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UMI
THE CLONING AND CHARACTERIZATION OF TWO semaphorin GENES IN Caenorhabditis elegans

by

Peter John Roy

A thesis submitted in conformity with the requirements for the degree of Ph.D.
Graduate Department of Molecular and Medical Genetics
University of Toronto

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THE CLONING AND CHARACTERIZATION OF TWO *semaphorin* GENES
IN *Caenorhabditis elegans*

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Graduate Department of Molecular and Medical Genetics
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Abstract

Cells and axons require molecules to guide them along stereotypical trajectories towards their target destination. Well-characterized guidance molecules such as the Netrins and the Semaphorins attract and repel migrations over both a long and short-range throughout development. While the Netrins guide the migrations of both cells and axons, the Semaphorins have been shown to act as axon guidance molecules only. Although the Semaphorins may be required for organ development, immunity, and tumor suppression, their role in these processes is not yet clear. In addition, down-stream-signaling components of the Semaphorin receptors that modulate the actin cytoskeleton during growth cone migration are still unknown.

To investigate if the Semaphorins have broad roles throughout development, I cloned and characterized two *semaphorin* genes in the nematode *Caenorhabditis elegans*. First, I have used a transgenic approach to demonstrate that *semaphorin I* may function in the development of the neuromuscular system that regulates defecation. Not only are animals carrying putative dominant-negative transgenes constipated, but *semaphorin I* reporters are also expressed in many cells that control defecation.

Second, I cloned, characterized, and created a putative null mutation in *C. elegans* *semaphorin II* (*ev574*), that failed to complement previously isolated *mab-20* mutations, for which the gene had not yet been cloned. That is, *mab-20* encodes *C. elegans* Semaphorin II. I show that *mab-20(ev574)* confers errors in axon and cell guidance during migrations. The most penetrant *mab-20* phenotypes, however, occur within the epidermis during embryonic and larval morphogenesis. These phenotypes can be explained by ectopic contact formation between a
small sub-set of epidermal cells during their migrations and rearrangements before and/or during epidermal morphogenesis.

These results show for the first time that the Semaphorins can not only guide migrating axons, but cells as well. In addition, the detailed descriptions of the semaphorin mutant phenotypes and a preliminary genetic screen have laid the foundation for further genetic screens to identify components within the Semaphorin pathway.

Finally, in pursuing loss-of-function mutations in C. elegans semaphorins, I have improved the efficiency and techniques for obtaining Tc1-transposon-mediated mutations. To date, this work has enabled the isolation of over 40 alleles for various laboratories.
To the memory of John Tom "Popo" Roy
who wondered at the majesty of nature
and showed me how to do the same
Acknowledgments

First and foremost, I would like to thank my wife, Kathleen J. Craigie, for her understanding, friendship, and love.

Next, I would like to thank my family, including my parents Jean-Robert Roy and Anne-Marie MacGregor, my grandfather John Tom Roy, my sister Carol Anne Scott and her family Michel, Larissa, and Jacob Scott, my in-laws Dr. James and Joan Craigie, and finally a inspirational man, my friend and my teacher, Dr. Thomas MacRae.

The work described herein is the product of numerous fruitful and insightful collaborations. For that, I would sincerely like to thank Hong Zheng, Dr. Charles E. Warren, Dr. Xiangmin Wang, Dr. Gratien Dalpe, Bruce Nash, Lijia Zhang, and Doug Holmyard.

This work could not have been possible without many gifts of reagents and tools from very generous members of the scientific community to whom I am grateful. In addition, I would like to thank Dr. A. Colavita for providing the neural-specific unc-129 reporter construct and strain, T. Stiernagle of the Caenorhabditis Genetics Center for providing strains. I would also like to express my appreciation to Dr. Y. Andachi and Dr. Y. Kohara for sharing unpublished results and EST fragments; to C. So and S. Chopra for helping build the Tc1 library; to all our collaborators for providing primers and requests to screen the Tc1 library and for sharing unpublished results. I would also like to thank those who have poured plates for our lab over the years, including Paul, Ona, and Chantal. Much of this work was supported by a grant from the Canadian NRC to J.G.C., and a Canadian NSERC postgraduate scholarship to me.

