuronal populations in the hindbrain,
midbrain, and forebrain (Fearon et al., 1990; Keino-Masu et al., 1996). In contrast, neogenin is expressed in a pattern that is nearly complementary to that of DCC in the developing spinal cord. Neogenin, however, is not expressed in commissural neurons, suggesting that neogenin may not be important in vivo for netrin-1-mediated guidance (Keino-Masu et al., 1996).

The in vivo function of DCC was determined by disruption of the Dcc gene locus in mouse by homologous recombination (Fazeli et al., 1997). Dcc−/− mice display behavioural defects and die within 24 hours after birth. Extensive histological examination of Dcc−/− mice did not reveal any evidence for gastrointestinal tumors. Such tumors were expected given the hypothesized role of DCC in colorectal cancers. However, Dcc−/− mice did exhibit commissural axon guidance defects in the developing spinal cord that are qualitatively similar to those found in netrin-1 deficient mice. As in the netrin-1 mutant, a fraction of commissural axons appear foreshortened or misrouted along aberrant trajectories. However these defects are not fully penetrant; many axons project normally to the floor plate. Furthermore, in both Dcc−/− and netrin-1 mutant mice, motor column axons that express DCC are normal (Serafini et al., 1996; Fazeli et al., 1997). Dcc−/− mice also display the same defects as netrin-1 deficient mice in the formation of the corpus callosum, hippocampal, and anterior commissures and the absence of certain migrating cells in the developing brain (Serafini et al., 1996; Fazeli et al., 1997). Both mutants also exhibit similar defects in retinal ganglion cell (RGC) axon guidance in the developing eye. In netrin-1 and DCC deficient mice, a similar fraction of RGC axons fail to exit the eye resulting in a reduction in the size of the optic nerve (Deiner et al., 1997). It was shown that RGC axons express DCC while the neuroepithelial cells at the entrance to the optic nerve express netrin-1. Together, these findings strongly support the view that DCC and netrin-1 act in the same axon guidance and cell migration pathway and that DCC may encode a netrin-1 receptor.

Recently, UNC5H1, UNC5H2 and the product of the mouse rostral cerebellar malformation gene, RCM (UNC5H3), have been identified as vertebrate homologues of C. elegans UNC-5 (Leonardo et al., 1997; Ackerman et al., 1997). The domain structures of these proteins are similar, and include a motif that had not been previously identified in C. elegans UNC-5. This motif has similarity with a unique carboxy-terminal region of Zona Occludens-1 (ZO-1). ZO-1 is a component of tight junctions at sites of cell-cell contact, but the sequence that
is found in the UNC-5 homologues is of unknown function (Willott et al., 1993; Leonardo et al., 1997).

UNC5H1 and UNC5H2 transcripts are detected in the developing spinal cord and brain during periods of axon outgrowth in regions that correspond to neuronal populations including commissural, trochlear, dorsal root ganglion (DRG), and motor neurons (Leonardo et al., 1997). During rat E11-E13, UNC5H1 transcripts are primarily detected in the ventral half of the spinal cord, excluding the floor plate. UNC5H2 transcripts appear at about E13 and are primarily found in dorsal regions of the spinal cord. UNC5 mRNA is also present in distinct regions of the developing brain.

*rcm* mutant mice exhibit aberrant migration of neuronal precursor cells in the cerebellum (Ackerman et al., 1997). In particular, granule and Purkinje cells fail to migrate to correct layers of the cerebellum in these mutants. A cell-autonomous role for RCM in this process is supported by the observation that *rcm* transcripts are detected in these cells during periods of normal migration in wild-type mice. However, *rcm* mutants do not exhibit defects in all RCM expressing cells. This result may be explained by genetic redundancy as regions of UNC5H1, UNC5H2, and RCM expression partially overlap in the developing brain. Furthermore, a role for netrin-1 in these migrations has not been demonstrated, although netrin-1 transcripts are found in the cerebellum (Ackerman et al., 1997).

Genetic studies in *C. elegans* and the receptor-like structure of DCC/UNC-40 and UNC-5 suggested that they could act as netrin/UNC-6 binding proteins. Recently, vertebrate UNC-5 and UNC-40 homologues expressed on the surface of a heterologous cell line have been shown to bind directly to netrin-1 (Keino-Masu et al., 1996; Leonardo et al., 1997). The netrin-1 domains that mediate interaction have not been determined; however, binding studies with chimeric netrin-1 proteins indicate that domains VI and V are required while domain C is dispensable for these interactions (Keino-Masu et al., 1996; Leonardo et al., 1997). These results are consistent with the finding that domains VI and V in *C. elegans* UNC-6 are altered in some mutants that disrupt specific guidance functions (Wadsworth et al., 1996).

The qualitatively similar axon guidance defects in *netrin-1* and *Dcc* mutant mice is consistent with a netrin-1-DCC interaction *in vivo*. However, there is no *in vivo* evidence for a role for UNC5H1 and UNC5H2 in netrin-1 mediated guidance. The ability of both UNC5 and DCC to interact directly with netrin-1 suggests the possibility that netrin-1 responses may be
mediated by a receptor complex involving UNC5 and DCC. This possibility is supported by the observation that UNC5 and DCC transcripts in the brain and spinal cord partially overlap in the same neuronal populations (Keino-Masu et al., 1996; Leonardo et al., 1997) and with genetic studies in *C. elegans* discussed above. Given the structural and functional conservation of components of the netrin/UNC-6 pathway, the identification of DCC and UNC5 as candidate receptors for netrin-1 in vertebrates implicates UNC-40 and UNC-5 as receptors for UNC-6/netrin in *C. elegans*. A summary of the results of *in vitro* and *in vivo* observations concerning the netrin, DCC, and UNC5 gene families is provided in Table 1.3. Figure 1.3 illustrates the apparent conservation of function observed between the UNC-6/netrin pathway components in *C. elegans* and those of vertebrates.

A potential difficulty arises when trying to understand mechanistically how a putative UNC-5/UNC-40 receptor complex can be involved in both dorsal and ventral migrations in response to the same guidance cue formed by UNC-6. Recently, Song *et al.* (1997) demonstrated that an attractive turning response of growth cones towards a chemotropic gradient of brain-derived neurotrophic factor (BDNF) can be changed into a repulsive turning response by reduction of cytosolic cAMP levels or by changes in Ca\(^{2+}\) levels. Both cAMP (Lohof *et al.*, 1992) and Ca\(^{2+}\) (Rehder and Kater, 1992) 2\(^{nd}\) messenger systems have been shown previously to regulate growth cone motility. Similarly, attractive or repulsive chemotropic responses elicited by netrin-1 on embryonic *Xenopus* spinal cord neurons were shown to depend on changes in cytosolic cAMP and Ca\(^{2+}\) levels (Ming *et al.*, 1997). These results suggest that modulation of second messenger systems by other factors, perhaps provided by path or target cells, can alter receptor-mediated growth cone responses to the same guidance cue. Ming *et al.* (1997) speculate that, among other possibilities, modulation of 2\(^{nd}\) messenger systems may convert a heteromeric DCC/UNC5 receptor complex that mediates repulsive responses to one that mediates attractive responses by reduction of UNC5 protein levels or activity.

**F. Other Families of Axon Guidance Molecules**

Recently, mutations have been identified in *C. elegans* genes that encode members of the semaphorin and EPH receptor-tyrosine kinase families of axon guidance molecules (P. Roy, and J.G. Culotti, unpublished results; A. Chisholm, personal communication). As genetic and
### Table 1.3. Summary of the Functions of Netrin, DCC, and UNC5 in Vertebrates.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression in Nervous System</th>
<th>In Vitro Observations</th>
<th>In Vivo Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Netrin-1 &amp; -2</strong></td>
<td><strong>Netrin-1</strong> RNA detected in floor plate cells, netrin-2 RNA detected in ventral two-thirds of spinal cord, excluding floor plate cells(^1). Netrin-1 (mouse) expressed in floor plate cells and ventral two-thirds of spinal cord(^2). Expression detected at the ventral midline along the entire length of the developing spinal cord and into the caudal diencephalon (forebrain)(^3). Netrin-1 (mouse) also expressed in telencephalon (forebrain) and retina (optic disc)(^2,3,4).</td>
<td>Diffusible chemoattractant that promotes outgrowth and reorientation of ventrally projecting commissural axons from rat dorsal spinal cord explants(^1). Diffusible chemorepellent for dorsally projecting trochlear motor and branchiomotor axons from rat midbrain/hindbrain and hindbrain explants respectively(^5,6). Chemoattractant for cortical axons from forebrain explants(^4). Netrin-1 can promote outgrowth of mouse retinal ganglion cell axons from retinal explants(^8).</td>
<td>netrin-1 mutant mice (hypomorph) exhibit strong but incompletely penetrant commissural axon outgrowth and guidance defects(^5), normal trochlear motor axon projections(^6), severe defects in formation of specific commissures in the forebrain(^2), failure of retinal ganglion cell axons to exit the eye and enter the optic nerve(^7), and absence of pontine nuclei from the base of the rostral hindbrain (possible migration defect)(^3).</td>
</tr>
<tr>
<td><strong>Netrin-1</strong> (mouse, rat)</td>
<td><strong>DCC</strong> RNA is detected on the cell bodies of commissural and motor neurons in the dorsal and ventral regions of the developing rat spinal cord. DCC protein is localized to the axons of these neurons. Expression also detected in neurons in the hindbrain, midbrain, and forebrain and retinal ganglion cells(^3,7).</td>
<td>Netrin-1 binds to DCC expressed on the surface of heterologous cells(^7). Antibodies to DCC block the outgrowth-promoting but not the reorienting effects of netrin-1 on commissural axons(^7). Antibodies to DCC block retinal growth cone outgrowth and turning elicited by netrin-1(^11).</td>
<td>Dcc(^c) mice exhibit commissural axon outgrowth and guidance defects(^7), severe defects in formation of specific commissures in the forebrain(^8), failure of retinal ganglion cell axons to exit the eye and enter the optic nerve(^9), and absence of pontine nuclei(^8). These defects are similar to those observed in netrin-1-deficient mice.</td>
</tr>
<tr>
<td><strong>Dcc</strong> (mouse, rat)</td>
<td><strong>Unc5h1, Unc5h2</strong> RNA is detected in the ventral spinal cord in a region containing motor neurons and transiently in dorsal regions containing commissural neurons(^9). RNA is not found in floor plate cells. <strong>Unc5h2</strong> RNA is detected in the roof plate of developing spinal cord(^9). mRNA for both is detected in the hindbrain, midbrain, and forebrain. <strong>rcm</strong> transcripts are detected in the hindbrain and cerebellum(^10,12).</td>
<td>Netrin-1 binds to UNC5H1, UNC5H2, and RCM expressed on the surface of heterologous cells(^9).</td>
<td>rcm(^c) mice exhibit aberrant migration of neuronal precursor cells in the cerebellum(^10,12). Mutations in Unc5h1 and Unc5h2 have not been reported.</td>
</tr>
</tbody>
</table>

References: \(^1\)Kennedy et al., 1994, \(^2\)Serafini et al., 1996, \(^3\)Deiner et al., 1997, \(^4\)Metin et al., 1997, \(^5\)Colamarino and Tessier-Lavigne, 1995, \(^6\)Varela-Echavarria et al., 1997, \(^7\)Deiner et al., 1997, \(^8\)Keino-Masu et al., 1996, \(^9\)Fazeli et al., 1997, \(^10\)Leonardo et al., 1997, \(^11\)de la Torre et al., 1997, \(^12\)Przyborski et al., 1998.
Figure 1.3. The UNC-6/netrin pathway defines a phylogenetically conserved mechanism for axon guidance. In *C. elegans*, UNC-6 is concentrated at the ventral midline where it acts to attract UNC-40 expressing axons (ventral guidance) and repel axons that express UNC-5 and UNC-40 (dorsal guidance). UNC-40 is also required for dorsal guidance, although genetic studies indicate that it plays a less important role compared to UNC-5. For simplicity, UNC-6 guidance information is shown as a smooth gradient. In vertebrates, netrin-1 is distributed as a gradient of highest concentration at the ventral midline (floor plate) where it acts to repel dorsally projecting motor neurons and attract ventrally projecting commissural neurons. DCC involvement in mediating the attractive functions of netrin-1 has been demonstrated; however a role for the vertebrate UNC5 homologues in mediating repulsive functions remains to be determined.
UNC-5 + UNC-40

C. elegans

UNC5H1

Netrin-1

Vertebrate

UNC-6

DCC
molecular analyses of these molecules are at initial stages in *C. elegans*, their well-defined roles in vertebrate axon guidance will be discussed below.

**Semaphorins: Contact-Dependent and Diffusible Repulsive Axon Guidance Cues**

Semaphorins are a phylogenetically conserved family of secreted and transmembrane proteins that function as contact-dependent or diffusible repulsive guidance cues for axon growth cones. Semaphorins have been grouped into five subfamilies based on amino acid sequence conservation and overall domain structure. All semaphorins contain a 500 amino acid semaphorin domain characterized by 14 to 16 highly conserved cysteines. Subgroups are distinguished by the presence or absence of Ig or thrombospondin domains, and C-terminal basic or transmembrane regions (Kolodkin *et al.*, 1993; Puschel *et al.*, 1995; Adams *et al.*, 1996).

The first described member of this family, the transmembrane protein semaphorin I (Sema I, a.k.a. fasciclin IV), was identified in monoclonal antibody screens for surface proteins preferentially expressed along nerve fascicles in the CNS of grasshopper (Kolodkin *et al.*, 1992). In addition to CNS expression, Sema I is also expressed in circumferential stripes of epithelial cells in the developing grasshopper limb bud. These stripes are located in a region that corresponds to a stereotypical turn made by a nerve fascicle as it traverses the limb bud on its way to targets in the CNS. Upon contacting a Sema I expressing stripe, growth cones stop migrating and eventually turn to grow ventrally along the stripe boundary. Antibody blocking experiments showed that Sema I may be involved in mediating this trajectory change. Addition of antibodies against Sema I to cultured embryos or limb buds during the period of axon outgrowth caused inappropriate crossing of the Sema I-demarcated boundary, defasciculation of axon bundles, and excess branching (Kolodkin *et al.*, 1992). These results suggested that Sema I functions as a contact-dependent inhibitor of axon growth.

*Drosophila* Sema II (D-Sema II) is a putative secreted protein of 724 amino acids containing a single Ig domain (Kolodkin *et al.*, 1993). D-Sema II is expressed in a subset of neurons and muscle during embryogenesis. Sema II is an essential gene as putative *sema II* null mutants die in early adulthood. Rare flies that escape lethality show various behavioural phenotypes such as flightlessness and visual orientation defects. *sema II* mutants, however, do not appear to display any visible axon guidance defects (Kolodkin *et al.*, 1993).
To further investigate the role of D-Sema II, a gain-of-function phenotype was created in transgenic *Drosophila* embryos by expressing *sema II* ectopically. Heterologous enhancer elements were used to promote expression of Sema II in a wider subset of muscles than in wild-type embryos (Matthes *et al.*, 1995). In these transgenic embryos, muscles appear normal but defects occur in a subset of axon pathways. Specifically, some axons, but not others, are inhibited from forming synaptic connections with muscles that ectopically express *sema II*. However, axons are not perturbed from growing on, or into, regions of high *sema II* expression. This suggests that D-Sema II does not act as a general repulsive cue, or convert permissive environments into non-permissive environments for axon outgrowth (Matthes *et al.*, 1995). D-Sema II may function as a target-derived factor that specifically inhibits the formation of synaptic arborizations (Matthes *et al.*, 1995).

The collapsin/Sema III subgroup is distinguished by an Ig domain and a highly basic C-terminal region in addition to the semaphorin domain (Kolodkin *et al.*, 1993). Collapsin was originally purified from adult chicken brain as a substance that can cause the collapse of axonal growth cones when applied uniformly to neuronal explants (Luo *et al.*, 1993). Fan and Raper (1995) subsequently found that collapsin presented from a localized source steers away, or repels, growth cones. Their observations of the turning response show that lamellar and filopodial extensions on growth cones collapse selectively on the side that is exposed to collapsin activity. This has the affect of biasing growth cone extension in the direction of the remaining, unaffected filopodia.

Collapsin-1 and -2 are structurally similar and share strong sequence identity but display different biological activities in *in vitro* functional assays. Collapsin-1 constructs missing specific regions and chimeric proteins consisting of specific collapsin-1 segments transferred to collapsin-2 were used to identify domains that confer biological activity and specificity (Koppel *et al.*, 1997). These studies revealed that the semaphorin domain is essential for biological activity and is the principle determinant of specificity. Furthermore, dimerization of the semaphorin domain was demonstrated to be essential for activity (Koppel *et al.*, 1997).

Sema III, a murine homologue of chick collapsin, was shown to act as a diffusible chemorepellent that is present in ventral spinal cord and is able to repel a subset of DRG sensory axons *in vitro* (Puschel *et al.*, 1995; Messersmith *et al.*, 1995). This property is consistent with the temporal and spatial expression pattern of Sema III in embryonic mouse spinal cord. Sema
III transcripts are present in ventral spinal cord during E11.5 to E18. These developmental stages correspond to the time that Sema III responsive-DRG axons enter and terminate at targets in the dorsal spinal cord (Puschel et al., 1995; Messersmith et al., 1995). Sema III was therefore hypothesized to act as a repulsive substance that prevents axons from entering Sema III-expressing regions. This prediction was confirmed by two different groups by targeted disruption of the mouse sema III gene (Behar et al., 1996; Taniguchi et al., 1997). Unexpectedly, these groups obtained slightly different phenotypes that were attributed to different genetic backgrounds (Taniguchi et al., 1997). In the sema III<sup>−/−</sup> mice generated by Behar et al., DRG axons exhibit inappropriate penetration of Sema III-expressing regions in the ventral spinal cord. In contrast, mice generated by Taniguchi et al. display apparently normal DRG axon guidance in the spinal cord, but display significant abnormalities in peripheral nerve pathways. These defects are caused by ectopic innervation of Sema III-expressing regions (Taniguchi et al., 1997). In addition, defects are not confined to the CNS or PNS, as abnormalities, which varied depending on genetic background, were also observed in bone and cardiovascular tissue.

Recently, expression cloning experiments have identified the first semaphorin receptors as the transmembrane proteins neuropilin and neuropilin-2. Neuropilin is a high affinity receptor for Sema III (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), whereas neuropilin-2 is a high affinity receptor for Sema E and Sema IV, but not Sema III (Chen et al., 1997). Neuropilin was originally identified as a cell surface protein expressed in specific regions of the nervous system of Xenopus tadpoles (Takagi et al., 1987). It is composed of five known extracellular motifs, a transmembrane domain, and a short cytoplasmic region (Takagi et al., 1991). The extracellular motifs are related to two domains found in C1 complement proteins (CUB domains), two domains found in coagulation factors V and VIII, and a domain found in some receptor protein-tyrosine phosphatases (MAM domain) that are predicted to mediate protein-protein interaction (Takagi et al., 1991; He and Tessier-Lavigne, 1997). It is not known which of these domains mediate the interaction between neuropilin and Sema III. However, the semaphorin domain and C-terminal regions of Sema III can each bind neuropilin independently (He and Tessier-Lavigne, 1997). This property, combined with the finding that semaphorin is active as a dimer and that the short cytoplasmic region lacks any known signaling or catalytic
domains has led He and Tessier-Lavigne (1997) to speculate on the existence of an unidentified neuropilin co-receptor that is important for signaling.

The expression pattern of neuropilin and neuropilin-2 in mouse and rat is consistent with their roles as putative semaphorin receptors. Neuropilin is expressed in a subset of DRG axons and spinal cord motor neurons during developmental stages that correspond to periods of axonal outgrowth (Kawakami et al., 1996; Kolodkin et al., 1997). Neuropilin-2 is also expressed in motor neurons in the spinal cord, but is not detected in DRG neurons (Kolodkin et al., 1997). In addition, both proteins are also present in the cardiovascular system and bone precursor tissue (Kawakami et al., 1996; Kolodkin et al., 1997).

Furthermore, in vitro antibody-blocking studies indicate that the ability of ventral spinal cord explants to repel growth cones of DRG axons and to induce collapse of growth cones is dependent upon functional neuropilin (He and Tessier-Lavigne, 1997). As an initial step to ascertain the in vivo role of neuropilin, chimeric mice were generated that constitutively express neuropilin in a wide variety of tissues during embryogenesis (Kitsukawa et al., 1995). These mice display defects in the morphogenesis of cardiovascular tissue, including heart, as well as skeletal defects in limbs such as extra digits. In the nervous system, excess sprouting and defasciculation of axons occurs at several places in the CNS and PNS. DRG neurons in the spinal cord are highly disorganized. These phenotypes are similar to defects found in sema III<sup>−/−</sup> mice, a result consistent with Sema III and neuropilin acting in the same pathway (Behar et al., 1996; Taniguchi et al., 1997).

The components of the signaling pathway(s) downstream of semaphorin receptors are not understood. Collapsin response mediator protein (CRMP-62) may be one such component (Goshima et al., 1995). CRMP-62 was identified as a factor from chick DRG neurons that is required for collapsin-induced currents when it is expressed in Xenopus oocytes. CRMP-62 is a cytoplasmic protein related to UNC-33, a C. elegans protein implicated in axon guidance (Li et al., 1992). CRMP-62 expression in brain, retina, spinal cord, and DRG overlaps the expression domains of Sema III/collapsin and neuropilin (Goshima et al., 1995). Antibody perturbation studies indicate that CRMP-62 is required in vitro to mediate the growth cone collapsing activity of collapsin on DRG neurons (Goshima et al., 1995). However, the mechanism by which this is accomplished is not known.
Eph Receptors and Ephrins: Axon Guidance via Contact-Dependent Axon Repulsion

Eph type receptor protein tyrosine kinases (RPTKs) and their ligands constitute a large family that is widely expressed during vertebrate embryonic development, especially in the nervous system (Gale et al., 1996). Eph RPTKs are composed of an extracellular Ig-like domain, a cysteine rich region, two FNIII repeats, a membrane spanning region, and a conserved intracellular tyrosine kinase domain (Pandey et al., 1995). These receptors can be classified into two subgroups, EphA and EphB, based on amino acid similarity between their extracellular domains (Orioli and Klein, 1997). Eph ligands, designated ephrins, are characterized by a conserved 130-150 amino acid receptor-binding domain and a site for membrane attachment (Pandey et al., 1995). Ephrins are classified into two subgroups. Members of the ephrin-A subgroup are attached to the cell surface via a glycosylphosphatidylinositol (GPI) anchor, while the ephrin-B subgroup consists of transmembrane proteins (Orioli and Klein, 1997). Membrane attachment of ephrins is essential for their normal function. Only surface bound-ligands or ligands that have been artificially clustered are able to activate Eph RPTKs (Davis et al., 1994). Therefore, Eph receptors and ephrins are believed to act locally at sites of cell-to-cell contact.

In general, members of the EphA receptor subgroup bind GPI-linked ephrin-A ligands and members of the EphB receptor subgroup bind transmembrane ephrin-B ligands (Gale et al., 1996). These interactions are of low specificity as receptors are known to bind more than one ligand within a subgroup and vice versa (Gale et al., 1996). A striking observation is that the overall protein distribution of members of a particular receptor subgroup and its corresponding ligand subgroup are often in complementary and mutually exclusive domains (Gale et al., 1996). The complementarity of receptor and ligand expression patterns has provided insight, discussed below, into the mechanisms by which these molecules act to guide axons.

Eph receptors and ephrins were initially implicated in axon guidance by their actions in establishing the topographic connections in the retinotectal system of vertebrates (Tessier-Lavigne, 1995). A topographic map in the context of axon guidance, is one in which the spatial relationships of axons along the A/P and D/V axes from the originating field are maintained along corresponding axes of the target field. In the chick retinotectal system, for example, retinal neurons project to their targets in the midbrain region, known as the optic tectum, such that nasal (anterior) retinal axons project to the posterior tectum whereas temporal (posterior) retinal axons
project to the anterior tectum. Similarly, dorsal retinal axons project to the ventral tectum and ventral retinal axons project to the dorsal tectum (Tessier-Lavigne, 1995).

The chemoaffinity model, proposed by R.W. Sperry (1963), described a molecular mechanism to explain the formation of the retinotectal map. In this model, positional information is encoded in the form of complementary "chemoaffinity" gradients on the projecting axons and on their target cells. Stereotypical neuronal connections are therefore formed when an axon carrying a specific level of a receptor "label" locates its target position by recognizing a complementary level of ligand "label" on the target cell. Recent findings appear to support this theory (see below).

*In vitro* axon guidance assays were instrumental in showing a difference between anterior and posterior tectal membranes on the outgrowth and guidance of nasal versus temporal retinal axons. Anterior and posterior tectal membranes from chick were shown to be permissive substrates for nasal and temporal axon outgrowth when assayed separately (Walter *et al*., 1987a). However, when presented to retinal axons as alternating stripes of anterior or posterior membranes, temporal axons clearly preferred to grow on anterior membranes, whereas nasal axons showed no preference. Additionally, posterior tectal membranes were able to induce the collapse of temporal axon growth cones far more efficiently than anterior membranes, suggesting the presence of a repellent substance on posterior membranes as opposed to an attractive substance on anterior membranes (Walter *et al*., 1987b). The observation that phosphatidylinositol-specific phospholipase treatment destroyed the activity of this putative repellent suggested that it is preferentially attached to posterior membranes via a GPI-linkage. Furthermore the repellent is only present in the optic tectum during developmental periods when retinotectal connections are made (Walter *et al*., 1987a, 1990).

The GPI-linked ligands ephrin-A2 (ELF-1) and ephrin-A5 (RAGS) were the first molecules that met the above criteria for potential ligands responsible for the differential axon guidance effects of anterior versus posterior optic tectum (Cheng and Flanagan, 1994; Drescher *et al*., 1995; Cheng *et al*., 1995). Both ligands are expressed in the tectum during the period of axon innervation and are found in A/P gradients with the highest levels in the posterior tectum (Drescher *et al*., 1995; Cheng *et al*., 1995). Ephrin-A5 however is expressed in a steeper, more posterior gradient than ephrin-A2 (Monschau *et al*., 1997). Furthermore, one of their receptors,
EphA3 (Mek4, Cek4) is expressed in retinal axons in a complementary nasal-to-temporal gradient with highest levels temporally (Cheng et al., 1995, Monschau et al., 1997).

Both ephrin-A2 and ephrin-A5 mimic the axon growth cone repelling and collapse-inducing properties of posterior tectal membranes on temporal axons in the in vitro stripe and collapse assays (Drescher et al., 1995; Monschau et al., 1997). Ephrin-A5 however, is only able to preferentially repel or collapse growth cones of temporal axons when presented at low concentrations. At higher concentrations it repels or induces growth cone collapse in both nasal and temporal axons (Drescher et al., 1995; Monschau et al., 1997). In addition, ephrin-A2 is able to repel retinal axons that contain high levels of ephrin-A2 receptors (i.e. temporal axons) when ectopically expressed in the optic tectum in vivo (Monschau et al., 1997). Reciprocal gradients of ephrin-A2 and ephrin-A5 on the tectum and EphA3 on the retina are therefore believed to cooperate to ensure that temporal axons map to anterior tectum and nasal axons to posterior tectum. In other words, temporal axons but not nasal axons are repelled and therefore excluded from progressively more posterior regions of the tectum. Similarly, complementary D/V gradients are believed to form the topographic map along the D/V axis of the optic tectum (Holash and Pasquale, 1995).

Recently, the generation of “knockout” mice deficient in Eph receptors has provided the strongest functional evidence for a role for these RPTKs and their ligands in axon guidance. Disruption of the mouse EphA8 gene (Eek) causes a subset of axons, which are from a region of the midbrain called the superior colliculus and which normally express EphA8, to be misrouted from their normal targets in the adjacent inferior colliculus (Park et al., 1997). Similarly, disruption of EphB2 (Nuk) in mice causes defects in the formation of the anterior commissure, a major axon tract that links the left and right cerebral cortices (Henkemeyer et al., 1996). In EphB2<sup>−/−</sup> mice, part of the commissure is misrouted to enter regions that would normally express the EphB2 receptor. This result suggests a non cell-autonomous role as EphB2 is normally expressed in cells that demarcate the pathway over which these axons migrate rather than in the axons themselves. This finding is consistent with EphB2 acting to repel or exclude axons from regions that express its ligand. In fact, ligands for EphB2 are expressed in the affected axons. Interestingly, an EphB2 mutant that specifically lacks tyrosine kinase activity does not display any axon guidance defects (Henkemeyer et al, 1996). This result shows that the tyrosine kinase catalytic activity of EphB2 is not essential for guidance and led Henkemeyer et al. (1996) to
suggest that the EphB2 receptor may transduce a signal in the ligand-expressing axons upon ligand interaction. Evidence for the bidirectional-signaling hypothesis was strengthened by the demonstration that EphB2 can induce phosphorylation of its ligands in neuronal cells. Phosphorylation leads to the recruitment of downstream signaling proteins (Holland et al., 1996, 1997).

G. Summary

Axon growth cones navigate in response to environmental cues that include ECM components, cell adhesion molecules, and diffusible chemotropic factors. Directed growth cone migration involves receptor-activated mechanisms to translate extracellular guidance information into regulated and polarized changes in the actin cytoskeleton. In this Chapter, I have reviewed three families of axon guidance molecules: the netrins, the semaphorins, and the ephrins. Whereas, much is beginning to be known about ligand and receptor components of axon guidance mechanisms, less is known about downstream targets or signaling components that transduce information from the cell surface to the cytoskeleton. Approaches that take advantage of the powerful genetics of C. elegans can be used to identify the genes that encode these components. In the following Chapters, I will describe and discuss the results of a genetic screen to identify mutations in new components of the netrin/UNC-6 axon guidance pathway.
CHAPTER TWO

Suppressors of Ectopic UNC-5 Growth Cone Steering Identify Eight Genes Involved in Axon Guidance in Caenorhabditis elegans

I did all of the experiments reported in this Chapter.

Chapter Two is a modified version of a published manuscript:

INTRODUCTION

Axons are able to navigate long distances through a complex extracellular environment to reach their proper synaptic targets. Elucidating the molecular mechanisms underlying this process has become a major focus of developmental neurobiology. Recently, several mechanisms have been described that involve both attractive and repulsive cues to guide axonal growth cones to target tissues (reviewed in Tessier-Lavigne and Goodman, 1996). I have taken a genetic approach to further our understanding of these processes by identifying new components involved in cell and growth cone migrations in the nematode Caenorhabditis elegans.

Three genes, unc-5, unc-6, and unc-40, are required for migrations of axon growth cones and motile cells along the D/V axis of C. elegans (Hedgecock et al., 1990). UNC-6 is a secreted laminin-related molecule expressed in neuroglia and neurons along the ventral midline (Ishii et al., 1992, Wadsworth et al., 1996). UNC-5 and UNC-40 are cell surface receptors that act cell autonomously to orient migrating cells in response to polarity information encoded by the UNC-6 cue (Leung-Hagesteijn et al., 1992, Chan et al., 1996). Genetic studies indicate that dorsal migrations require UNC-5, while ventral migrations and, to a lesser extent, dorsal migrations require UNC-40 (Hedgecock et al., 1990). However, the signaling mechanisms that cells use to integrate guidance cue information and regulate cytoskeletal organization and motor activities required for directed outgrowth are poorly understood.

Vertebrate homologues of UNC-5, UNC-6, and UNC-40 have been identified, and their functions appear to be conserved. UNC-6 homologues, designated netrins, are diffusible molecules that can act as either attractive or repulsive guidance cues for different populations of neurons (Serafini et al., 1994, Kennedy et al., 1994, Colamarino et al., 1995). UNC5H1, UNC5H2, and the product of the murine rostral cerebellar malformation (rcm) gene are homologues of UNC-5 (Leonardo et al., 1997, Ackerman et al., 1997) and the products of the deleted in colorectal cancer (dcc) gene and neogenin are homologues of UNC-40 (Keino-Masu et al., 1996, Fazeli et al., 1997). These proteins have been implicated as candidate netrin receptors (Keino-Masu et al., 1996, Leonardo et al., 1997).

C. elegans unc-5 is able to induce dorsally directed axon outgrowth when ectopically expressed in the touch-sensitive mechanosensory neurons which normally extend along longitudinal or ventral trajectories (Hamelin et al., 1993). This dorsal reorientation requires
UNC-6 and is therefore consistent with a mechanism in which UNC-5 expression in the touch receptors makes their growth cones responsive to UNC-6 in the same way that motoneurons that normally express UNC-5 are responsive to UNC-6. I have used the ability of UNC-5 to steer growth cones as the basis for a suppressor screen to identify additional genes involved in unc-5-mediated guidance. In this Chapter, I describe mutations in eight genes, including unc-6, that are suppressors of ectopic UNC-5 function in the touch neurons. I propose that several of these genes encode previously unknown components of a developmentally important mechanism of UNC-5 receptor function and signaling.

MATERIALS AND METHODS

General Techniques and Strains

General techniques for the culture and handling of worms have been described (Brenner, 1974). The C. elegans Bristol (N2) stock was used as the wild type strain. The phenotypes of mutations used in this study are Bli (blister), Dpy (dumppy), Fem (feminization), Lin (lineage abnormal), Mes (maternal effect sterile), Pag (pattern of reporter gene expression), Seu (suppressor of ectopic unc-5), and Unc (uncoordinated). The mutations and rearrangements used were as follows:

Linkage group I (LG I): dpy-5(e61), unc-40(e1430).
LG II: dpy-10(e128).
LG III: dpy-17(e164), pag-1(ls2).
LG IV: dpy-13(e184), unc-5(e53), fem-1(e1991), unc-44(e362), bli-6(sc16), unc-24(e138), mes-6(bn66), fem-3(e2006), dpy-20(s1282), unc-22(s12), stDf8, eDf18.
LG V: unc-34(e566), unc-60(m35), lin-40(e2173), unc-62(e644), unc-46(e177), dpy-11(e224), sDf34, nDf32.
LG X: unc-6(ev400).

Mutant strains that were not derived in our laboratory were provided by A. Spence (University of Toronto), E. Aamodt (Louisiana State University) or the Caenorhabditis Genetics Center. Unless stated otherwise, worms were handled and maintained at 20°.
Construction of Transgenic Lines

Standard germline transformation techniques were used as described by Mello and Fire (1995). mec-7::unc-5 transgenic worms were generated by injecting a mixture of 100 µg/ml mec-7::unc-5 plasmid DNA (Hamelin et al., 1993), 50 µg/ml mec-7::lacZ plasmid DNA (Hamelin et al., 1992), and 20 µg/ml of plasmid pMH86 containing the wild-type dpy-20 gene (Han and Sternberg, 1991) into the distal gonad arms of dpy-20 hermaphrodites. evls41 and evls68 were independently derived by integrating an extrachromosomal array carrying these constructs at random genomic sites (Mello and Fire, 1995). Integration was promoted by irradiation of worms with approximately 4000 rads from a 137Cs source. The absence of Dpy segregants among the progeny of irradiated worms was used as evidence for a successful integration. mec-7::lacZ (evls55) worms were created in the same way except mec-7::unc-5 plasmid DNA was not included in the injection mixture. evls41, evls68, and evls55 were passed into a pag-1(ls2) background by mating to establish lines with increased levels of lacZ expression (Xie et al., 1995). unc-6(ev400) was also passed into the evls41; pag-1(ls2) background by mating.

Identification of Suppressors of Ectopic UNC-5

evls41; pag-1(ls2) worms were mutagenized with 50 mM ethyl methanesulfonate (EMS) as described by Brenner (1974). Upon overnight recovery, subpopulations of 25-50 worms were transferred to 100 mm diameter seeded NGM plates and allowed to lay eggs overnight. A semi-synchronized population of F1 eggs was established by washing off the mutagenized P0 adults and newly hatched worms with M9 buffer. After two to three days, approximately 200 F1 adults were transferred to each of approximately 20 large (100 mm diameter) seeded NGM plates per screen and allowed to lay eggs. After three days, approximately 1000-2000 gravid F2 worms were collected from each plate and stained for lacZ activity. Animals were fixed, permeabilized, and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) using a technique described by Xie et al. (1995) that allows for the histochemical staining of gravid worms without killing the majority of their eggs.

Strains carrying mutations that suppressed unc-5-induced growth cone guidance were identified by visual inspection of touch neurons with a Leitz Wild M3B microscope under conditions of 40x magnification and diffuse illumination. Worms in which at least two of the
three anterior touch neurons projected longitudinally were identified as containing putative suppressor mutations. To facilitate visualization of the lacZ-stained touch neurons and recovery of suppressors, stained worms were first transferred to 60 mm diameter tissue culture plates containing a thin pad of 1% agarose on their bottom surface and partially filled with water. Once identified, an animal carrying a putative suppressor was picked using forceps and transferred to a 35 mm diameter seeded NGM plate to establish a line from the living embryos trapped in its uterus. Animals from each line were subsequently restained for lacZ to confirm the presence of a suppressor. To ensure that mutations were of independent origin, only one suppressor was retained from each subpopulation of mutagenized animals.

A strain was defined as containing a suppressor mutation if at least 70% of the anterior touch neurons did not project in a dorsal direction. The strongest suppressor from each complementation group (see below) was scored for the penetrance of suppression of ectopic unc-5-growth cone steering. Populations of 400-500 worms were fixed and stained for lacZ as described, mounted on 1% agarose pads on glass slides, and observed using bright field optics with a Leitz DMRB microscope. The axonal processes of the anterior touch neurons, ALML/R and AVM, were scored as dorsalized if they displayed a dorsally oriented trajectory and entered the dorsal cord.

**Genetic Mapping and Complementation Tests**

Determination of linkage groups (LG), complementation tests, and mapping by three-factor crosses were performed using standard methods (Brenner, 1974).

Those suppressors that caused an obvious Unc phenotype were assigned to linkage groups by following the segregation of the uncoordinated defect in progeny from crosses with the reference mutants dpy-5 (LG I), dpy-10 (II), dpy-17 (III), dpy-13 (IV), and dpy-11 (V). Suppressors were assigned to LG X if the resultant phenotype was observed in hemizygous F1 males. Once assigned to a LG, these mutations were tested for complementation of canonical alleles of known genes on the same LG that mutate to cause similar phenotypes.