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Finally, I would especially like to thank Dr. Marcus Perry, Dr. Andrew Spence, and my supervisor Dr. Joe G. Culotti for many enlightening and inspirational conversations.
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Chapter One

An Introduction to Axon Guidance Molecules
1.1 A General Introduction

Without cues to guide cell and axon migrations, the invariability of form and function of individuals within a given animal species would not exist as we know it. Stereotypical cell migrations are essential to many stages of non-spongiform animal development, including gastrulation, morphogenesis, and organogenesis. In addition, billions of neurons in higher animals, and those of lower orders as well, extend axons and dendrites that make invariant trajectories and connections. We now know that the invariance of migratory paths depends on spatially and temporally regulated guidance cues. What are these cues and how do they guide migrations? Ramon y Cajal (1892) first suggested that chemotropic cues guide axons (Kennedy et al., 1994) and ever since, neuroscientists have been searching for such candidate molecules. In 1963, fish retinotectal regeneration experiments performed by R. W. Sperry extended Cajal’s ideas on chemotropism and were formalized as the chemoaffinity hypothesis. Sperry suggested that a limited number of guidance cues could provide each migrating axon with target coordinates to reach their respective destination. Although the guidance capabilities of proteins were inferred from these and a variety of other studies (Tessier-Lavigne et al., 1988; Raper and Kapfhammer, 1990; Hedgecock et al., 1990), it was not until the cloning of the unc-6 gene in C. elegans that an instructive guidance molecule was identified (Ishii et al., 1992). A plethora of guidance molecules have since been shown to fall within four broad groups, including long range attractive and repulsive cues that act via diffusion, and short range attractive and repulsive cues that act via contact between cell and substrate (Figure 1.1). Some of these cues, however, not only guide growth cones, which are specialized structures on the leading edge of migrating axons, but also have a global role in directing a large group of cell migrations. For example, C. elegans UNC-6 is not only required for the circumferential guidance of pioneer axons, but is used for the direction of mesodermal and other ectodermal cell migrations as well. Is this example the exception or the rule? A fundamental question of development and neurobiology is
whether the same cues are used repeatedly throughout development to guide migrations of different cell types.

Figure 1.1 Pioneer axon guidance cues
Illustrated is an axon that has defasciculated (yellow) from two fascicles (blue and red) and is beginning a pioneer migration. The organelle on the leading edge of the pioneer axon is the growth cone that is extending filopodia to sample the environment. Long-range repulsive cues (black circles) such as UNC-6, the Netrins, SemaphorinIII homologues, and possibly MAG may induce localized collapse of growth cone extensions at a distance from their source of expression. Similarly, short-range repulsive molecules (black ellipses) such as the Ephrins, Semaphorin I and II, and Slit can induce localized growth cone collapse, but in a contact-dependent manner. Short-range repulsive cues may be used to prevent ectopic target acquisition and to defasciculate axons at specific choice points. Short-range attractive cues (white ellipses) such as UNC-6, the Netrins, Semaphorin I, Fasciculin II, and Connectin may stabilize the adhesion between a migrating axon and its substrate in a contact dependent manner. Short-range attractive cues may be used to channel axons through otherwise non-permissive substrate, or be required for fasciculation. Finally, long-range attractive cues (white circles) such as UNC-6, the Netrins, FGFs, and possibly UNC-129 and the Neurotrophins may also stabilize growth cone filopodial adhesion to the substrate, but can do so at a distance from the source of their expression.

To address this question, I have studied Semaphorin homologues in the nematode, C. elegans. Members of the Semaphorin family of secreted and transmembrane guidance molecules are excellent candidates for playing a broad role in guiding migrations throughout development. Semaphorin mutants not only have axon guidance errors, but they also have pleiotropic developmental defects that are just beginning to be understood.