Other suppressors were assigned to LGs by scoring segregation of the suppressor of ectopic unc-5 (Seu) phenotype by staining recombinants from heterozygotes of genotype + seu/dpy +; evIs4I; pag-1, where dpy was one of the tester mutations listed above. If the seu mutation is linked to dpy, Dpy Seu recombinants should be difficult to recover. The segregation
of the Seu phenotypes among the progeny of the above heterozygotes was consistent with recessive or loss-of-function mutations (data not shown).

The suppressors, ev520, ev529, and ev572 were designated alleles of seu-1 based on failure to complement each other for a mild uncoordinated movement defect and were subsequently found to be linked to LG IV. Mapping was carried out by identifying and staining recombinants among progeny of genotype + seu +/a + b; evIs41; pag-1, where a and b are tester mutations listed above (see General Techniques and Strains). seu-2 was mapped in a similar manner.

Mapping of seu-3 on LG V was complicated by the presence of the evIs41 array, which also mapped to the left arm of LG V. To map seu-3 in the unc-60 to lin-40 interval, seu-3 + + + evIs41/+ unc-60 lin-40 dpy-11 +; pag-1/+ animals were produced by crossing homozygous seu-3 evIs41; pag-1 males to unc-60 lin-40 dpy-11/eT1, and Unc non-Lin non-Dpy recombinants were identified among the F2. Those recombinants that did not carry evIs41, as determined by lacZ staining, were mated to evIs68; pag-1 homozygous males and the progeny of the resulting heterozygous worms tested for the presence of the Seu phenotype by staining for lacZ. seu-3 was mapped in the unc-34 dpy-11 interval in the same manner, with the exception that mating to evIs68; pag-1 was not necessary as all recombinants carried the evIs41 array.

Complementation Tests using Deficiencies

unc-129 and seu mutants were placed in trans to deficiencies once their map positions had been determined by 3-factor mapping. Heterozygous unc-129(ev554) dpy-20/+ males were mated with eDf18/unc-24 dpy-20 hermaphrodites and the resulting unc-129 dpy-20/eDf18 animals identified by non-complementation for the unc-129 defect and subsequent segregation of the expected marker mutations among their self progeny. Homozygous seu-1(ev520) males were crossed to fem-1 unc-24 unc-22/stDf8 hermaphrodites and seu-1/stDf8 animals identified by their failure to segregate Fem Unc animals. seu-1/stDf8 animals were also compared to control +/stDf8 animals generated by genetic crosses involving N2 instead of seu-1 males. seu-2 and seu-3 were placed over deficiencies nDf32 and sDf34, respectively in the same way.
Construction of Worms Transgenic for Neuronal lacZ and GFP Reporters

Extrachromosomal arrays containing either lacZ or the gene for GFP fused to a neuronal-specific promoter were generated by standard germline transformation techniques described in Mello and Fire (1995). The unc-5::lacZ transgene was used to visualize the axonal trajectories of the DD and VD classes of motoneurons. unc-129 dpy20 and dpy-20 hermaphrodites were co-injected with 100 μg/ml of the unc-5::lacZ reporter plasmid pYZ129 (M.W. Su, Y. Zhou, and J.G. Culotti, unpublished results) and 31 μg/ml of pMH86 [dpy-20(+)]. seu-1, seu-2, and seu-3 hermaphrodites were each transformed with a mixture containing 100 μg/ml of pYZ129 and 33 μg/ml of plasmid pRF4 containing the dominant co-transformation marker rol-6(su1006) (Kramer et al., 1990). Transgenic animals were fixed and stained for lacZ as described in Xie et al. (1995).

Worms transgenic for the cytoplasmic GFP transcriptional reporter pAC12 (see Chapter III) were generated to visualize the axonal processes of the DA and DB classes of motoneurons. An extrachromosomal array containing the pAC12 reporter was generated by co-injecting 60 μg/ml of pAC12 and 20 μg/ml of pMH86 into the distal gonads of dpy-20 hermaphrodites. Random integration of this array into the genome was promoted by gamma irradiation of the injected worms. Two independent lines designated evIs82A and evIs82B were obtained that contained the integrated array. N2, seu-1(ev520), seu-2, and seu-3 worms were co-injected with 60 μg/ml of pAC12 and 44 μg/ml of pRF4 to generate stable transformed lines that express the pAC12 GFP reporter from extrachromosomal arrays. In all cases, the seu mutants were back-crossed to evIs41; pag-1(ls2) worms at least twice before establishing homozygous lines.

Construction of Double Mutants

unc-129(ev554) mutants are readily distinguished from unc-6(ev400) and unc-5(e53) mutants as they are much less uncoordinated. unc-6(ev400); unc-129(ev554) double mutants were constructed by cloning unc-129 mutants from the self-progeny of unc-6(ev400) +/- unc-129(ev554) hermaphrodites. As unc-6 and unc-129 are not linked, two-thirds of the unc-129 homozygotes segregate unc-6 mutant progeny, some of which were cloned to identify the double.

unc-5(e53) unc-129(ev554) double mutants were constructed by cloning non-Dpy Unc-129 recombinants from the self progeny of dpy-13(e184) + unc-129(ev554)/+ unc-5(e53) +
worms and then screening for Unc-5 among their progeny to identify the double. Complementation tests were used to confirm the genotype.

**Scoring Axon Guidance Defects**

Integrated arrays containing pAC12 were crossed into lines carrying suppressors of ectopic unc-5 that cause an Unc phenotype. These mutants had been back-crossed to wild type males at least twice. The progeny of 7-8 transgenic worms were immobilized with 10 mM levamisole and transferred to a 1% agarose pad on a glass slide. GFP was visualized using epifluorescence. DA and DB axon morphologies were examined in 30 randomly chosen late larval and adult hermaphrodites. A representative subset of DA and DB motoneurons consisting of DA3, DA4, DA5, and DA6 of the DA class and DB4, DB5, DB6, and DB7 of the DB class were scored. The data from these neurons were subsequently pooled. Motoneurons were scored as having an outgrowth defect if a cell body was clearly visualized but the axon could not be seen. Motoneurons were scored as having an axon guidance defect if an axon failed to reach the dorsal cord and instead extended longitudinally along the lateral epidermis.

**RESULTS**

**Identification of Eight Genes Required for unc-5-Induced Dorsal Steering of the Touch Neurons**

Ectopic expression of unc-5 in the touch neurons is sufficient to steer their ventrally or longitudinally directed axons in a dorsal direction (Figure 2.1) (Hamelin et al., 1993). The integrated array, evls41, contains multiple copies of a mec-7::unc-5 construct and a mec-7::lacZ reporter. In animals carrying evls41 and pag-1(ls2), a mutation that increases expression from the mec-7 promoter (Xie et al., 1995), 86% (n=296) of the anterior touch neurons, ALML/R and AVM, have dorsally directed axons. In contrast, when unc-6(ev400), a null mutation, was introduced into this genetic background, 0% (n=100) of the anterior touch neuron axons extended in a dorsal direction.

The ability of ectopically expressed unc-5 to steer touch receptor axons dorsally provided the basis of a genetic screen to identify additional components of the unc-5-mediated dorsal
Figure 2.1. Ectopic expression of *unc-5* in the touch receptor neurons steers their growth cones dorsally. Touch neurons are visualized in adult hermaphrodites by expression of *lacZ* from a *mec-7::**lacZ* reporter gene. A *pag-I*(ls2) mutant background is used as defects in *pag-I* cause increased expression from the *mec-7* promoter. Anterior is to the left and dorsal is towards the top of each panel. (A) *pag-I*(ls2) mutant transgenic for a *mec-7::**lacZ*-containing array (*evIs55*) showing ALML/R and AVM touch neurons. (B) *pag-I*(ls2) mutant transgenic for a *mec-7::**lacZ* and *mec-7::**unc-5* containing array (*evIs41*) showing dorsally directed ALML/R and AVM touch neurons. (C) *unc-6*(ev400) suppresses the guidance defects caused by ectopic *unc-5*. Scale bar, 50 μm.
A

pag-1(is2); evls55[mec-7::lacZ]

B

pag-1(is2); evls41[mec-7::unc-5, mec-7::lacZ]

C

unc-6(ev400); pag-1(is2); evls41[mec-7::unc-5, mec-7::lacZ]
guidance pathway. Mutations in such components would, like *unc-6(ev400)*, disrupt ectopic *unc-5* function in the touch neurons (Figure 2.2). The F₂ progeny of EMS-mutagenized *evIs41; pag-1* worms were stained for *lacZ* to visualize the touch neurons and a visual screen was performed to identify worms with normal touch neuron trajectories.

In a screen of approximately 30-40,000 mutagenized genomes, I identified 33 mutations in eight genes that suppressed the guidance defects induced by ectopic *unc-5* expression in the touch neurons (Table 2.1). Two of these genes, *unc-6* and *unc-40*, like *unc-5*, are known to play a key role in axon guidance and cell migrations along the D/V axis (Hedgecock *et al.*, 1987, 1990). Mutations in two other genes, *unc-34* and *unc-44*, are known to cause axon and cell migration defects along both the A/P and D/V axes, but have not previously been shown to be required for *unc-5* function (Siddiqui and Culotti, 1991; McIntire *et al.*, 1992; Forrester and Garriga, 1997). Finally, I identified alleles of four new genes, *unc-129*, *seu-1*, *seu-2*, and *seu-3* (suppressor of ectopic *unc-5*) that potentially define new components of an *unc-5* signaling pathway. Genetic and phenotypic characterization during outcrossing and mapping indicated that all of the mutations were recessive, consistent with the suggestion that they cause a loss of gene function.

The gene that was identified most frequently in this screen was *unc-6*; twelve new alleles of this gene were recovered as suppressors. These mutations resulted in nearly complete suppression (99%) of ectopic *unc-5* function (Table 2.1). Since positional information encoded by *unc-6* is required for *unc-5*-induced dorsal guidance (Hamelin *et al.*, 1993), mutations in *unc-6* were expected if the screen were working as designed to identify components of the *unc-5* guidance pathway. An unexpected result was the observation that *unc-6* was haplo-insufficient in its ability to mediate dorsal guidance of the touch neurons by ectopic *unc-5*. Three *unc-6* loss-of-function alleles, *ev517*, *ev568*, and *ev569*, which gave rise to phenotypes less severe than an *unc-6* null mutant (data not shown), were identified as dominant suppressors in this screen. In contrast, the visible phenotypic defects present in *unc-6* mutants, which include uncoordination, egg-laying defects, and distal tip cell migration defects, are completely recessive (Hedgecock *et al.*, 1990). These results indicate that *evIs41; pag-1* represents a sensitized background from which to recover mutations affecting components of the *unc-5* signaling pathway. Dorsally directed touch neurons may be more sensitive to the dose of UNC-6 because their cell bodies are located along the lateral body wall. At this position the cells are exposed to lower levels of
Figure 2.2. Schematic outline of the genetic screen used to identify suppressors of ectopic unc-5. Worms in which the touch neurons express unc-5 and extend axons in a dorsal direction were mutagenized with EMS. The F2 progeny were then stained for lacZ using a technique that does not kill eggs, and a visual screen was performed to identify worms with normal touch neuron trajectories. The candidate suppressor was isolated and the viable eggs trapped in utero were used to establish a mutant line.
Transgene:

\[ evi55 \sim5 ~ evi41 ~ VISQI \sim41 \]

Genotype:

\[ \text{[m-7::iw mef::laiZ]} \sim7::unc-S \text{[m-7::unw]} \text{[m-7::unc-5]} \]

EMS mutagenesis

\[ lacZ \text{staining of F2} \]

visual screen for normal touch neurons
### Table 2.1. Suppressors of *unc-5* Induced Reorientation of the Touch Neurons.

<table>
<thead>
<tr>
<th>Strain Background</th>
<th>Suppressor</th>
<th>Alleles</th>
<th>% Anterior Touch Neurons with Normal Trajectories</th>
</tr>
</thead>
<tbody>
<tr>
<td>evIs55; pag-l</td>
<td>None</td>
<td>--------</td>
<td>100</td>
</tr>
<tr>
<td>evIs41; pag-l</td>
<td>None</td>
<td>--------</td>
<td>14</td>
</tr>
<tr>
<td>evIs41; pag-l</td>
<td>unc-40</td>
<td><code>ev541, ev542, ev543, ev544, ev545, ev546, ev547</code></td>
<td>91</td>
</tr>
<tr>
<td>evIs41; pag-l</td>
<td>unc-34</td>
<td><code>ev553, ev561, ev562, ev564</code></td>
<td>72</td>
</tr>
<tr>
<td>evIs41; pag-l</td>
<td>unc-44</td>
<td><code>ev570, ev571</code></td>
<td>85</td>
</tr>
<tr>
<td>evIs41; pag-l</td>
<td>unc-129</td>
<td><code>ev554, ev557, ev566</code></td>
<td>94</td>
</tr>
<tr>
<td>evIs41; pag-l</td>
<td>seu-2</td>
<td><code>ev520, ev529, ev572</code></td>
<td>92</td>
</tr>
<tr>
<td>evIs41; pag-l</td>
<td>seu-3</td>
<td><code>ev555</code></td>
<td>82</td>
</tr>
</tbody>
</table>

1. All suppressors are in a genetic background containing `evIs41 [mec-7::unc-5 mec-7::lacZ dpy-20(+)] and pag-l(l52)`. The evIs55 array contains `[mec-7::lacZ dpy-20(+)]`.
2. Alleles that were scored for suppression of ectopic *unc-5* function are indicated in **Bold**.
3. Total number of axons scored include only the anterior touch neurons ALML/R and AVM.
UNC-6, than cells at the ventral midline where UNC-6 is at its highest concentration (Wadsworth et al., 1996).

I identified seven mutations in unc-40, several of which were among the weakest so far identified for axon and cell migration defects (Hedgecock et al., 1990, D. Merz and J.G. Culotti, unpublished results). unc-40(ev542), a mutation which resulted in one of the most severe uncoordinated unc-40 phenotypes identified in this screen, was 91% (n=338) effective at suppressing unc-5-induced dorsal reorientation of the touch neurons (Table 2.1).

Suppression of ectopic unc-5-induced dorsal reorientation varied from nearly complete suppression in unc-6 (99%) and unc-129 (97%) mutants to less effective suppression in unc-34 (72%) and seu-2 (72%) mutants (Table 2.1). This variation in the penetrance of suppression may be due to identification of partial loss-of-function instead of null mutations at some loci, redundant mechanisms where another gene or signaling pathway may partially compensate, or a combination of both.

Identification and Mapping of Four New Genes

Complementation testing and genetic mapping, summarized in Table 2.2, indicated that mutations in unc-129, seu-1, seu-2, and seu-3 identified new genes. The positions of these genes on the C. elegans genetic map are shown in Figure 2.3.

unc-129 mutants displayed a fully penetrant uncoordinated movement defect that manifested itself as a kink in the normal sinusoidal wavelike motion of wild type. This kink is especially evident during backward movement. Whereas all unc-129 worms were uncoordinated, the severity of the movement defect varied from worm to worm. Examination of axon morphology in unc-129 mutants, described below, indicated that uncoordination was most likely caused by variable axon guidance defects that prevented motorneurons from reaching their final targets in the DNC. Observation of unc-129 mutants with Nomarski DIC optics did not reveal any other visible muscle defects or gross morphological defects that could account for the uncoordinated phenotype. Similar observations of seu-1, seu-2, and seu-3 mutants did not reveal any visible morphological or movement defects except for a mildly uncoordinated phenotype in seu-1 mutants.
Table 2.2. Three-factor Map Data for *unc-129* and the *seu* Genes.

<table>
<thead>
<tr>
<th>Suppressor&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Parental genotype</th>
<th>Recombinant Phenotype&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Genotype of Recombinant Chromosome</th>
<th># of Recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>unc-129</em></td>
<td><em>unc-24 dpy-20/unc-129</em></td>
<td>Dpy non-Unc</td>
<td>+ <em>unc-129</em> dpy-20</td>
<td>15/20</td>
</tr>
<tr>
<td></td>
<td><em>unc-24 fem-3/unc-129</em></td>
<td>Fem non-Unc</td>
<td>+ <em>unc-129</em> fem-3</td>
<td>1/4</td>
</tr>
<tr>
<td><em>seu-1</em></td>
<td><em>unc-44 bli-6/seu-1(ev520)</em></td>
<td>Bli non-Unc</td>
<td>+ <em>seu-1</em> bli-6</td>
<td>2/35</td>
</tr>
<tr>
<td></td>
<td><em>bli-6 unc-24/seu-1(ev520)</em></td>
<td>Bli non-Unc</td>
<td>+ <em>seu-1</em> bli-6</td>
<td>33/35</td>
</tr>
<tr>
<td></td>
<td><em>unc-44 bli-6/seu-1(ev572)</em></td>
<td>Bli non-Unc</td>
<td>+ <em>seu-1</em> bli-6</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td><em>bli-6 unc-24/seu-1(ev572)</em></td>
<td>Bli non-Unc</td>
<td>+ <em>bli-6</em></td>
<td>5/5</td>
</tr>
<tr>
<td><em>dpy-13 unc-24/seu-1(ev520)</em></td>
<td>Unc non-Dpy</td>
<td>+ <em>seu-1</em> unc-24</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>dpy-13 unc-24/seu-1(ev529)</em></td>
<td>Unc non-Dpy</td>
<td>+ <em>seu-1</em> unc-24</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td><em>dpy-13 unc-24/seu-1(ev529)</em></td>
<td>Unc non-Dpy</td>
<td>+ <em>unc-24</em></td>
<td>5/6</td>
</tr>
<tr>
<td><em>seu-2</em></td>
<td><em>unc-46 dpy-11/seu-2</em></td>
<td>Dpy non-Unc</td>
<td>+ <em>seu-2</em> dpy-11</td>
<td>22/26</td>
</tr>
<tr>
<td></td>
<td><em>seu-2</em></td>
<td>Dpy non-Unc</td>
<td>+ <em>dpy-11</em></td>
<td>4/26</td>
</tr>
<tr>
<td><em>seu-3</em></td>
<td><em>unc-62 dpy-11/seu-3</em></td>
<td>Dpy non-Unc</td>
<td>+ <em>dpy-11</em></td>
<td>0/37</td>
</tr>
<tr>
<td></td>
<td><em>lin-40 dpy-11/seu-3</em></td>
<td>Dpy non-Lin</td>
<td><em>seu-3</em> + <em>dpy-11</em></td>
<td>37/37</td>
</tr>
<tr>
<td></td>
<td><em>unc-60 lin-40 dpy-11/seu-3</em></td>
<td>Unc non-Lin non-Dpy</td>
<td><em>seu-3</em> + <em>dpy-11</em></td>
<td>19/19</td>
</tr>
<tr>
<td></td>
<td><em>unc-34 dpy-11/seu-3</em></td>
<td>Dpy non-Unc</td>
<td>+ <em>dpy-11</em></td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td><em>seu-3</em></td>
<td>Dpy non-Unc</td>
<td>+ <em>dpy-11</em></td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td><em>seu-3</em></td>
<td>Dpy non-Unc</td>
<td>+ <em>dpy-11</em></td>
<td>16/17</td>
</tr>
</tbody>
</table>

<sup>1</sup> *unc-129* was mapped based on its Unc phenotype. The *seu* mutants were mapped based on direct visualization of touch neuron morphology by *lacZ* staining.

<sup>2</sup> *seu* genes were mapped in a genetic background containing the *evls41[meic-7::unc-5 meic-7::lacZ dpy-20(+)]* transgene and *pag-1* (see Materials and Methods).
**Figure 2.3.** Genetic map positions of the genes identified in this study and the markers and deficiencies used for mapping. New genes are highlighted in **bold**. The map position of *seu-3* is approximated by a bar since its position relative to *unc-60* is not known. Bar below each map represents 1 map unit (mu).
To test whether these mutations caused a complete loss of gene function, they were made hemizygous using genomic rearrangements that resulted in chromosomal deficiencies. These tests may reveal residual gene function if the phenotype of the mutation in trans to a deficiency is more severe than the mutant phenotype on its own. The visible appearance and movement of seu-1, seu-2, and seu-3 hemizygotes did not appear to differ from those of homozygous mutants. However, unc-129(ev554) in trans to a deficiency appeared to be slightly more uncoordinated than unc-129(ev554) homozygotes. This may suggest that ev554 is not a null mutation or alternatively that loss of another gene uncovered by the deficiency may be additive with unc-129 resulting in an apparently stronger phenotype.

Axon Guidance Defects: A Potential Role for unc-34 and unc-129 in unc-5-Mediated Guidance

The unc-5 gene is specifically required to guide pioneer growth cones and migrating cells in a dorsal direction on the basal surface of the epidermis (Hedgecock et al., 1990). Five classes of motorneurons with cell bodies in the VNC (AS, DA, DB, DD, and VD) send commissural processes dorsally to synapse with muscle targets in the dorsal cord (White et al., 1986). These neurons can be identified by their position in the ventral cord, axon morphology, and connectivity (White et al., 1986). A large proportion of these axons are misguided in unc-5 mutants, often extending along aberrant longitudinal trajectories at lateral or dorsolateral axial positions (Hedgecock et al., 1990, Siddiqui and Culotti, 1991). A similar phenotype is observed in unc-6 mutants (Hedgecock et al., 1987, 1990). If the ectopic unc-5 suppressors identified in this study function in a physiologically relevant unc-5 signaling pathway, then they might be expected to have defects similar to those found in unc-5 mutants. To determine if this is the case, neurons were observed in late larval and adult hermaphrodites. An unc-5::lacZ reporter was used to characterize the DD and VD motorneurons and pAC12, a DA and DB neuron specific GFP reporter, was used to characterize the DA and DB motorneurons.

Axon morphologies of the DD and VD motorneurons in unc-40, unc-34, and unc-44 mutants have been described elsewhere (Hedgecock et al., 1987; Siddiqui and Culotti, 1991; McIntire et al., 1992; Forrester and Garriga, 1997). Qualitative observation of DD and VD axons in unc-129(ev554) mutants revealed a large number of axons that failed to reach the dorsal cord and instead extended along longitudinal paths at aberrant axial levels (Figure 2.4). In some
Figure 2.4. DD and VD axon morphology in wild-type and unc-129(ev554) hermaphrodites. Axons were visualized using lacZ expression from an unc-5::lacZ transgene. Anterior is to the left and dorsal is towards the top of each panel. (A) Schematic drawing of the DD and VD class of motorneurons in an adult hermaphrodite. (B) DD and VD axon morphology in a wild-type adult hermaphrodite. (C) DD and VD axons in an unc-129(ev554) adult hermaphrodite deviate from their normal trajectories to extend longitudinally at lateral axial positions (arrow). Arrowheads indicate the excretory cell process. Scale bar, 50 μm.
wild type

unc-129(ev554)
cases, these motorneurons displayed excessive branching and very disorganized trajectories. Similar, but more penetrant defects were seen with the same reporter in unc-5 and unc-6 mutants (data not shown). Observation of DD and VD axons in seu-1, seu-2, and seu-3 mutants did not reveal any defects.

The pAC12 GFP reporter is expressed in the DA and DB class of motorneurons as well as a discrete set of other cells. The strong fluorescence that is observed in the axons of living animals expressing this reporter make it an ideal marker for studying the axon morphologies of the DA and DB neurons. Visualization of these neurons in Unc mutants that were suppressors of ectopic unc-5 (Figure 2.5) showed that unc-6, unc-40, unc-34, and unc-129 were required for proper circumferential growth of the DA and DB motorneurons. In these mutants, a significant fraction of the DA and DB axons failed to grow to their targets in the dorsal cord and instead extended along longitudinal paths at dorsal lateral or lateral positions (Table 2.3 and Figure 2.5A-G). For example, in unc-129(ev554) mutants 39% (n=120) of DA and 63% (n=120) of DB processes failed to reach the dorsal cord. These defects were qualitatively identical to those seen in unc-5 and unc-6 mutants, but were not as penetrant. The morphology of DA and DB axon appeared normal in seu-1, seu-2, and seu-3 mutants (Table 2.3). The touch neurons in outcrossed unc-129 mutants appeared morphologically wild type (data not shown), indicating that ventral and longitudinal axon guidance, at least for these cells, did not require unc-129. This is also the case in unc-5 mutants, although ventral guidance of AVM and PVM axons is perturbed in unc-6 and unc-40 mutants (Hedgecock et al., 1990).

To examine possible genetic interactions between unc-5 and either unc-34 or unc-129, and between unc-6 and unc-129, double mutant combinations were constructed and the DA and DB motorneuron axons were examined. Dominant enhancement of axon guidance defects was not observed in trans heterozygous combinations of the above mutants (data not shown). I also did not observe any additional synthetic defects in unc-5 unc-129 or unc-6; unc-129 double mutants compared to unc-5 and unc-6 single mutants (Figure 2.5H-I). Determination of additive genetic effects in these double mutants was complicated by the nearly complete penetrance of DA and DB guidance defects seen in the unc-5 and unc-6 single mutants. Worms homozygous for unc-5(e53) plus unc-34(ev553) were very abnormal and difficult to interpret.

Mutations in unc-5, unc-6, and unc-34 also appeared to cause less frequent defects in initial axon outgrowth as brightly fluorescing cell bodies were sometimes observed to lack a
Figure 2.5. DA and DB axon morphology in wild-type and unc hermaphrodites. Axons are visualized using GFP expression from a transgenic array containing pAC12, a reporter gene expressed in a discrete set of cells including the DA and DB class of motorneurons. Anterior is to the left and dorsal is towards the top of each panel. (A) Schematic drawing of the DA and DB class of motorneurons in an adult hermaphrodite. (B-I) Fluorescence micrographs of the middle portion of L4 stage larvae showing the axon morphology of the set of DA and DB motorneurons characterized in this study. In the unc mutants, axons deviate from their wild-type trajectories and extend along subdorsal longitudinal trajectories. Note that the dorsal and ventral nerve cords are not in the plane of focus. (B) wild-type, arrowhead indicates row of seam cells, arrow indicates spermatheca. (C) unc-129(ev554), arrow indicates subdorsal longitudinal cord. (D) unc-40(e1430), (E) unc-34(ev553), (F) unc-5(e53), (G) unc-6(ev400). (H-I) DA and DB axon morphology in unc5 unc-129 and unc-6 unc-129 double mutants is qualitatively indistinguishable from that in the single mutants. Scale bar, 50 μm.
Table 2.3. Summary of DA and DB Axon Guidance Defects in Seu Mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DA motoneurons</th>
<th>DB motoneurons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>defects (%)</td>
<td>defects (%)</td>
</tr>
<tr>
<td></td>
<td>wt</td>
<td>outgrowth</td>
</tr>
<tr>
<td>evls82A</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>evls82B</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>unc-129(ev554)</td>
<td>60.8</td>
<td>0</td>
</tr>
<tr>
<td>unc-129(ev557)</td>
<td>59.2</td>
<td>0</td>
</tr>
<tr>
<td>unc-129(ev566)</td>
<td>77.5</td>
<td>0</td>
</tr>
<tr>
<td>unc-40(e1430)</td>
<td>71.5</td>
<td>0.9</td>
</tr>
<tr>
<td>unc-34(ev553)</td>
<td>85.8</td>
<td>4.2</td>
</tr>
<tr>
<td>unc-34(ev566)</td>
<td>85.8</td>
<td>4.2</td>
</tr>
<tr>
<td>unc-5(e53)</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>unc-6(ev400)</td>
<td>0.8</td>
<td>5</td>
</tr>
<tr>
<td>unc-5(e53) unc-129(ev554)</td>
<td>0</td>
<td>2.5</td>
</tr>
<tr>
<td>unc-6(ev400): unc-129(ev554)</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td>seu-1(ev520)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>seu-2(ev523)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>seu-3(ev555)</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

1 All strains are in an evls82A transgenic background, except for unc-40(e1430), which carries evls82B, and the seu mutants, which are transgenic for an extrachromosomal array containing pAC12 and pRF4. All transgenic arrays contain multiple copies of pAC12, a GFP reporter expressed in DA and DB motoneurons. Axon morphologies in unc-44 mutants were not determined (nd) as it was difficult to unambiguously identify neurons because of missing and mispositioned cell bodies.

2 Axon morphologies of DA3, DA4, DA5, and DA6 of the DA class of motoneurons and DB4, DB5, DB6, and DB7 of the DB class of motoneurons were examined by fluorescence microscopy. Motoneurons were scored as having an outgrowth defect if a cell body was clearly visualized but not the axon. Axon guidance defects were scored if an axon failed to reach the dorsal cord and instead extended longitudinally along the lateral epidermis. wt, wild-type.
process exiting the ventral cord (Table 2.3). Alternatively, these axon growth cones may have failed to migrate dorsally, or they may have done so and then retracted and migrated longitudinally along other axons in the VNC. The observation of axon outgrowth defects in unc-6 mutants suggests that UNC-6 may act, in part, as a permissive guidance cue to promote outgrowth in addition to its role as an instructive guidance cue to impart direction. In a like manner, axon outgrowth defects have also been observed in netrin-1 deficient mice (Serafini et al., 1996).

The morphological defects of the DA and DB neurons in unc-44 mutants were not quantified as the ventral cord was often disorganized due to missing or mispositioned neuronal cell bodies. When commissures were observed, they appeared to reach the DNC but were often branched and took indirect paths.

Neuronal cell bodies in the ventral cord were occasionally mispositioned in unc-5, unc-6, and unc-34 mutants. In unc-40 mutants these defects were slightly more common (data not shown). These defects were not observed in unc-129, seu-1, seu-2, or seu-3 mutants.

DA and DB neurons appeared to differ in their requirement for functions of certain of these genes. Mutations in unc-34, unc-40, and unc-129 caused the DB neurons to be misguided more frequently than the DA neurons (Table 2.3). For example, in unc-34(ev553) mutants 10% (n=120) of DA neurons showed axon guidance defects compared to 45% (n=120) of DB neurons. This observation also extended to the apparent axon outgrowth defects observed in unc-5, unc-6, and unc-34 mutants (Table 2.3). For example, in unc-34(ev553) mutants, 4% (n=120) of DA neurons showed apparent axon outgrowth defects compared to 16% (n=120) of DB neurons. DA and DB neurons have similar morphologies, appearing to differ only in the decision to turn and elongate anteriorly (DA) or posteriorly (DB) upon reaching the dorsal cord and in their pattern of synapses (White et al., 1986). The differential requirement for these genes may therefore reflect underlying differences in guidance mechanisms or the presence of DA-specific genes that confer partial redundancy.
DISCUSSION

In a screen for suppressors of touch receptor axon guidance defects induced by ectopic UNC-5 expression, I have identified eight genes involved in pioneer axon guidance in C. elegans. These include four genes previously known to be required for axon guidance, unc-6, unc-40, unc-34, and unc-44, as well as four new genes, unc-129, seu-1, seu-2, and seu-3. I propose that some or all of these genes are involved in axon guidance mediated by the UNC-5 receptor.

As unc-5 is not normally expressed in the touch neurons (Hamelin et al., 1993) these genes were identified under artificially induced conditions. However, the ability of unc-5 to cause unc-6-mediated dorsal guidance of touch neuron axons indicates that some or all of the components necessary for dorsally directed axon outgrowth are present in the touch neurons. Presumably, the addition of the UNC-5 receptor to the touch neurons is sufficient to allow UNC-6 to trigger a mechanism that results in reorganization of the cytoskeleton and regulation of molecular motors involved in directed growth cone migration. The observation that ectopic unc-5 guidance requires the path cue molecule UNC-6 is consistent with this mechanism being the same or similar to that used by endogenous UNC-5 (Hamelin et al., 1993). Furthermore, the observation that suppressors of ectopic unc-5 produce, in the absence of ectopic unc-5, the same axon guidance defects that are observed in unc-5 and unc-6 mutants (see below) suggests that a common mechanism is being revealed.

Suppressors of ectopic unc-5 may impinge directly on the unc-5 pathway as is believed for the products of unc-6 and unc-40, or alternatively they may identify components of a partially redundant or parallel pathway. The incomplete penetrance of many of the axon and cell migration defects observed in mutants containing null alleles of unc-5, unc-6, and unc-40 is evidence for a second, unc-5-independent, pathway that is involved in dorsal migrations (Hedgecock et al., 1990). Mutations in an alternative pathway may cause suppression of ectopic unc-5 if signaling from both pathways contributes to a common mechanism for dorsally directed outgrowth. The observation that unc-6 is haplo-insufficient for unc-5-mediated reorientation of the touch neurons suggests that suppressors were identified in a sensitized background and is consistent with the identification of genes with weak effects on dorsal outgrowth.
This screen was limited in the sense that only those mutations that did not adversely affect the fecundity of the founder parental worm or the viability of its progeny could be detected as suppressors. As a result, even though most of the suppressors were identified multiple times, I do not believe that I have identified all of the genes that can be mutated to cause suppression of ectopic unc-5 function in the touch neurons.

**unc-40 is Required for Guidance Mediated by Ectopic UNC-5**

Vertebrate homologues of UNC-5 (UNC5H1, UNC5H2, UNC5H3 (RCM)) and UNC-40 (DCC) have been shown to bind directly to netrins, providing a mechanism for their involvement in axon guidance (Keino-Masu et al., 1996, Leonardo et al., 1997). By inference, given the phylogenetic conservation of these genes, unc-5 and unc-40 in *C. elegans* are also likely to encode netrin receptors.

The UNC-40 receptor, unlike the UNC-5 receptor, is expressed in the touch neurons and has been shown to act cell autonomously in the AVM and PVM touch neurons to guide their axons towards ventral sources of UNC-6 (Chan et al., 1996). However, the identification of unc-40 as a suppressor of ectopic unc-5 function is evidence that these receptors can also be required together to guide axons and motile cells away from ventral sources of UNC-6. This combined instructive role is consistent with the findings that unc-5 and unc-40 mutants share similar axon and cell migration defects and that unc-5 and unc-40 are co-expressed in all motile cells in which they are known to function in dorsal migrations (Hedgecock et al., 1990, Chan et al., 1996). Furthermore, the observation that the phenotypes of double and triple mutant combinations of unc-5, unc-6, and unc-40 are no more severe than the phenotypes of the single mutants suggests that these genes may act in the same pathway (Hedgecock et al., 1990).

I propose two simple, non-mutually exclusive models to account for the involvement of both receptors in mediating dorsal guidance. In the first model, UNC-5 and UNC-40 may associate laterally or multimerize on the cell membrane to form a single receptor complex that leads to localized cytoskeletal reorganization and outgrowth in an UNC-6 dependent manner. In this model the UNC-40 receptor might be recruited by UNC-5 (or vice versa) in order to respond to UNC-6 as a repulsive guidance cue. Alternatively, UNC-5 and UNC-40 may exist as separate receptor complexes that signal independently to downstream components. In this case, dorsally directed growth would require the combined input from both receptors. The important
observation that dorsal guidance defects in unc-40 mutants are much less severe than those in unc-5 mutants suggests that UNC-5 is also able to signal in an UNC-40 independent manner. The UNC-40 receptor may therefore act to increase the efficacy of UNC-5-mediated signaling, possibly by presenting UNC-6 in a more favorable context on the cell surface or modulating some common downstream component. Similar models have been advanced by Leonardo et al. (1997).

**UNC-44 (ankyrin): a Putative Link between UNC-5 and the Cytoskeleton**

*unc-44*, identified in this study as a suppressor of ectopic unc-5-mediated guidance, encodes a series of ankyrin-related proteins (Otsuka *et al.*, 1995). Ankyrins are spectrin-binding proteins that link integral membrane proteins such as ion channels and some cell adhesion molecules to the underlying actin cytoskeleton (Bennett, 1992). Mutations in *unc-44* result in axon outgrowth and guidance defects in many classes of neurons (Hedgecock *et al.*, 1985, Siddiqui and Culotti, 1991, McIntire *et al.*, 1992), consistent with the ubiquitous expression of *unc-44* in the nervous system, including the touch neurons (A. Otsuka, personal communication). However, whereas *unc-44* mutants display aberrant DA and DB motor axon morphologies, they usually do not exhibit axon guidance errors of the kind found in *unc-5* or *unc-6* mutants. Similar observations have been made concerning the DD and VD classes of neurons (McIntire *et al.*, 1992). These results may be explained if other genes, perhaps other ankyrin homologues, are able to partially compensate for the loss of UNC-44 function, or UNC-44 has other functions in addition to UNC-5 signaling, and these complicate the phenotype.

Cell adhesion molecules that have been shown to associate with ankyrinB, the major isoform of ankyrin in brain, include L1, neurofascin, and NrCAM (Davis *et al.*, 1993, Davis and Bennett, 1994). These members of the Ig superfamily of cell adhesion molecules have been implicated in multiple aspects of neurite outgrowth and guidance (Doherty *et al.*, 1995, Stoeckli and Landmesser, 1995). Similarly, an ankyrinB isoform is present in axons in the developing brain during the time of axonogenesis (Kunimoto, 1995a). This isoform of ankyrinB is able to promote neurite outgrowth in cell culture (Kunimoto, 1995b).

While *unc-5* and *unc-40* are also members of the Ig superfamily, they do not possess the conserved cytoplasmic ankyrin binding domain that is present in the cell adhesion molecules mentioned above. However, both *unc-5* and *unc-44* share a conserved death domain (DD)
sequence located at their carboxy ends, a motif first identified in proteins involved in apoptosis (Hofmann and Tschopp, 1995). DD motifs have been implicated as sites for direct interaction involving other DD containing proteins (reviewed in Hofmann and Tschopp, 1995). Therefore, it is possible that the genetic interaction observed between *unc-5* and *unc-44* is attributable to a direct physical interaction mediated by their DD motifs.

A possible model to explain the role of *unc-44* in *unc-5*-mediated guidance is that UNC-44 associates with either UNC-5 or UNC-40 (or both), linking them directly to the actin cytoskeleton. If activated UNC-5 binds UNC-44, this association may constitute part of a mechanism to generate localized actin polymerization or depolymerization and subsequent directed outgrowth of filopodia and lamellipodia. Evidence for a similar mechanism has recently been demonstrated in which activated neuroglian, a cell adhesion molecule, recruits ankyrin to the membrane, resulting in a localized distribution of ankyrin at sites of cell-cell adhesion (Dubreuil et al., 1996). Alternatively, ankyrin may simply act as a structural element or scaffold stabilizing UNC-5 or UNC-40 at specific sites and preventing its lateral diffusion on the membrane. A similar role has been proposed for ankyrin in localizing or stabilizing sodium channels at specialized axonal structures called the nodes of Ranvier (Kordeli et al., 1990).