This thesis describes and discusses the characterization of C. elegans semaphorins throughout development using a transgenic and reverse genetic approach. In chapter I, I explain how a cell migrates and uses four types of guidance cues to reach a target destination. Therein, I
highlight the effectiveness of using *C. elegans* as a model to study axon guidance. Also, I argue that the Semaphorins may affect the migrations of cells other than neurons. Finally, I describe the rationale behind using *C. elegans* as a model to investigate the developmental role of a guidance molecule that is found in all animals so far investigated. Next, in chapter II, I report the results of the cloning and characterization of *C. elegans semaphorin I*, and discuss how it may play a role in the development of the neuromusculature system that controls defecation. In chapter III, I describe and discuss the results of the construction and screening of a frozen library of worms that contains Tcl transposable elements randomly inserted throughout the genome. In the last data chapter, IV, I describe and discuss the finding that *mab-20*, a mutant for which the gene had not been cloned, encodes a *C. elegans* homologue of *Drosophila semaphorin II*. Not only do *mab-20* mutants have errors in axon guidance, they have several morphological defects that occur throughout development that are attributable to errors in the guidance of epidermal cell contacts. Finally, in chapter V, I discuss the general implications of the data collected on the *C. elegans semaphorins* and propose several experiments that could be done to further our understanding of cell and axon guidance throughout development.

1.2 How Does a Cell Migrate?

In order to understand how guidance cues can direct the migration of cells or axons, it is important to know the basic mechanics of migration. Although some of the details of cell migration still need elucidation, how migration occurs at a morphological level is well understood. To migrate in any direction, a cell must coordinate three major events; extension, traction, and retraction. The first event in initiating locomotion is the extension of cytoplasmic protrusions, namely, needle-like filopodia or fan-shaped lamellipodia. The major structural component of the both extensions is filamentous (F)-actin. F-actin in filopodia is tightly bound into bundles, while in lamellipodia, F-actin is cross-linked, giving each their respective
characteristic form. Membrane protrusion and extension at the leading edge of a cell is driven through the growth of F-actin at the barbed end from a pool of monomeric (G)-actin. The barbed, or growing ends of the filaments are generally pointed in the direction of the protrusion. Caps generated by proteins such as gelsolin and CapZ (Schafer and Cooper, 1995) prevent extension of the barbed end. F-actin extension is also regulated by the availability of free G-actin, which is sequestered by proteins such as profilin and thymosin β4 (Sun et al., 1995). Controversy still remains, however, as to exactly how F-actin extension occurs. Three mechanisms have been proposed to explain microfilament extension, although none are mutually exclusive. First, Rho family GTPases have been shown to mediate F-actin uncapping, allowing growth of existing filaments (Hartwig et al., 1995). Second, uncapped barbed ends could be created by severing existing microfilaments (Carlier, 1998). Finally, microfilaments may be created de novo by nucleation complexes that include actin-related proteins Arp2 and Arp3 (Kelleher et al., 1995). Room for F-actin extension within the membrane protrusion is thought to be provided by either thermal fluctuations (the Brownian ratchet model; Peskin et al., 1993) or by a local influx of water (the cortical expansion model; Condeelis, 1993).

Accompanying the production of membrane extensions at the leading edge of the cell is the formation of new attachments to the substratum, which in turn engages cell traction (Regen and Horwitz, 1992). An important player in cell attachment is the family of integrin receptors, although members of the cadherin and immunoglobulin families may also play a role in cell adhesion during migration (Felsenfeld et al., 1994; Gumbiner, 1996). The clustering of integrins and their interaction with ligands recruits a plethora of signaling and cytoskeletal components to the adhesion complex, including focal adhesion kinase (FAK), Src kinases, Ras, Raf, and MAP kinases, tensin, α-actinin, talin, and vinculin (Yamada and Geiger, 1997). The adhesion complex works to engage a direct link between the actin cytoskeleton, integrins, and the extracellular matrix (ECM) of the substrate (Hynes, 1992). The actin cytoskeletal network is therefore fixed
relative to the ECM as the cell moves forward. Relative to the rest of the cell however, the basal F-actin moves in a retrograde direction. In contrast to membrane extension, which requires actin polymerization and is independent of myosin motors (Forscher and Smith, 1988), the forward movement of the cell depends on myosin I and II and does not require continuous actin polymerization (Lin et al., 1996). The stationary microfilament network is used as tracks by the myosins to move the cell forward relative to the substrate.