*unc-34*, *unc-129*, *seu-1*, *seu-2* and *seu-3* encode Potential New Components of *unc-5*-Mediated Guidance

The identification in this study of *unc-34* as a suppressor of ectopic *unc-5* suggests a potential role in UNC-5-mediated signaling. This hypothesis is supported by the identification of DA and DB axon guidance defects in *unc-34* mutants that are qualitatively similar to those found in *unc-5* mutants. Similar defects in the circumferential axon growth of the DD and VD class of motorneurons have been described previously (Siddiqui and Culotti, 1991; Forrester and Garriga, 1997).

A possible role for the involvement of *unc-34* in the interpretation of environmental cues has been suggested by McIntire et al. (1992). In that study, the HSN axon in *unc-34* mutants was shown to be defective in fasiculative outgrowth along longitudinal nerve fascicles but grew to normal length when forced to elongate longitudinally along the lateral epidermis. In addition, DD and VD motorneurons in *unc-34* mutants were shown to be defective in fasiculation and longitudinal outgrowth along the DNC. This result, combined with our own, indicates that *unc-
34 is required for both fasiculative and pioneer guidance of the same axons over different substrates and along both body axes. If a common mechanism mediates these processes, it may involve a role in regulation of membrane or intracellular components that respond to environmental cues rather than one that establishes or is part of an extracellular guidance cue. If this assumption is valid and unc-34 is involved in unc-5-mediated processes, these results are consistent with a model in which UNC-34 acts at the same genetic level as the UNC-5 receptor or downstream, as part of an intracellular signaling mechanism that regulates the cytoskeleton.

Three alleles identified in this study define a new axon guidance gene designated unc-129. Mutations in this gene disrupt dorsally directed axon growth cone migrations. A significant fraction of commissural axons fail to reach their targets in the dorsal cord and instead elongate along longitudinal trajectories at various D/V axial positions. These defects, while less penetrant, are similar to those found in unc-5 and unc-6 mutants and are therefore consistent with unc-129 functioning as part of an unc-5 pathway. This idea is supported by examination of unc-5 unc-129 and unc-129; unc-6 double mutants in which these axon guidance defects do not appear qualitatively different from unc-5 and unc-6 single mutants. Furthermore, mutations in unc-129 did not reveal any ventral guidance defects like those caused by mutations in unc-6 and unc-40; therefore, like unc-5, unc-129 appears to be specifically required for dorsal migrations. However, unc-129 differs from unc-5, in that it does not appear to function in the migration of the distal tip cells, mesodermal cells that display frequent dorsal guidance defects in unc-5, unc-6, and unc-40 mutants (Hedgecock et al., 1990). Molecular analysis of unc-129 will be described in Chapter III.

I have identified mutations in three other new genes, seu-1, seu-2, and seu-3, that are strong suppressors of unc-5-induced guidance of the touch neurons. However, apart from their effect on ectopic unc-5 function, they seem to have no other readily identifiable phenotypes. These genes may therefore represent redundant components involved in unc-5-mediated signaling. The touch neurons, presumably contain at least one functional pathway that allows the UNC-5 receptor to transduce external cues into directed axon outgrowth. This pathway may be present in cells that normally require unc-5 guidance functions, such as the commissural motoneurons, but the presence of other components in these cells may render it redundant. If this assumption is valid, then the seu genes may have been difficult to identify in a conventional unc-5 enhancer or suppressor screen, as other genes may have been able to compensate for their
loss in the motorneurons. Alternatively, the *seu* mutations may represent hypomorphs of essential genes or recessive gain-of-function mutations. These possibilities would appear to be most likely for *seu-2* and *seu-3*, which were each identified by a single mutant allele.

I have shown that mutations in eight genes, which include four new genes, disrupt the ability of *unc-5* to steer touch neurons in a dorsal direction on the epidermis of *C. elegans* and in some cases cause motor axon guidance defects that are similar to a subset of defects found in *unc-5* mutants. The latter observation provides good evidence that each gene functions in a physiologically important guidance mechanism that either involves the *unc-5* signaling pathway or acts in parallel with it. Characterization of the genes identified in this study will likely lead to greater insight into the mechanisms involved in pioneer axon guidance including those mediated by *unc-5*. These insights could have broad implications given the phylogenetic conservation of other genes in the *unc-5* pathway such as *unc-6* and *unc-40*. 
CHAPTER THREE

unc-129 Encodes a Novel TGF-β Required for Axon Guidance along the Dorsoventral Axis of Caenorhabditis elegans

I did all of the experiments reported in this Chapter except for isolation of the unc-129 cDNA and sequencing the mutant alleles of unc-129 (performed by Srikant Krishna in the laboratory of R.W. Padgett). The multiple sequence alignment of the C-terminal regions of UNC-129 and other TGF-β family members was generated by Srikant Krishna using the computer programs Pileup and Prettyplot (GCG) and I made minor changes to the alignment by hand. HA-tagged UNC-129 and myo-3::UNC-129HA ectopic expression constructs were made with the technical assistance of Hong Zheng.
INTRODUCTION

Neuronal growth cones navigate by interpreting positional information in their local environment via a receptor-mediated process that induces reorganization of cytoskeletal components and directed outgrowth. Axonal growth cones and cells migrate circumferentially on the epidermis of C. elegans by expressing UNC-5 and/or UNC-40 guidance receptors on their leading edge (Leung-Hagesteijn et al., 1992; Hamelin et al., 1993; Chan et al., 1996). These receptors respond to ventral sources of UNC-6/netrin path cue molecules. UNC-5 is a transmembrane receptor with extracellular immunoglobulin (Ig) and thrombospondin (TSP) type I domains and an intracellular death domain motif (Leung-Hagesteijn et al., 1992; Hofmann and Tschopp, 1995). UNC-40 is a transmembrane receptor with extracellular Ig and fibronectin type III domains and a largely novel intracellular moiety (Chan et al., 1996). UNC-6 is a laminin-related molecule, concentrated at the ventral midline, that acts as either a chemoattractant or chemorepellent for different classes of neurons (Ishii et al., 1992; Wadsworth et al., 1996).

Genetic studies in C. elegans revealed that both UNC-5 and UNC-40 are required to repel motile cells or axons away from UNC-6, whereas UNC-40 is required to attract motile cells or axons toward ventral sources of UNC-6 (Hedgecock et al., 1990). Vertebrate homologues of these genes have been identified (reviewed in Tessier-Lavigne and Goodman, 1996), and those in which loss-of-function mutations exist appear to act in an analogous manner to guide axons along the D/V axis of the developing spinal cord (Serafini et al., 1996; Fazeli et al., 1997). The C. elegans genes unc-5, unc-6, and unc-40 therefore define components of an axon pathfinding mechanism that has been conserved across animal phyla.

unc-129 was identified in a genetic screen for suppressors of the errors in dorsal axon routing that are caused by ectopic expression of unc-5 in a subset of mechanosensory neurons in C. elegans (see Chapter II). unc-129 mutants exhibit an uncoordinated movement behaviour attributable to defects in motor axon guidance. Visualization of motor axons in unc-129 mutants with the use of neuronal specific reporter genes revealed significant axon guidance defects affecting the dorsalward projections of the DA, DB, DD, and VD classes of motorneurons. These defects are qualitatively similar to the axon guidance defects exhibited by unc-5, unc-6, and unc-40 mutants (Hedgecock et al., 1990; see Chapter II). The unc-129 gene may therefore encode a new component of the netrin/UNC-6 pathway.
In this Chapter, I report the cloning and molecular analysis of the \textit{unc-129} gene. Sequence inspection revealed that \textit{unc-129} encodes a novel member of the transforming growth factor-\(\beta\) superfamily. I show that the promoter of \textit{unc-129} is active in a subset of motoneurons and all muscle of the dorsal body wall. By further restricting expression of \textit{unc-129}, I show that expression from dorsal body wall muscle suffices for normal movement. Moreover, analysis of the effects of ectopic expression of \textit{unc-129} indicated that the spatially restricted expression of UNC-129 protein along the dorsal midline is essential for normal axon and cell migrations. I propose several models for the role of UNC-129 in mediating normal axon guidance, including its possible involvement in the netrin/UNC-6 pathway.

\section*{MATERIALS AND METHODS}

\subsection*{General Techniques and Strains}

General techniques for the culture and handling of worms have been described (Brenner, 1974). The \textit{C. elegans} Bristol (N2) stock was used as the wild type strain. The phenotypes of mutations used in this study are Daf (\textit{dauer} larva formation abnormal), Sma (small), and Unc (\textit{uncoordinated}).

The mutations used were as follows:

- Linkage group II (LG II): \textit{sma-6}(\textit{wk8}).
- LG III: \textit{daf-4}(\textit{e1364ts}).
- LG IV: \textit{daf-1}(\textit{m213ts}), \textit{unc-129}(\textit{ev554, ev557, ev566}), \textit{dpy-20}(\textit{e1282ts}).

Strains that were not isolated in the laboratory of J.G. Culotti were provided by R.W. Padgett (Rutgers University) or the \textit{C. elegans} Genetics Stock Center (University of Minnesota).

\subsection*{Positional Cloning of \textit{unc-129}}

Overlapping cosmids spanning the interval between the physically mapped loci \textit{mes-6} and \textit{fem-3} were obtained from A. Coulson (Sanger Centre). Standard germline transformation techniques (Mello and Fire, 1995) were used to generate transgenic animals carrying individual or pooled cosmids on extrachromosomal arrays. Distal gonad arms of \textit{dpy-20}(\textit{e1282}) \textit{unc-129}(\textit{ev554}) hermaphrodites were injected with a DNA mixture consisting of 20 \(\mu\)g/ml per cosmid, 20 \(\mu\)g/ml of plasmid pMH86 containing the wild type \textit{dpy-20} gene (Han and Sternberg,
1991), and pBS(SK+) (Stratagene) carrier DNA for a total concentration of 75 μg/ml. Independently derived populations that carried an inheritable extrachromosomal array were scored as rescued for the unc-129 movement defect if greater than 80% of non-Dpy worms showed wild-type movement.

General molecular biology techniques were performed according to protocols in Sambrook et al. (1989). A cloned 10 kilobase (kb) ClaI fragment (pAC2) derived from cosmid C53D6 was used to generate smaller fragments by deleting internal and flanking genomic DNA using appropriate restriction enzyme sites. These fragments were used in germline transformation experiments as part of a DNA mixture consisting of 30 μg/ml of the fragment to be tested, 20 μg/ml of pMH86, and approximately 25 μg/ml plasmid carrier DNA.

**Identification of an unc-129 cDNA and Northern Analysis**

The primer pair 5'CTAAAGTTAATCATACTCTCGG and 5'ACATTTTTACTTTATCAGTG GG was used to amplify a 1 kb fragment corresponding to the coding region for the conserved C-terminal region of unc-129. A mixed-staged cDNA library in λ Zap (Barstead and Waterston, 1989) (3x10⁵ pfu) was probed at high stringency (Church's solution, hybridization at 65°, wash at 65° in 1 mM EDTA, 40 mM Na phosphate, 1% SDS) with the 1 kb fragment. Eight independent cDNA clones in pBS SK+ (Stratagene) were sequenced at the 5' and 3' termini. A 1.5 kb cDNA coding for the expected secretion signal and carboxyl-terminal conserved region was judged to be full-length, and was subsequently sequenced multiple times on both strands using overlapping primers. Comparison with cosmid C53D6, sequenced by the *C. elegans* genome sequencing consortium, was used to verify the sequence. Sequence analysis was performed using the Wisconsin package (GCG group), and the alignment was generated by slight modifications of the output from Pileup (GCG) and Prettyplot (GCG).

The unc-129 minigene (pAC7) was constructed by subcloning a BglII-SalI fragment derived from the cDNA between the BglII-SalI sites of pAC2.

A Northern blot containing mRNA derived from mixed staged wild-type hermaphrodites (courtesy of R. Steven) was hybridized with a full length cDNA probe. The probe had been gel-purified with GeneClean (Bio101) and labeled with ³²P by a random primer protocol (Boehringer Mannheim). Hybridization was in 6x SSC, 5x Denhardt's solution, 0.5% SDS, and 100 μg/ml of
salmon sperm DNA at 65° for 12 hours. The blot was washed once in 2x SSC, 0.1% SDS at 65° for 30 minutes, rinsed three times with 2x SSC and autoradiographed.

**DNA Sequencing of unc-129 Mutant Alleles**

The primer pair 5'GCGGCGGATCCAGGAAAATTTGGAAGAGCAAGCAAG and 5'GCGGCTGCAGTTACTTTATT'CAG'TTGGTTCTATG was used to amplify the coding region of unc-129 from DNA of ev554, ev557, and ev566 mutant worms and from DNA of wild-type worms. Fragments were subcloned in pBS(SK+) (Stratagene) and sequenced from both termini in a LI-COR automated sequencer. *HindIII* deletions within the unc-129 constructs were generated to facilitate sequencing of the ev557 and ev566 alleles. DNA alterations were confirmed by manual sequencing and verified against the wild-type sequence.

**GFP Reporter Constructs and Transgenic Arrays**

A fragment of the unc-129 5' regulatory region extending 4.8 kb upstream from the initiator methionine codon was amplified by PCR from a genomic clone using the primer pair 5'CATTCTCTTGCTTGTCCCTT-CC and T7. The PCR product was subcloned directly into a T-tailed EcoRV site of pBSKS (Stratagene) (pAC8). Transcriptional reporter genes were made by inserting the full length 4.8 kb regulatory region upstream of, and in frame with, the GFP coding sequence contained in vectors pPD95.79 (with no nuclear localization sequence (NLS)) and pPD95.70 (+ NLS) (courtesy of A. Fire) to create pAC9 and pAC10 respectively. pAC9 was cut with either XbaI, SmaI, or both BstEII and XbaI and religated to generate GFP reporters containing 3 kb (pAC11), 2.5 kb (pAC12), or 3 kb with an internal deletion of 1.2 kb (pAC13) of unc-129 regulatory sequence, respectively.

Extrachromosomal arrays carrying unc-129::GFP reporters were generated by coinjecting a DNA mixture consisting of 50 µg/ml reporter construct and 25 µg/ml of pMH86 into dpy-20 hermaphrodites. pAC9, pAC10, and pAC12 containing arrays were randomly integrated by γ irradiation as described in Mello and Fire (1995) to generate evIs79, evIs78, and evIs82B respectively. GFP was visualized with fluorescence optics on a Leitz DMRB microscope.
Construction of HA-tagged UNC-129 Protein and Ectopic Expression Constructs

UNC-129 was tagged with a single copy of an epitope of hemagglutinin (HA) (YPYDVPDYASL) immediately after residue 291, which follows the putative dibasic cleavage site. Two PCRs were performed using the 5' primer ACSpeI (5'TCTACTAGTCTCTTCTCCA-AAACC) and 3' primer T7 to introduce a new SpeI site and the primer pair consisting of the overlapping 3' primers ACHA1 (5'GAGACTAGTAGAGAAAGAGAAAGATATCC-GTATGATGTCCTGATTATGCTAGCTAC-CTC) and ACHA2 (5'TATCCGTATGATGTTCTCCTGATTATGCTAGCCTCATTGCAAATGAAGAAGCTGGCT) and 5' primer T3 to introduce a SpeI site and the HA-tag. The first PCR mixture contained 20 pmol each of primers ACSpeI and T7, 5 μl of 10x PCR buffer (Perkin-Elmer), 1 μl of a 10 mM dNTP mix, 5 ng pSK20 template DNA, and 5 units of Taq DNA polymerase (Perkin-Elmer) in a total volume of 50 μl. The second PCR mixture consisted of the same except that 20 pmol of ACHA1, 2 pmol of ACHA2 (1/10 the concentration of ACHA1), and 20 pmol of T3 primers were used. PCR conditions were as follows: an initial single cycle at 95° for 5 min, followed by 36 cycles of 95° for 1 min, 55° for 30 sec, and 72° for 1 min, followed by a final single cycle at 72° for 10 min. PCR products were ligated in frame after digesting with SpeI and unique restriction sites in pBC(KS-) (Stratagene) to create pZH46. A full-length HA-containing unc-129 cDNA was released from pZH46 by restriction digest and subcloned downstream of the myo-3 promoter found in pPD96.52 (courtesy of A. Fire).

Extrachromosomal arrays carrying myo3::unc-129HA were created by coinjecting a DNA mixture consisting of 50 μg/ml of myo-3::unc-129HA plasmid and 15 μg/ml of pMH86 into dpy-20 hermaphrodites. Control injections were performed by replacing the myo-3::unc-129HA plasmid with pPD96.52. A myo-3::unc-129HA containing array was integrated at independent sites to generate evIs87A, evIs87B, and evIs87C.

Immunohistochemistry

Indirect immunofluorescence using the anti-HA mAb 12CA5 was performed as follows (Finney and Ruvkun, 1990). Mixed stage populations of worms from 100 mm diameter seeded NGM plates were collected in Eppendorf tubes and washed three times with water. Worms were fixed with 2% paraformaldehyde and 25% methanol in 1x MRWB [80 mM KCl, 20 mM NaCl, 10 mM Na2EGTA, 5 mM spermidine HCl, 30 mM PIPES, pH 7.4] at 4° for 45 min. Tubes
containing worms were then frozen in dry ice-ethanol followed by thawing at room temperature for 20 min. Worms were permeabilized further at room temperature as follows. After washing once in 1x TTB [100 mM Tris HCl pH 7.4, 1% Triton X-100, 1 mM EDTA], worms were incubated in 1x TTB containing 1% β-mercaptoethanol for 15 min, washed once with borate buffer (25 mM H₃BO₃, 12.5 mM NaOH, pH 9.5), incubated in borate buffer containing 10 mM DTT for 15 min, washed again in borate buffer, and incubated with borate buffer containing 0.3% H₂O₂ for 15 min. After permeabilization, worms were washed once in antibody buffer A (AbA; 1x PBS containing 1% BSA, 0.5% Triton X-100, 0.05% sodium azide and 1 mM EDTA). Incubation with anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim) was done overnight using a 1:1000 dilution of antibody in AbA. Worms were then washed over 6 hrs with at least three changes of AbB (1x PBS containing 0.1% BSA, 0.5% Triton X-100, 0.05% sodium azide and 1 mM EDTA) and incubated overnight with a 1:50 dilution in AbA of fluorescein-conjugated goat anti-mouse IgG (Boehringer Mannheim) secondary antibody. After washing once in AbB, worms were mounted on 1% agarose pads on glass slides, and observed with fluorescence optics on a Leitz DMRB microscope.