Finally, a migrating cell must disengage the cytoskeleton-integrin-ECM link in the rear end, resulting in the retraction of the trailing pole of the cell relative to the substrate. It is thought that the affinity of the integrins for F-actin decreases from front to rear (Schmidt et al., 1993), possibly by regulating the activity of the aforementioned signaling proteins within the adhesion complex (Miura et al., 1993). In addition, myosin II has been suggested to mechanically break the cytoskeletal-integrin connection in the trailing end of the cell (Jay et al., 1995). In support of this notion, rear release of a migrating cell requires Ca\(^{2+}\), which is essential for myosin activity (Maxfield, 1993) and is highest in concentration at the trailing end of the cell (Hahn et al., 1992). In contrast to the weakened intracellular actin-integrin link at the rear end of the migrating cell, the extracellular integrin-ECM affinity remains so strong that the substrate tears off fragments of integrin containing membrane at the trailing end of the advancing cell (Regen and Horwitz, 1992). In solitary migrating cells, the difference in the cytoskeletal support of the leading versus trailing end result in a larger leading edge than trailing edge. In axons, however, the weakened cytoskeletal support of the membrane at the rear end of the growth cone, in combination with microtubule bundling (Drechel et al., 1992), results in the formation of the axon tube.

The path taken by a migrating cell is dependent upon microtubule invasion of extensions that have made attachments to the substrate. When a growth cone is presented with a discrete attractive cue, microtubules will invade only the extension that contacts the cue, and therefore
predicts the path followed by the axon (Sabry et al., 1991). If the ability of microtubules to extend into the membrane protrusion is blocked, however, the path taken by an axon meanders (Tanaka et al., 1995; Challacombe et al., 1997). Microtubule reorientation and bundling near the membrane during pathfinding is dependent on F-actin (Challacombe et al., 1996). What specific components within adhesion complexes provide a signal for microtubule reorganization, however, is not known.

1.3 How Do Cues Guide Migrations?

In vitro observations made by Grandville Harrison over eighty years ago suggested that the growth cone is a highly dynamic organelle that is instrumental in leading the migration of axons (Tanaka and Sabry, 1995). How do extracellular cues influence growth cone dynamics to guide migrations? In other words, are the membrane extensions on the growth cone formed at random sites and later modulated by guidance cues, or are the sites of cellular protrusions somehow selected a priori by guidance molecules? The grasshopper limb bud has been an extremely useful tool to study the properties of growth cone dynamics in vivo. Work done by O'Connor et al. (1990) has shown that the two Ti1 pioneer growth cones can indeed extend processes randomly, given a relatively uniform substrate. Upon contact of a single filopodium with a guidepost cell, however, the growth cone grows into the filopodium that made the first contact. In addition, Myers and Bastiani (1993) have shown that the Q1 pioneer growth cone is rapidly translocated over the grasshopper midline through a process called filopodial dilation; only the growth cone extension that contacts the Q1 contralateral homologue mediates translocation. Conversely, in vitro studies have demonstrated that contact between a single filopodium and a discrete repulsive cue can mediate localized filopodial collapse and retraction, deflecting the growth cone in oblique directions via filopodia that have not collapsed (Fan and Raper, 1995).
Evidently, the modulation of apparently random growth cone extensions is the prevalent mechanism by which growth cones are guided.

The two types of guidance cues, namely attractive and repulsive, may modulate the mechanics of cellular extension at different levels. For example, ubiquitous attractive cues have inhibiting effects on axon outgrowth and extension from spinal cord or retinal explants when the cue concentration exceeds a certain threshold level (Ebondal, 1989; Serafini et al., 1994; Deiner et al., 1997; de la Torre et al., 1997). Repulsive cues, however, can induce continued growth cone collapse at any concentration above threshold levels (Raper and Kaphammer, 1990; Luo et al., 1993; Koppel et al., 1997). One model to explain the difference in the effects of attractive versus repulsive cues on neurite extension is that attractive cues stabilize the adherence of existing extensions to the substrate, while repulsive cues function to disassemble the microfilaments within extensions. If attractive cues simply increased the formation of growth cone extension through microfilament assembly, then the ability of the cue to cause neurite outgrowth from explants should not decrease upon increasing the cue concentration past the maximum effective concentration, but might be expected to remain the same. If, however, attractive cues function to stabilize the adhesion between membrane extensions and the substrate, then ubiquitously increasing the concentration of the attractive cue will increase the traction between the growth cone and substrate. Increasing the amount of attractive cue past the maximum effective concentration could make the traction between migrating growth cones and the substrate difficult for the growth cone to overcome, resulting in stationary adhesion to the substratum and the observed decrease in neurite outgrowth. Direct observation of the number and length of growth cone extensions in response to localized and ubiquitous attractive cues might help to resolve this question.
1.4 What Are the Types of Molecules that Guide Migrations?

As mentioned in the general introduction, a long list of instructive guidance cues have been described since the early 1990s. Of course, it is beyond the scope of this introduction to describe every characterized putative guidance molecule (for review see Goodman and Tessier-Lavigne, 1996). Instead, this section aims to discuss molecules that typify the four broad functional groups of guidance cues, namely long-range repellents, long-range attractants, short-range attractants, and short-range repellents. Since several types of cues have distinct and separable guidance functions that fall within the range of two or more groups, they will be repeatedly discussed within those contexts.

It should be noted that groups working on various model organisms use different conventions to name genes, RNA, and their protein products. All genes and RNAs described herein are in lower case form and italicized, whereas their respective protein products are capitalized. In the case of C. elegans acronyms, the entire three-letter code that represents the mature gene product are capitalized.

1.4.1 Long-Range Repulsive Cues

Long range repulsive cues may function to push growth cones away from the source of cue expression and/or create barriers inhospitable for migrating axons. Representatives of this group include the Semaphorins and Collapsins, UNC-6 and the Netrins, and possibly myelin-associated glycoprotein (MAG). Although fragments of recombinant MAG can repel axons in vitro (Song et al., 1998), mouse knock-outs of MAG have not been observed to have any axon guidance defects in vivo (Li et al., 1996) and will therefore not be discussed further.
The Semaphorins can Mediate Long-Range Repulsion of Vertebrate Spinal Cord Afferents

The Semaphorins are a large group of secreted and transmembrane ligands that are encoded in all metazoan genomes examined to date, including those of worms, humans, and intriguingly, mammalian viruses (as reviewed by Cook et al., 1998; Culotti and Kolodkin, 1996). Figure 1.2 illustrates the various protein domains found in Semaphorin family members, including immunoglobulin (Ig), thrombospondin (TSP), and glycosylphosphatidylinositol (GPI) anchorage domains. To date, invertebrate Semaphorins seem to be restricted to class I, which is characterized by a semaphorin domain, followed by a transmembrane domain and a short cytoplasmic tail, and class II, which are secreted and have a semaphorin domain, followed by an Ig domain, and a short carboxy(C) tail. The vertebrate Semaphorins contain representatives of all seven classes, including class III, which are similar in structure to class II but have a basic carboxy tail. This disparate class distinction suggests that the vertebrate semaphorins have evolutionarily modified the rudimentary domain structure found in invertebrates. Nevertheless, each member of the family has a conserved ~500 amino acid "semaphorin" domain that defines the family (Kolodkin et al., 1993). Since the discovery of the first member in 1992 (Kolodkin et al.), the Semaphorin family has increased to over 30 identified members, several of which are present in any one species. Several investigations have demonstrated that the Semaphorins are long-range repulsive cues used to guide axons.