**Scoring Axon Guidance and Cell Migration Defects**

DA and DB motor axon morphology was visualized using the *unc-129* neuronal-specific reporter (pAC12). The mechanosensory neurons, AVM and PVM, were visualized using a *mec-4::GFP* reporter gene (M. Driscoll, unpublished materials). Integrated arrays containing pAC12 or *mec-4::GFP* were crossed into wild-type, *unc-129*, *daf-1*, *daf-4*, and *sma-6* strains, and into lines carrying *myo-3::unc-129HA* arrays by using standard genetic techniques (Brenner, 1974). The progeny of 7-8 transgenic worms were immobilized using 10 mM levamisole and transferred to a 1% agarose pad on a glass slide. GFP was visualized by epifluorescence on a Leitz DMRB microscope. DA and DB axon morphologies were examined in 30 randomly chosen late larval and adult hermaphrodites. A representative subset of DA and DB motorneurons consisting of DA3, DA4, DA5, and DA6 of the DA class and DB4, DB5, DB6, and DB7 of the DB class were scored. The data from these neurons were subsequently pooled. Motorneurons were scored as having an axon guidance defect if an axon failed to reach the dorsal cord and instead extended longitudinally along the lateral epidermis. Axons were scored as defective in ventral guidance if AVM and PVM axons were routed longitudinally instead of
ventrally along the lateral hypodermis. DTCs were scored as defective in dorsal migration if they failed to migrate dorsally and instead migrated longitudinally along the ventral side. These defects were scored under a dissection microscope by counting the characteristic ventral clear patch caused by displacement of the intestine by the ventrally misplaced gonad arm. Axon guidance defects were scored at 20° except for counts made on daf-1(m213ts), daf-4(e1364ts), and sma-6(wk8), which took place at room temperature.

RESULTS

Positional Cloning of the unc-129 Gene

The unc-129 gene was previously mapped between mes-6 and fem-3 on linkage group IV (see Chapter II). To gain insight into the molecular functions of unc-129, I initiated a molecular analysis of the gene. Cosmids containing genomic DNA spanning the mes-6 to fem-3 interval were assayed individually or as pools for the ability to complement the unc-129 mutant phenotype. Two overlapping cosmids, M01D10 and C53D6, were able to rescue the uncoordinated phenotype of unc-129 mutants after germline transformation (Figure 3.1A). The rescuing region was further demarcated by the identification of a 6.5 kb region from cosmid C53D6 that retained rescuing activity (Figure 3.1B).

This region of the genome had been previously sequenced by the C. elegans genome sequencing consortium (Wilson et al., 1994). Sequence analysis by the consortium revealed a single open reading frame (ORF) on the 6.5 kb fragment. It encodes a protein that is related to the TGF-β superfamily. A genomic fragment containing an internal 1.7 kb deletion within this ORF failed to rescue the mutant phenotype. The ORF was confirmed by the isolation and sequencing of a corresponding 1.5 kb cDNA. Translational initiation from the first methionine of this cDNA was predicted to generate a protein of 407 amino acids (Figure 3.2). The genomic structure of unc-129 consists of 5 exons, 34 bp of 5'UTR and 281 bp of 3'UTR. The 6.5 kb fragment includes at least 3 kb of 5' regulatory sequence. A minigene consisting of 4.5 kb of 5' promoter sequence fused to the unc-129 cDNA was able to rescue the phenotype of unc-129 mutants, indicating that there were no essential intronic or 3' regulatory elements. Northern analysis of wild-type mRNA revealed a single approximately 1.9 kb transcript that was consistent with the size of the cDNA (Figure 3.3).
Figure 3.1. Genetic and physical map of the unc-129 region and results of transformation rescue experiments. (A) Cosmids spanning the mes-6-fem-3 interval were assayed for unc-129 rescuing activity. A partial genetic map is indicated on top. Cosmids used in transformation experiments are indicated by solid lines. mu, map units. (B) Cosmids C53D6 and M01D10 contain unc-129 rescuing activity. The rescuing region was delimited by testing genomic subclones and an unc-129 minigene. Rescue (+), no rescue (-). The restriction map of genomic DNA and exon structure of the unc-129 gene are indicated. Solid boxes represent coding region, open box represents 3'UTR, and the direction of transcription is indicated by an arrow. The curved arrow indicates that the orientation of cosmids in (B) is reversed compared to (A). Restriction sites: AhdI (a), ClaI (c), BspMI (bm), BstEII (b), SmaI (s), XbaI (x).
A

mes-6 0.08 mu  unc-129  fem-3 0.03 mu

C33D9  B0483  D1046  C53D6  fem-3 JK2
C22G4  T28G12  C06F7  C14D9  M01D10  W05G10

B

unc-129 rescue

cosmid:
C53D6  +
M01D10  +
C53B4  -

subclone:
Clal  +
△AhdI  -
SmaI  -
Clal/BspMI  +
XbaI/BspMI  +
minigene  +

1 kb
Figure 3.2. Nucleotide sequence of the *unc-129* cDNA and predicted amino acid sequence of UNC-129. Proposed signal sequence is underlined, putative cleavage site is boxed, the carboxy-terminal domain that is most highly conserved in TGF-β family members is shaded and the conserved cysteines are highlighted. The position of introns are indicated by slashes and the termination codon is indicated by an asterisk. Potential N-linked glycosylation sites are indicated by boxed asparagines. The proposed polyadenylation signal is indicated by double underlines.
Figure 3.3. Northern blot of mRNA derived from mixed-staged wild-type hermaphrodites probed with an *unc-129* cDNA. The *unc-129* mRNA and RNA size markers (BRL) are indicated in kb.
UNC-129 Defines a New Subfamily of the TGF-β Superfamily

UNC-129 has all the hallmarks of a member of the TGF-β superfamily of dimeric proteins. These include a putative signal sequence (residues 1-26), a prodomain (residues 27-289), and a conserved C-terminal region (residues 290-407) that contains seven highly conserved cysteines involved in the formation of intra and interchain disulphide bonds (Kingsley, 1994) (Figure 3.4). Based on their extent of sequence conservation members of the TGF-β family have been grouped into several subfamilies, of which the best characterized are the TGF-β, activin, and BMP/dpp (bone morphogenetic protein/decapentaplegic) subfamilies (Heldin et al., 1997). To determine if UNC-129 could be placed in a TGF-β subfamily, the sequence of the conserved C-terminal region of UNC-129 was compared with the corresponding sequence of several members of the TGF-β superfamily (Figure 3.5). The C-terminal region of UNC-129 shared 33% amino acid identity with human BMP-7 and 24% amino acid identity with human TGF-β2. As members of different TGF-β subfamilies generally show about 30-40% amino acid identity with each other (Kingsley, 1994), this result suggested that unc-129 defined a new subfamily of the TGF-β superfamily.

A striking feature that was revealed through sequence alignment with other members of the TGF-β family was that UNC-129 lacked a sequence of residues that would normally be present between a highly conserved α-helical region (H3) and β-sheet (Figure 3.5A). Based on the crystal structures of TGF-β2 (Schlunegger and Grutter, 1992) and BMP-7 (Griffith et al., 1996), this interdomain region forms a β-turn with a solvent accessible protruding loop structure (Figure 3.5B). However, given the uncertainty with making deductions based solely on sequence alignment, the residues missing in UNC-129 may instead delete some of the residues that form the C-terminal end of the long α-helix (Figure 3.5A). Notwithstanding, the absence of this region in UNC-129 may have functional implications for protein-protein interactions.

unc-129 (ev554) and (ev557) are Putative Null Mutations

Three mutations in unc-129 were previously isolated in a genetic screen for suppressors of ectopic UNC-5 function (see Chapter II). Sequence alterations were identified in DNA obtained from unc-129 mutants. Single base-pair changes converted D327 in unc-129(ev554) and Y242 in unc-129(ev557) to stop codons (Figure 3.4). These mutant alleles are strong
Figure 3.4. *unc-129* encodes a TGF-β-related protein. The top section shows the genomic organization of the *unc-129* gene. The bottom section illustrates the structural map of *unc-129* showing the major hallmarks of a TGF-β family member. Solid boxes represent coding region, open boxes represent untranslated regions (UTR). The approximate positions of the proposed dibasic cleavage site and conserved cysteines are indicated by RXXR and C respectively. The approximate positions of amino acid substitutions in *unc-129* alleles are indicated by arrows. Asterisks indicate stop codons. Restriction sites: *Bsp*MI (bm), *Bst*EI (b), *Sma*I (s), *Xba*I (x).
Figure 3.5. Comparison of the carboxy-terminal domain of UNC-129 with members of the TGF-β superfamily. (A) Multiple sequence alignment of UNC-129 and other TGF-β family members. Secondary structure resolved from the crystal structures of TGF-β2 (Schlunegger and Grutter, 1992) and BMP-7 (Griffith et al., 1996) is aligned with the primary sequence. Asterisks indicate stop codons, box indicates region predicted to be absent from UNC-129. DPP (Drosophila), 60A (Drosophila); BMP-2 (chicken); Nodal (mouse); BMP-7 (human), MIS (human), GDF-1 (human), TGF-β2 (human), DAF-7 (C. elegans). (B) A ribbon diagram of a human TGF-β2 monomer showing region predicted to be missing in UNC-129 by a bracket. Figure B is reprinted with permission from Nature [Schlunegger and Grutter (1992)] Copyright (1992) Macmillan Magazines Limited.
candidates for molecular nulls as translation is predicted to eliminate or severely truncate the conserved C-terminal domain of UNC-129. The C-terminal domain mediates the biological functions of TGF-βs (Qian et al., 1996). The mutation in unc-129(ev566) converts D163 to an asparagine in the prodomain region. The molecular lesions in ev557, ev554, and ev566 are consistent with the severity of the mutant phenotypes that they have been previously shown to generate. The putative null alleles, ev557 and ev554, cause axon guidance defects with higher penetrance than the hypomorph, ev566 (see Chapter II).

**unc-129 Promoter Activity is Detected in Motorneurons and Dorsal Muscle**

The expression pattern of unc-129 was assessed by using unc-129::GFP transcriptional reporter genes expressed from transgenic arrays (Figure 3.6A; Table 3.1). Promoter activity was first detected at late gastrulation stage in many cells that likely included descendents of the AB and E lineages (Figure 3.6B) (Sulston et al., 1983). Only the intestinal precursor cells (from the E lineage) have been unambiguously identified at this stage. Expression continued through embryonic elongation in some of these cells. Approximately 450-520 min after the first cleavage, expression was seen in a subset of cells in the head, including in one ventral muscle in the head, and in all muscles of the dorsal body wall (Figure 3.6C,D). Between 520 min after first cleavage and hatching, GFP expression was detected in the DA and DB classes of motorneurons, excluding DA8 and DA9 (Figure 3.6E). This pattern of expression persisted into the adult stage. In addition, expression was also detected in a subset of cells in the head and in pharyngeal neurons and muscle. Of these, only interneuron I4 and muscles m5 and m8 were unambiguously identified. In late larval stages, expression was detected in the spermatheca, seam cells, CAN, PDE socket, and four cells that encircle the vulva. Among cells that expressed unc-129 promoter activity, only DA and DB motorneurons appeared to display morphological or axon guidance defects.

**UNC-129 Activity is Required in Dorsal Muscle for Normal Axon Guidance**

The expression of unc-129 in dorsal muscles and in DA and DB motorneurons suggested a connection to the phenotype of unc-129 mutants, in which DA, DB, DD, and VD motorneurons fail to reach their dorsal muscle targets (see Chapter II). To identify the cells in which unc-129 expression is required, deletions were made within the full-length unc-129 promoter and
Figure 3.6. *unc-129* promoter activity was assessed using *unc-129::GFP* transcriptional reporters expressed from transgenic arrays. (A) *unc-129::GFP* transcriptional reporter used in (B-E) compared to *unc-129* rescuing genomic DNA. (B-E) Fluorescence micrographs showing *unc-129::GFP* expression pattern at approximately (B), 280 min, (C), 430 min, (D), 550 min after first cleavage, and (E) L1 larva. Expression in dorsal body wall muscle (arrowheads in D and E) is observed before DA and DB expression. GFP expression vectors used in (B-D) contain a nuclear localization signal (NLS). The absence of a NLS in the reporter used in (E) does not allow individual muscles to be distinguished. Restriction sites: *BspMI* (bm), *BstEII* (b), *SmaI* (s), *XbaI* (x). Scale bar, 10μm.
Table 3.1. *unc-129::GFP* Promoter Activity.

<table>
<thead>
<tr>
<th>unc-129 Promoter&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Cells Expressing unc-129 Promoter Activity&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 kb <em>XbaI</em></td>
<td>expression in subset of cells during embryogenesis&lt;sup&gt;3&lt;/sup&gt;, intestinal cells, DA and DB motorneurons, dorsal body wall muscle, ventral muscle in head, subset of cells in head (variable expression), inner labial sheaths, subset of pharyngeal neurons&lt;sup&gt;3&lt;/sup&gt; (l4), subset of pharyngeal muscles (m5, m8), spermatheca&lt;sup&gt;4&lt;/sup&gt;, PDE socket cell&lt;sup&gt;4&lt;/sup&gt;, seam cells (weak)&lt;sup&gt;4&lt;/sup&gt;, four cells encircling vulva&lt;sup&gt;3,4&lt;/sup&gt;.</td>
</tr>
<tr>
<td>2.5 kb <em>SmaI</em></td>
<td>expression in subset of cells during embryogenesis&lt;sup&gt;3&lt;/sup&gt;, intestinal cells, DA and DB motorneurons, subset of cells in head (variable expression)&lt;sup&gt;3&lt;/sup&gt;, inner labial sheaths, subset of pharyngeal neurons&lt;sup&gt;3&lt;/sup&gt; (l4), spermatheca&lt;sup&gt;4&lt;/sup&gt;, PDE socket cell&lt;sup&gt;4&lt;/sup&gt;, seam cells (weak)&lt;sup&gt;4&lt;/sup&gt;.</td>
</tr>
<tr>
<td>3 kb <em>XbaI</em> with 1.2 kb <em>BstEIIΔ</em></td>
<td>expression in subset of cells during embryogenesis&lt;sup&gt;3,5&lt;/sup&gt;, intestinal cells (weak), dorsal body wall muscle, ventral muscle in head, subset of cells in head (variable expression)&lt;sup&gt;3,2&lt;/sup&gt;, subset of pharyngeal muscles (m5, m8), spermatheca&lt;sup&gt;4&lt;/sup&gt;, four cells encircling vulva&lt;sup&gt;3,4&lt;/sup&gt;.</td>
</tr>
</tbody>
</table>

<sup>1</sup>Length of *unc-129 5’* regulatory sequence beginning with the initiator methionine of *unc-129* fused in frame to GFP coding sequence found in pPD95.79.

<sup>2</sup>Bold indicates important motor axon and dorsal muscle expression (see Results).

<sup>3</sup>Cells have not been identified.

<sup>4</sup>Late larval onset of expression.

<sup>5</sup>The 1.2 kb *BstEIIΔ XbaI* promoter is active in a smaller subset of cells in the head compared to the 2.5 kb *SmaI* promoter.
fragments were identified which drove muscle- or motorneuron-specific GFP expression (Figure 3.7A). A GFP reporter containing 2.5 kb of unc-129 promoter sequence primarily expressed GFP in neurons whereas a GFP reporter containing a 1.2 kb internal deletion in the full-length promoter primarily expressed GFP in dorsal muscle (Figure 3.7B,C).

These truncated promoters were used to test whether UNC-129 expressed only in dorsal muscle or only in DA and DB motorneurons could rescue the unc-129 mutant phenotype. UNC-129 expression from dorsal body wall muscle, but not from DA and DB neurons, was sufficient to completely rescue the uncoordinated movement defect of unc-129 mutants (Figure 3.7D). Expression in dorsal muscle appeared to be the only significant difference in expression observed during the period of axon outgrowth that differed between the neuronal and muscle-specific promoter derivatives of unc-129 (Table 3.1). Expression in embryonic lineages was detected from the neuronal promoter or with both the neuronal and muscle-specific promoters, and thus probably did not account for the rescuing ability of the muscle specific promoter.

Axon Guidance in TGF-β type I and type II Receptor Mutants Appears Normal

Strains that encode mutant forms of components of TGF-β signal transduction mechanisms in C. elegans were examined for axon guidance defects by using the unc-129 motorneuron specific GFP reporter. daf-1(m213ts) and sma-6(wk8) are mutant alleles of genes encoding type I serine/threonine kinase receptors (Georgi et al., 1990; S. Krishna and R.W. Padgett, personal communication) and daf-4(e1364ts) is a mutant allele of a gene encoding a type II serine/threonine kinase receptor (Estevez et al., 1993). These mutations did not cause any defects in DA (n=120) or DB (n=120) axon guidance.

Ectopic UNC-129 Expression Causes Axon and Cell Migration Defects

To further investigate whether dorsal-specific expression of unc-129 was important for its function, I expressed unc-129 both in dorsal and ectopically in ventral rows of body wall muscle. Functional HA-tagged UNC-129 was expressed under the control of the body wall muscle-specific myo-3 (myosin) promoter (Okkema et al., 1993) from integrated transgenic arrays in three independent lines. Immunostaining with anti-HA confirmed the expression of UNC-129 in both dorsal and ventral rows of body wall muscle (Figure 3.8). Axon morphologies of the DA and DB neurons in the ectopic unc-129 expressing animals were examined using the neuronal
Figure 3.7. Potential cell non-autonomous activity of \textit{unc-129}. (A) \textit{unc-129::GFP} transcriptional reporters used in this study compared to \textit{unc-129} rescuing genomic DNA. (B, C) \textit{unc129::GFP} reporter derivatives expressed predominantly in (B) DA and DB motorneurons (arrow indicates dorsal nerve cord) and (C) dorsal body wall muscle in L1 larvae. (D) \textit{unc-129} expression from the predominantly dorsal muscle specific promoter is sufficient to completely rescue the \textit{unc-129} phenotype. Restriction sites: \textit{BspMI} (bm), \textit{BstEII} (b), \textit{SmaI} (s), \textit{XbaI} (x). Scale bar, 10\textmu m.
A

Expression
neurons muscle

| XbaI/GFP | + | + |
| Smal/GFP | + | - |
| ΔBstEII/GFP | - | + |

B

neuronal
Smal/GFP

C

muscle
ΔBstEII/GFP

D

unc-129 rescue

| XbaI | + |
| Smal | - |
| ΔBstEII | + |
| ΔBstEII/cDNA | + |
Figure 3.8. UNC-129::HA expressed ectopically using a myo-3 promoter is localized to both dorsal and ventral rows of body wall muscle. Fluorescence micrographs of the same L1 larvae transgenic for myo-3::unc-129HA stained with fluorescein-conjugated anti-HA (A) and DAPI (B). Arrowheads in (B) mark ventral cord motorneurons. Scale bar, 10μm.
specific derivative of the \textit{unc-129} promoter. Wild type worms transgenic for \textit{myo-3::unc-129HA} displayed errors in axon pathfinding in these neurons that were qualitatively similar to the defects seen in \textit{unc-129} mutants (Figure 3.9; Table 3.2). The DB neurons in particular exhibited the same penetrance of axon guidance errors as observed in \textit{unc-129} mutants. Approximately 60\% of DB motor axons were misrouted along the lateral hypodermis in both the \textit{unc-129} null mutants and the \textit{myo-3::unc-129HA} transgenic lines (Table 3.2, Figure 3.10A). Expression of UNC-129 in both dorsal and ventral muscles therefore mimics the effect of the \textit{unc-129} null mutation in causing defects in axon guidance in the DB, and, to a lesser extent, the DA neurons.

Ectopic \textit{unc-129} expression also causes axon and cell migration defects that were not observed in \textit{unc-129} null mutants (Figure 3.10B,C; Table 3.2). To determine if ventralward guidance is perturbed in animals ectopically expressing \textit{unc-129}, the AVM and PVM mechanosensory neurons were visualized using a \textit{mec-4::GFP} reporter gene (M. Driscoll, unpublished materials). These neurons are located at lateral positions and send a single process ventrally to enter and run within the VNC. In both wild type and \textit{unc-129} mutant worms, these neurons appeared normal. In ectopic \textit{unc-129} lines, however, approximately 30\% of AVM and 10\% of PVM axons failed to grow ventrally and were instead misrouted along longitudinal trajectories (Figure 3.10B; Table 3.2). Similar ventral guidance defects are also present in \textit{unc-6} and \textit{unc-40} mutants (Hedgecock \textit{et al.}, 1990). Therefore \textit{unc-129} expression from all body wall muscles perturbed guidance of axon growth cones in both dorsal and ventral directions.

UNC-129 expression from all body wall muscle also affected the dorsalward migrations of the distal tip cells (DTC), which are mesodermal cells that normally follow a C-shaped trajectory along the epidermis (Figure 3.10C; Table 3.2). All lines transgenic for the \textit{myo-3::unc-129HA} construct exhibited defects in the dorsalward component of the DTC trajectory. In these worms, a significant fraction of DTCs failed to migrate dorsally and instead migrated longitudinally at a ventral position. Dorsal migration defects displayed by the posterior DTCs (approximately 80\%) were more severe than those displayed by anterior DTCs (approximately 20\%). A similar penetrance of DTC migration defects is exhibited by \textit{unc-5} and \textit{unc-6} mutants and to a milder degree by \textit{unc-40} mutants (Hedgecock \textit{et al.}, 1990).
Figure 3.9. Ectopic expression of unc-129 in dorsal and ventral body wall muscle phenocopies the axon guidance defects of an unc-129 null mutant. Motor axons are misrouted longitudinally at lateral positions (arrows). DA and DB neurons are visualized in L4 stage animals using the neuronal unc-129::GFP reporter. (A) wild type; (B) unc-129(ev557); and (C) unc-129(+) animal carrying an integrated myo-3::unc-129HA transgene. Arrowhead indicates row of lateral seam cells. Scale bar, 50μm.
wild type

unc-129 (ev557)

myo-3::unc-129HA
<table>
<thead>
<tr>
<th>Strain</th>
<th>Dorsal Axon Guidance</th>
<th>Ventral Axon Guidance</th>
<th>Dorsal DTC Guidance</th>
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<tr>
<td></td>
<td>DA</td>
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<td>AVM</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>evIs82A (^1)</td>
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<td>0 (119)</td>
<td>-</td>
</tr>
<tr>
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<tr>
<td>ev554</td>
<td>39±5</td>
<td>62±4</td>
<td>0 (83)</td>
</tr>
<tr>
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<td>nd</td>
<td>nd</td>
</tr>
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<td>57±5</td>
<td>29±5</td>
</tr>
</tbody>
</table>

The number of axons or cells examined is indicated in parentheses. Means include ± standard error (SE) of a binomial distribution of the same sample size and observed mean (Zar, 1984).

\(^1\) evls82A [unc-129::GFP (pAC12) Dpy-20(+)]. Strains that were examined for DA and DB guidance defects were transgenic for evIs82A.

\(^2\) Strains that were examined for AVM and PVM guidance defects were transgenic for an integrated mec-4::GFP array. evIs87B transgenic animals were not scored for AVM and PVM axon guidance defects because the mec-4::GFP array could not be successfully introduced by mating.

\(^3\) The myo-3 vector containing strain is dpy-20(-) and transgenic for an extrachromosomal array consisting of pPD96.52 (myo-3 plasmid) and Dpy-20(+) This strain did not exhibit uncoordinated movement.

\(^4\) evIs87 strains are dpy-20(-) and transgenic for independently integrated arrays consisting of myo-3::unc-129HA and Dpy-20(+). These strains exhibit uncoordinated movement.

Abbreviations: nd, not determined.
Figure 3.10. Ectopic expression of *unc-129* in all body wall muscle causes axon and cell migration defects. Data from Table 3.2 is presented in the form of histograms. (A) DA and DB axon guidance defects in wild type, *unc-129* mutant, and three independent lines carrying integrated *myo-3::unc-129HA* transgenes. All strains are transgenic for the neuronal *unc-129::GFP* reporter. (B) Ventral guidance defects in the AVM and PVM neurons. Axons were scored as ventral guidance defective if AVM and PVM axons, which normally grow ventrally, were misrouted longitudinally along the lateral hypodermis. All strains are transgenic for a *mec-4::GFP* reporter. (C) DTC dorsal migration defects in wild type and *unc-5* mutants expressing *myo-3::unc-129HA* transgenes. The *myo-3* vector strain carries an extrachromosomal array containing the *myo-3* expression vector. Bars represent ±SE of a binomial distribution of the same sample size and observed mean.
% DTC migration defects

% Axon guidance defects

Panel A: Bar graph showing % Axon guidance defects with different strains.

Panel B: Bar graph showing % Axon guidance defects with different subtypes.

Panel C: Bar graph showing % DTC migration defects with different subtypes.
DISCUSSION

The TGF-β family members have been shown to play diverse roles in developmental programs such as those involved in cell growth, differentiation and pattern formation (reviewed in Kingsley, 1994; Hogan, 1996; and Mehler et al., 1997). TGF-β signaling pathways are known to mediate cell-cell interactions by regulating the expression of extracellular matrix components, cell adhesion molecules and cytokines (Baghdassarian et al., 1993; Perides et al., 1994; Takeshita et al., 1995). These functions may underlie the ability of TGF-β family members to promote cell motility. For example, TGF-β1 is known to act as a potent chemotactic factor that may be involved in inflammatory responses by attracting monocytes, neutrophils, and fibroblasts to sites of wound repair (Wahl et al., 1987; Parekh et al., 1994). In addition, decapentaplegic (dpp) and TGF-β1 have been implicated in tracheal cell migration in Drosophila and vertebrates, respectively (Boland et al., 1996; Vincent et al., 1997).

The C-terminal domain of a TGF-β consists of two antiparallel pairs of β-strands that form a β-sheet structure and a long α-helical region stabilized by intrachain disulphide bonds. The bioactive dimer is formed so that the α-helix of one monomer associates with the β-sheet of the other and the two monomers are connected via a single interchain disulphide bond (Schlunegger and Grutter, 1992). TGF-βs are synthesized and secreted in an inactive form consisting of a dimer of the C-terminal region non-covalently associated with a dimer of the prodomain (Kingsley, 1994). They are released from this latent state and thereby activated through protease action (Lyons et al., 1990) or non-enzymatically by association with TSP type I domains (Schultz-Cherry et al., 1994, 1995).

TGF-β family members perform their diverse biological functions by activating a phylogenetically conserved signal transduction pathway to control gene expression. TGF-βs bind and activate a heteromeric receptor complex consisting of two transmembrane serine/threonine receptors named type I and type II (Wrana et al., 1994; Heldin et al., 1997). These receptors activate downstream Smad signal transducer molecules that subsequently enter the nucleus to regulate expression of target genes (Heldin et al., 1997).
unc-129 Encodes a TGF-β-Related Molecule Required for Axon Guidance

I have cloned and characterized unc-129, a gene involved in the dorsal migrations of motor axon growth cones. UNC-129 contains all the conserved structural features of a TGF-β-related molecule and based on amino acid sequence comparisons appears to define a new subgroup of the TGF-β superfamily. However, a significant difference between UNC-129 and other TGF-β family members is the predicted absence of an exposed loop structure corresponding to amino acids 371-375 of human TGF-β2 (Schlunegger and Grutter, 1992).

The crystal structure of TGF-β2 reveals that the region spanning residues 371-375 is solvent accessible and combined with the sequence variance seen between different TGF-β subgroups is predicted to be a site of direct protein-to-protein interaction (Schlunegger and Grutter, 1992). Furthermore, comparison of the 3-D structures of TGF-β1 and TGF-β2 reveals a conformational difference at this site, which may, in part, be responsible for the differing receptor binding affinities and in vitro activities shown by these isoforms (Hinck et al., 1996). Structure-function analysis of TGF-β1 indicates that this loop structure participates in receptor interaction as a deletion of residues 371-375 (TGF-β2 numbering) abolishes a distinct set of TGF-β1 mediated responses, whereas it does not affect other TGF-β1 mediated responses (Qian et al., 1994). The in vitro responses of the mutated TGF-β1 appeared to mimic some of the properties of TGF-β2 suggesting a role for this region in determining receptor specificity for different TGF-β isoforms. However, the deletion mutant displayed unique binding properties that differed from both TGF-β1 and TGF-β2 (Qian et al., 1994). Subsequent studies showed that this region is not essential for receptor binding as the TGF-β1 deletion mutant retains high affinity binding to its type II receptor (Qian et al., 1996). Nevertheless, its absence in UNC-129 may have implications for receptor specificity or for the possibility of non-conventional TGF-β-protein interactions.

In C. elegans, type I receptors are encoded by daf-1 (Georgi et al., 1990) and sma-6 (S. Krishna and R.W. Padgett, personal communication) and a type II receptor is encoded by daf-4 (Estevez et al., 1993). daf-1 and daf-4 have been implicated in the regulation of dauer larvae formation while daf-4 and sma-6 function in body size and patterning of the male tail (Georgi et al., 1990; Estevez et al., 1993; Ren et al., 1996; Savage et al., 1996). As these functions appear to be normal in the putative unc-129 null mutants ev554 and ev557, UNC-129 may act in a
distinct axon guidance pathway. In addition, daf-1(m213ts), daf-4(e1364ts), and sma-6(wk8) mutants do not exhibit axon guidance defects, at least not among the DA and DB class of motorneurons, lending further support to a distinct role for unc-129 in axon guidance. It is interesting to speculate that the absence in UNC-129 of the sequence predicted to extend from the C-terminal end of α-helix H₃ to the β-turn prevents its binding to TGF-β receptors in C. elegans.

**unc-129 is Asymmetrically Expressed along the D/V Axis**

unc-129 promoter activity is primarily detected in all dorsal body wall muscle and in the DA and DB motorneurons. A complete correlation between unc-129 promoter activity in the nervous system and the motorneurons known to exhibit axon guidance errors in unc-129 mutants was not observed. Expression was not detected in the DD and VD classes of motorneurons, that, like DA and DB motorneurons, are misguided in unc-129 mutants. GFP expression from the unc-129::GFP reporter gene appears in motorneurons sometime during the 3-fold stage of embryogenesis (520 min after first cleavage-hatching), after the onset of pioneer motor axon outgrowth (480-515 min after first cleavage) towards the dorsal midline (Durbin, 1987). However, determining the onset of expression using a GFP reporter can only be a rough approximation, as there is an undetermined amount of time necessary between gene expression and chromophore activation (Cubitt et al., 1995).

Expression of unc-129 in muscle is striking as it is primarily found in the four longitudinal rows of dorsal body wall muscle but not in the four longitudinal rows of ventral muscle, excluding two ventral muscles (left/right symmetrical) in the head. Gross morphological defects in muscle were not observed in unc-129 mutants. The asymmetrical expression pattern in dorsal muscle at the 2-fold stage of embryogenesis (450-520 min after first cleavage) correlates with the onset of pioneer motor axon outgrowth (480-515 min after first cleavage) towards the dorsal midline (Durbin, 1987). The spatial and temporal pattern of expression of unc-129 is consistent with a role in dorsally directed motor axon guidance.

The asymmetric expression of unc-129 along the dorsal midline of C. elegans resembles the expression pattern of other TGF-β family members in Drosophila and vertebrates. dpp is expressed in dorsal regions of the early Drosophila embryo (Wharton et al., 1993) and TGF-β/BMP molecules are present in dorsal segments of the developing spinal cord (Liem et al.,
1995, 1997). These factors regulate gene expression to specify cell fates and patterning along the D/V axis. However, apart from UNC-129, a role for TGF-β/BMP molecules in axon guidance has not been demonstrated.

**UNC-129 Acts Cell Non-Automously**

Expression of UNC-129 from dorsal muscle, but not from DA and DB neurons, using cell-specific *unc-129* promoter derivatives, is sufficient to rescue the uncoordinated behaviour of *unc-129* mutants. These results suggest that UNC-129 may be secreted from dorsal muscle to act locally on dorsal midline cells or form a gradient along the D/V axis that either acts directly or indirectly on axonal growth cones to guide them in a dorsal direction. A caveat however, concerning this type of experiment, which is not a mosaic analysis, is that a low level of *unc-129* expression in motorneurons may have been present but simply not detected. Still, this result is consistent with the findings that *unc-129* encodes a member of a family of secreted signaling proteins, and that *unc-129* expression is not observed in all motor axons that are known to exhibit guidance defects, and with ectopic expression studies discussed below. Furthermore, a cell non-autonomous function is consistent with the initial identification of *unc-129* mutations as suppressors of ectopic UNC-5 function in six mechanosensory neurons that do not appear to express *unc-129* promoter activity.

**An Instructive Role for UNC-129 in Axon Guidance**

Ectopic UNC-129 expression from dorsal and ventral rows of body wall muscle in transgenic *myo-3::unc-129* strains causes axon guidance defects that are qualitatively similar to those exhibited by *unc-129* loss-of-function mutations. This finding indicates that the spatial distribution of UNC-129 along the D/V axis is important. However, the penetrance of defects exhibited by the DB neurons are more severe than those exhibited by DA neurons. This result parallels the finding that DB neurons are more frequently misguided than DA neurons in *unc-129* mutants and suggests that factors in addition to a spatially restricted distribution of UNC-129 contribute to DA guidance.

Ectopic UNC-129 expression also disrupts the ventral guidance of AVM and PVM axons and the dorsal migrations of the mesodermal DTC, guidance defects that are not observed in *unc-129* loss-of-function mutants. An approximately symmetric distribution of UNC-129 molecules
along the D/V axis may act to desensitize or confuse motile cells as they follow distinct guidance cues or to actively sequester or modify existing guidance cues. If UNC-129 does impinge on the netrin/UNC-6 guidance pathway as suggested by genetic studies reported in Chapter II, then the presence of ventral and dorsal guidance defects in *myo-3::unc-129* lines that are qualitatively similar to defects in *unc-6* mutants is consistent with either direct or indirect effects on UNC-6 activity. However, the finding that the penetrance of guidance defects in *myo-3::unc-129* lines or *unc-129* mutants are much less severe, especially for DA motor axons, than those in *unc-6* null mutants suggests that this mechanism would not fully abolish UNC-6 activity. Overall, these results indicate that the spatial distribution of UNC-129 is essential for normal activity, and they suggest an instructive role in axon guidance.

**Models for UNC-129 Activity**

I propose two general models that may explain the ability of *unc-129* expression from dorsal muscle to mediate dorsally directed axon guidance, one acting through conventional TGF-β serine/threonine kinase receptors, and a second in which UNC-129 acts directly on the netrin/UNC-6 pathway. As TGF-βs bind TSP type I domains, it is interesting to speculate in the following models that activation of UNC-129 may result from binding to the extracellular TSP type I domains present in UNC-5 (Leung-Hagesteijn *et al.*, 1992).

In the first model, UNC-129 may act indirectly by regulating gene expression through a conventional TGF-β signaling pathway. Three variations of this model can be envisioned. First, UNC-129 may function by inducing the expression of a substance in dorsal muscle or from the adjacent epidermis that functions as a diffusible chemoattractant or creates a more favorable migratory pathway to guide motor axons dorsally (Figure 3.11A). Alternatively, UNC-129 may repress the expression of an axon growth inhibiting or chemorepellent activity in dorsal regions. Finally, UNC-129 may act directly on TGF-β receptors expressed on the surface of growth cones to guide their migrations. In this scenario, growth cones may migrate dorsally by regulated changes in the levels or types of cell surface receptors or in cytoplasmic signaling molecules. For example, regulated changes in protein kinase A activity have been shown to act as a switch that can shift a growth cone’s turning response towards a chemotropic factor from attractive to repulsive (Song *et al.*, 1997; Ming *et al.*, 1997). At present however, no known TGF-β receptors or Smads have been demonstrated to be involved in motor axon guidance.
Figure 3.11. General models for UNC-129 involvement in axon guidance along the D/V axis of *C. elegans*. The upper and lower figures are schematics of a transverse section of a worm showing the location and trajectory of sensory and motor axons and the position of body wall muscle. (A) UNC-129 is acting indirectly by inducing the expression of another factor (chemoattractant X) that guides axons dorsally. (B) UNC-129 is acting directly as a chemoattractant to guide axons dorsally. In both models, UNC-129 is proposed to interact with the UNC-6/netrin guidance cue. For simplicity, all guidance cues are shown as smooth gradients emanating from either dorsal muscle (UNC-129, Chemoattractant X) or the ventral midline (UNC-6).
In the second model, UNC-129 may act directly with UNC-5, UNC-6, and/or UNC-40 to convey polarity information to the migrating growth cone. UNC-129 may act directly as a chemoattractant to guide motor axons dorsally (Figure 3.11B). Polarity information along the D/V axis may thus be encoded by two opposite gradients, consisting of an UNC-6 repellent ventrally and UNC-129 attractant dorsally. Both of these gradients may act independently, via distinct receptors and signaling pathways, but function together to guide motor axons dorsally. Alternatively, UNC-129 may bind to UNC-5, possibly to its TSP type I domains, and subsequently bind and modify the chemoattractant or chemorepellent properties of UNC-6. For example, at dorsal or lateral axial levels UNC-129 may synergize with UNC-6 resulting in a stronger chemoattractant that "pulls" axons dorsally or stronger chemorepellent that "pushes" axons dorsally. Conversely, UNC-129 may act as a sink for UNC-6 molecules by binding and sequestering UNC-6 in an inactive state as migrating growth cones advance dorsally. UNC-129 may in this way function as a final regulator of UNC-6 activity by acting to sharpen or establish a putative gradient of UNC-6 molecules along the hypodermis. However, the absence of more severe axon guidance defects, like those exhibited by unc-6 null mutants, in ectopic UNC-129 expressing lines suggest that UNC-6 is not entirely inactivated or that UNC-6 distribution in a gradient is not absolutely necessary for axon guidance. A partial precedent for this type of model is the sequestration and neutralization of BMP molecules by binding to the secreted proteins chordin and noggin during D/V pattern formation in vertebrates (Piccolo et al., 1996; Zimmerman et al., 1996).

The identification of unc-129 as a new member of the TGF-β superfamily provides the first direct evidence that a TGF-β is necessary for proper neuronal pathfinding. In addition, UNC-129 most likely exerts its effects, either directly or indirectly, by acting on the phylogenetically conserved netrin/UNC-6 pathway.
CHAPTER FOUR

General Discussion and Future Directions
A. A Screen for Suppressors of Dorsal Axon Guidance Caused by Ectopic UNC-5

The experiments reported in this thesis were performed in order to identify potential new components of the *unc-5* signaling pathway in *C. elegans*. The ability of ectopic UNC-5 to steer axons in a dorsal direction provides a system, albeit artificial, which is amenable to genetic dissection of UNC-5-mediated axon guidance mechanisms. I have identified eight genes in a screen for suppressors of the dorsal misrouting of touch receptor axons that is caused by ectopic expression of UNC-5. Four of these are the previously known axon guidance genes *unc-6*, *unc-40*, *unc-34*, and *unc-44* and in four others are the novel genes: *unc-129*, *seu-1*, *seu-2*, and *seu-3*. I propose that some or all of these genes may act in a physiologically important UNC-5/UNC-6 axon and cell guidance pathway. However, as these genes were identified under the artificial conditions caused by ectopic expression, their inclusion in the UNC-5/UNC-6 guidance pathway cannot be taken for granted.

**Ectopic Expression as a Tool to Study Axon Guidance**

Ectopic expression studies involve manipulating the expression of a gene in a specific temporal or spatial pattern in vivo to better understand its function. These studies are sometimes undertaken as an initial step to elucidate gene function before the identification of a loss-of-function mutation (Kitsukawa et al., 1995; Monschau et al., 1997). Ectopic expression studies may support roles for genes deduced from loss-of-function studies or reveal redundant functions that may otherwise have been undetectable (Matthes et al., 1995; Mitchell et al., 1996; Harris et al., 1996; Raghavan and White, 1997). However, all results deriving from ectopic expression experiments should be interpreted with caution as misexpression creates an artificial situation and therefore some findings may be the result of interactions that would not normally be physiologically relevant.

In *C. elegans*, ectopic expression studies to examine neuronal development have not been widely practiced, perhaps because of the nematode's limited number of cell types and cell-specific promoters. However, respecification of chemosensory function by ectopic expression of a specific olfactory receptor (Troemel et al., 1997) and of neuronal differentiation by ectopic expression of a specific transcription factor (Jin et al., 1994) has been accomplished in *C. elegans*. In addition, as discussed in Chapters I and II, expression of a single neuron-specific
growth cone receptor, UNC-5, ectopically in a set of axons and motile cells is able to alter the direction of axon and cell migration (Hamelin et al., 1993; M.W. Su and J.G. Culotti, unpublished results). These experiments demonstrate that UNC-5 is sufficient and acts instructively, by interpreting extracellular guidance cues, to guide axons and cells. This latter result provides a good example of the information that can be derived from ectopic expression studies that cannot easily be discerned by other means.

Interpretation of the Results from the Suppressor of Ectopic UNC-5 Screen

Ectopic UNC-5 expression in the touch receptor neurons is sufficient to redirect axon growth cone migration and therefore provides the basis for a screen to identify mutants that display normal touch receptor axon trajectories. Suppression of UNC-5 induced dorsal guidance in the touch neurons may result from three plausible events. First, suppression may result from interference with UNC-5 activity or localization as a secondary consequence of mutations that perturb the cytoskeleton or cellular function. Second, mutations that strengthen the normal longitudinal or ventral outgrowth of the touch neurons may override UNC-5-induced dorsal axon guidance. Finally, suppression may be the result of mutations in genuine components of the UNC-5-signaling pathway that disrupt dorsal axon guidance. Findings that are consistent with the latter possibility are the identification of known components of the unc-5 pathway, such as unc-6 and unc-40, among the suppressors and the identification of mutants that display axon guidance defects similar to those of unc-5 mutants.

unc-34 and the ankyrin proteins encoded by unc-44 have been shown to be required for general axon outgrowth and guidance of a number of neurons (McIntire et al., 1992; Otsuka et al., 1995; Forrester and Garriga, 1997). Among the new genes, only unc-129 mutants exhibit axon guidance defects. Mutations in unc-34 and unc-129 result in frequent misrouting of dorsally projecting motor axons that resembles defects in unc-5, unc-6, and unc-40. Therefore, unc-34 and unc-129 are likely to encode components that either act directly in, or indirectly impinge upon, the axon guidance mechanism encoded by the unc-5, unc-6, and unc-40 genes. The axon guidance defects in unc-44 mutants do not appear to resemble those found in unc-5 mutants, therefore its placement in an unc-5 signaling pathway cannot be assumed. As ankysins are known to link membrane proteins to the underlying cytoskeleton (Bennett, 1992), mutations in unc-44 may suppress ectopic UNC-5 function indirectly by disrupting the cytoskeleton in the
touch neurons. Nevertheless, its demonstrated involvement in axon guidance, its ubiquitous expression in the nervous system, and the presence of death domains (DD), conserved structural motifs that can mediate direct interaction involving other DD containing proteins, in both UNC-5 and UNC-44, at least provide for the possibility of a real interaction.

As seu-1, seu-2, and seu-3 mutants do not appear perturbed in dorsally directed axon guidance or have any other easily discernable visible phenotype, their involvement in unc-5-mediated dorsal guidance is uncertain. seu gene activity may be physiologically important for unc-5 mediated guidance, but is only detected under conditions of ectopic unc-5 expression in the touch axons, as seu gene activity in the motor axons may be masked by other genes. Another possibility is that the seu genes may simply encode touch neuron specific proteins that function in axon guidance or an unrelated process but have been recruited somehow by ectopic UNC-5 to redirect axons dorsally. If this is true, then the identification of the seu genes was an artifact of this particular suppressor screen.

B. UNC-129: A Role for a TGF-β-Related Molecule in Axon Guidance

Members of the TGF-β superfamily play fundamental roles in many diverse cellular and developmental processes. For example, in the vertebrate nervous system, TGF-β-related molecules act in inhibition of primary neural tissue induction during gastrulation, promotion of dorsal cell fates and pattern formation along the D/V axis of the developing spinal cord, determination of cell fate in the PNS, and the establishment of boundaries of gene expression during brain development (reviewed in Hogan, 1996 and Mehler et al., 1997). This functional diversity is likely due to the role of TGF-βs as secreted factors that bind and activate serine/threonine kinase receptors to regulate gene expression (Heldin et al., 1997). The finding that unc-129 encodes a TGF-β-related molecule represents the first functional evidence that TGF-βs also function in axon guidance. An important question remains unresolved: Does UNC-129 mediate its effects on dorsally directed axon guidance through a conventional TGF-β signaling cascade?
Tracheal Cell Migration in Drosophila: A Model for UNC-129 Action via a Conventional TGF-β Signaling Pathway

The tracheal network in Drosophila is a complex branching structure of tubes required for respiration (Klambt et al., 1992). Morphogenesis of the tracheal system involves stereotypical migrations of branches along the A/P and D/V axes. Loss-of-function mutations in the breathless (btl) and branchless (bnl) genes, which encode an FGF receptor and FGF-like ligand respectively, cause failure of branch migrations. BTL acts cell-autonomously in tracheal cells to respond to polarity information created by the dorsal and ventral distribution of BNL, which acts as a chemoattractant (Klambt et al., 1992; Sutherland et al. 1996). Interestingly, loss-of-function mutations in the DPP type I and II receptor genes thick veins (tkv) and punt exhibit migration defects similar to those of btl and bnl mutants, though restricted to the D/V axis (Ruberte et al., 1995). These findings implicate the DPP signaling pathway in tracheal cell migration.

Experiments performed by Vincent et al. (1997) demonstrate that DPP signaling controls tracheal cell migration via a combination of two mechanisms. First, DPP signaling induces the expression of BTL in dorsal regions of the embryo thereby establishing part of the guidance information required for tracheal migration along the D/V axis. Second, several observations indicate that DPP signaling acts in tracheal cells to program their responses to guidance information. Ubiquitous expression of DPP, normally expressed dorsally and ventrally of tracheal cells, or expression of a constitutively active form of the DPP receptor in tracheal cells cause failure of migrations along the A/P axis. Conversely, loss-of-function or dominant negative DPP receptor mutants specifically expressed in tracheal cells cause failure of migrations along the D/V axis. As DPP is normally expressed dorsally and ventrally, these results can best be explained if tracheal cells that receive a DPP signal are programmed, via changes in gene expression, to migrate along the D/V axis while those that do not are programmed to migrate along the A/P axis.

As cell migration mediated by DPP and FGF signaling is somewhat analogous to axon and cell migration mediated by UNC-129 and the UNC-6/netrin pathway, tracheal branch migration provides a good model for a direct or indirect role for UNC-129 in axon guidance. Like DPP signaling in tracheal cell migration, UNC-129 may induce the expression of polarity information along the D/V axis or act within growth cones to guide their migrations.
Does UNC-129 Mediate Axon Guidance via a Novel Mechanism?

Based on what is known about TGF-β-based signal transduction cascades (Wrana et al., 1994; Heldin et al., 1997), it is reasonable to think that UNC-129, a TGF-β-related molecule, should act through similar cascades. However, in the absence of empirical data indicating otherwise, the possibility that UNC-129 may be acting through a non-conventional mechanism should be considered.

Comparison of the conserved C-terminal domain of UNC-129 with the C-terminal domains of other TGF-β family members indicates that UNC-129 does not appear to belong to any particular subgroup and therefore probably defines a divergent member of the TGF-β superfamily. Indeed, UNC-129 appears unique among TGF-βs as it appears to lack a protruding loop structure formed by a β-turn that spans two well conserved TGF-β structural domains (see Figure 3.5). Structure-function analysis of TGF-β isoform mutants indicate that this region is not essential for TGF-β receptor binding (Qian et al., 1996), although it appears to be involved in determining the specificity of receptor binding (Qian et al., 1994). Therefore, one can speculate that the absence of this segment may prevent UNC-129 from recognizing the TGF-β receptors found in C. elegans.

Are there any known components of TGF-β signal transduction pathways in C. elegans that also function in axon guidance? As previously stated, the major components of a TGF-β signal transduction pathway are TGF-β ligand, a heteromeric receptor complex consisting of type I and type II serine/threonine kinase receptors, and downstream Smad molecules that act as signal transducers (Figure 4.1). Besides those in unc-129, mutations in two other genes encoding TGF-β-related molecules have been identified in C. elegans, daf-7 (Ren et al., 1996) and the BMP homologue dbl-1 (Y. Suzuki, P. Roy, J.G. Culotti, and W.B. Wood, unpublished results). daf-7 mutants are defective in regulating entry into the dauer larva state, an alternative to the normal L3 stage specialized for survival under adverse environmental conditions. dbl-1 mutants exhibit small body size and male tail patterning defects. As for receptors, only three genes encoding TGF-β receptors, daf-1, daf-4, and sma-6, have been identified in C. elegans (Georgi et al., 1990; Estevez et al., 1993; S. Krishna and R.W. Padgett, personal communication). Mutations in these genes cause defects in one or more of the control of dauer larva formation,
Figure 4.1. A simple TGF-β signal transduction pathway (Heldin et al., 1997). (A) In the absence of receptor activation by TGF-β, the heteromeric TGF-β receptor complex consisting of type I and type II serine/threonine kinases is not formed, SMAD proteins are inactive, and target gene expression is turned off. (B) TGF-β binding to type II receptor recruits type I receptor and activates it by trans-phosphorylation. Type I receptor then phosphorylates and activates Smads causing them to translocate to the nucleus where they act as transcriptional modulators to turn on target gene expression.
A

Cytoplasm

Receptors

type II

type I

Nucleus

DNA binding protein

B

TGF-β

Cytoplasm

type II

type II

type I

Nucleus

Target Gene Expression

DNA binding protein

TGF-β dimer

phosphorylation

phosphate

Smad
body size, and male tail morphogenesis. Smad mutants, such as sma-2, sma-3, sma-4, daf-3, daf-8, and daf-14 also share these phenotypes, consistent with their proposed involvement in a common pathway (Savage et al., 1996; Patterson et al., 1997; Ogg et al., 1997). However, the easily observed uncoordinated movement phenotype, like that displayed by unc-129 mutants, has not been reported among any of these TGF-β pathway mutants, suggesting that motor axon guidance is not perturbed. Moreover, unc-129 mutants do not share the phenotypes associated with known TGF-β pathway mutants.

Furthermore, considering that TGF-β signaling requires a heteromeric receptor complex consisting of a type I and a type II serine/threonine kinase receptor, a signaling pathway involving the existing TGF-β receptors in C. elegans can account for all of the functions deduced from genetic analysis. daf-1 encodes a type I receptor involved in dauer formation (Georgi et al., 1990), daf-4 encodes a type II receptor involved in dauer formation, body size, and male tail morphogenesis (Estevez et al., 1993; Savage et al., 1996), and sma-6 encodes a type I receptor involved in body size and male tail morphogenesis (S. Krishna and R.W. Padgett, personal communication). At present, no other type I or type II receptor genes have been identified in the C. elegans genome sequence, which is nearing completion (S. Krishna and R.W. Padgett, personal communication). If UNC-129 acts through a conventional TGF-β signaling pathway, then one must assume that UNC-129 receptors have not yet been identified, there is functional redundancy among known receptors, or a combination of both. The same argument can be made for the absence of uncoordinated movement phenotypes among Smad mutants.

The absence of uncoordinated phenotypes among TGF-β signaling mutants provides no obvious candidates for components of an UNC-129-based TGF-β pathway. Therefore, it is exciting to speculate that UNC-129 may act in a novel manner to guide axons through interaction with the UNC-6/netrin pathway.

C. Future Directions

I have identified six genes, unc-34, unc-44, unc-129, seu-1, seu-2, seu-3, that may represent new components of the unc-5/6/40-based mechanism for axon guidance. The genetic and molecular analysis of these genes and their products should provide new insights into the molecular mechanisms involved in growth cone and cell motility. The models of unc-129
function discussed in Chapter III include consideration of its genetic interactions with the unc-6/netrin pathway and the possibility that it may function via a conventional TGF-β signaling pathway. The identification of unc-129 mutations as suppressors of ectopic UNC-5-mediated growth cone guidance is consistent with either a direct role in the UNC-6/netrin pathway or a role in a parallel pathway of related function. Furthermore, UNC-129 may act directly as a guidance cue that provides polarity information to migrating growth cones, or indirectly, for example, by inducing neighboring cells (i.e. the epidermis) to form a guidance cue. The results of this thesis leave three important questions to be resolved: Do the genes identified in the suppressor of ectopic UNC-5 screen act in a physiologically important UNC-5/UNC-6 pathway? Does UNC-129 function through direct interactions with components of the UNC-6/netrin pathway? Does UNC-129 function through a conventional TGF-β signaling pathway? Several genetic and biochemical approaches can be attempted to address these questions.

The first question may be addressed by performing additional genetic screens to isolate new mutations in components of the unc-5 signaling pathway. In this study, I have isolated several alleles of unc-6 and unc-40 that are among the weakest so far identified for axon and cell migration defects (see Chapter II). These alleles may be used in genetic screens to isolate dominant or recessive enhancers of their weak axon guidance defects. These screens can be performed on living animals if GFP-based reporter genes, such as the neuronal unc-129::GFP reporter, are used to visualize axons. Some of the new mutations thus obtained may be in the same genes identified in this study. If this were the case, it would strengthen the argument that the genes that mutate to a Seu phenotype are physiologically relevant as gene function would have been addressed in cells that are known to require the UNC-6/netrin pathway.

Cloning and molecular analysis of unc-34 and the seu genes will also provide information from which to assess the relevance of these genes to the unc-5 signaling pathway. The identity of their gene products, their expression patterns, and their sites of action, will allow new molecular models to be created and tested. In addition, if a seu gene function appears to be restricted to the touch neurons, then further analysis of that gene as a component of the unc-5 pathway can be abandoned. Experiments to clone seu-l are in progress as I have recently rescued the seu-l mutant phenotype with a YAC subclone carrying 15 kb of genomic DNA.

Biochemical approaches to identify UNC-5 binding proteins should help elucidate the biochemical mechanism that translates extracellular guidance information into growth cone
steering and motility. UNC-5 binding proteins can be isolated by passing worm protein lysates over an UNC-5 affinity column, or alternatively, by using the cytoplasmic domain of UNC-5 as bait in yeast two-hybrid screens. In either case, a second, independent binding assay, such as co-immunoprecipitation, should be attempted to confirm the protein-protein interaction. These approaches have the potential to identify proteins that correspond to the products of genes identified in this study. For example, a finding that UNC-44 interacts directly with the cytoplasmic domain of UNC-5 would provide strong support for the models involving UNC-44 discussed in Chapter II. Affinity chromatography and/or yeast two-hybrid approaches should also be attempted to identify UNC-40 and UNC-44 binding proteins.

Understanding the molecular mechanisms involved in unc-129 signaling will require biochemical studies to identify UNC-129-binding proteins and genetics to identify suppressors and enhancers that encode additional components of the signaling cascade. For example, the identification of a type I or type II serine/threonine kinase receptor as an UNC-129 receptor or the identification of mutations in known TGF-β signaling components would provide strong evidence for a role in a conventional TGF-β pathway to regulate gene expression. Likewise, the finding that UNC-129 binds to UNC-5, possible to TSP type I repeats, and/or to UNC-6 would support a direct role in the netrin/UNC-6 axon guidance pathway.

UNC-129-binding proteins may be identified by passing labeled worm protein lysates or specific proteins over an UNC-129 affinity column. Specific protein-protein interactions like those proposed between UNC-129 and UNC-5 or UNC-129 and UNC-6 can be tested by co-immunoprecipitation or chemical cross-linking experiments. Alternatively, recombinant UNC-5, UNC-6, or UNC-129 proteins can be expressed separately on the surface of tissue culture cells. Protein interactions can then be assessed by detecting aggregation between two cell lines expressing different proteins or by detecting clustering of labeled protein on the surface of a cell expressing its putative binding partner. If proteins are found to interact, the domain that mediates the interaction may be determined by expressing constructs encoding isolated protein domains or encoding proteins with specific in frame deletions in the above binding assays.

It will also be important to determine if the expression or distribution of UNC-6 is altered in unc-129 mutants or in strains ectopically expressing UNC-129. However, assuming that an asymmetrical distribution of UNC-6 is absolutely necessary for axon guidance, then the mild axon guidance defects observed in an unc-129 mutant or ectopic unc-129 background compared
to those of unc-6 null mutants suggest that a drastic alteration in UNC-6 distribution probably does not occur. Nevertheless, the expression pattern of unc-6 in an unc-129 null mutant can be easily assessed by immunostaining wild type and unc-129(ev554) worms transformed with HA-epitope tagged UNC-6.

D. Conclusions

The unc-5, unc-6, and unc-40 genes are required to guide the circumferential migrations of pioneer axons and mesodermal cells in C. elegans. unc-5 and unc-40 encode members of a phylogenetically conserved family of guidance receptors and unc-6 encodes the defining member of the secreted, laminin-related, netrin/UNC-6 family. UNC-5 and UNC-40 are required to mediate the migration of growth cones and cells away from UNC-6, whereas UNC-40 is required to mediate migration towards ventral sources of UNC-6.

In a genetic screen for suppressors of ectopic UNC-5-mediated growth cone guidance, I have identified mutations in six genes, unc-34, unc-44, unc-129, seu-1, seu-2, seu-3, that may represent new components of the unc-5/6/40-based mechanism for axon guidance. Of these, unc-34 and unc-129 mutants display axon guidance defects that resemble those displayed by unc-5, unc-6, and unc-40 mutants and are therefore most likely to disrupt a physiologically relevant process in UNC-5-mediated guidance.

The unc-129 gene, like unc-5, is required to guide circumferential migrations of pioneer axons away from the ventral midline. I have shown that unc-129 encodes a novel member of the transforming growth factor-β (TGF-β) superfamily of secreted signaling molecules. unc-129 promoter activity is detected in a restricted pattern that includes dorsal, but not ventral, rows of body wall muscle. Furthermore, asymmetric expression of UNC-129 from dorsal body wall muscle is required for normal axon and cell migration. Thus, UNC-129 acts in a previously unknown TGF-β-mediated process in C. elegans that establishes or encodes polarity information along the D/V axis required for axon guidance.
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