During mammalian development, a large number of semaphorin family members are expressed in distinct regions (Puschel et al., 1995; Adams et al., 1996) and are thought to create domains that are inhibitory for certain axon migrations. For example, SemaIII homologues from various species including human (H-SemaIII), mouse (M-Sem D), and chick (Collapsin-1) are expressed in the ventral half of the spinal cord (Puschel et al., 1995; Messersmith et al., 1995; Shepherd et al., 1996). While neurotrophic factor 3 (NT-3)-responsive afferent axons of the dorsal root ganglia (DRG) invade the ventral half of the spinal cord, nerve growth factor (NGF)-
Figure 1.2 The Semaphorin superfamily
To date, there are seven classes of Semaphorins that are grouped according to domain structure. All members have a signal sequence (yellow box) and a semaphorin domain of about 500 amino acids (black and yellow striped box). Class I is characterized by a transmembrane (TM) domain (blue box) and a cytoplasmic tail (green box) of unknown function. Class I members include Grasshopper (G)-Sema, Drosophila (D)-Sema, Tribolium (T)-Sema, C. elegans (Ce)-Sema Ia, Ce-Semalb, Murine (M)-Sema VIa, and Rat (R)-Sema Z. Class II is secreted and has an immunoglobulin (Ig) domain (purple pac-man). Class II members include D-SemaII and Ce-SemaII. The third class of Semaphorins (III) is very similar to class II, except that it has a basic carboxy (C)-terminus that is cleaved by furin-like proteases. Members of class III include Zebrafish (Z)-Sema Z1a, M-SemaIII, A, D, E, and H, Human (H)-SemaIII, IV, A, E and F, and Collapsin 1,2,3, and 5. Class IV is similar to class I, except it contains an Ig domain between the semaphorin domain and the TM domain. Members of class IV include M-Sem B,C, F, and G, H-CD100, and Collapsin 4. Class V, whose members include M-Sem F and G, has seven thrombospondin repeats between the semaphorin domain and the TM domain. Three viral semaphorins exist, including alcelaphine herpesvirus I (AHV)-sema, vaccinia virus (A39R)-sema, and variola virus (Var V)-sema. The viral Semaphorins are essentially secreted semaphorin domains. Finally, a single glycosylphosphatidylinositol (GPI)-linked semaphorin is known to exist, called H-Sema K1, which also encodes an Ig domain. Any black box is sequence without significant homology.

Responsive afferents avoid the region of semaphorin expression, preferring to terminate migration in dorsal laminae I and II, near the dorsal horn (Figure 1.3). Puschel et al. (1995) and Messersmith et al. (1995) went on to demonstrate that recombinant SemaIII is sufficient to repel NGF responsive afferents in culture, but not NT-3 responsive afferents. Moreover, antibodies against SemaIII can block the repulsive activity of spinal cord explants on DRG sensory axons in culture (Shepherd et al., 1997). Together, these results suggest that SemaIII can instructively
guide neurons from a distance from the source of their expression via repulsion. Three additional lines of evidence support this assertion. First, Collapsin-1-coated beads placed in front of advancing DRG axons can induce localized collapse of DRG growth cones near the site of filopodial contact with the bead (figure 1.4; Fan and Raper, 1995). Upon growth cone recovery, the DRG axon trajectory is deflected from the bead, suggesting that SemalIII can steer axons. Furthermore, the growth cones of Xenopus spinal neurons turn away from microscopic gradients of SemalIII in suspension (Song et al., 1998). Second, disrupting a SemalIII receptor, called Neuropilin-1, eliminates the repulsive effects of SemalIII on NGF-responsive afferents (see below; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Finally, in mice homozygous for *semaphorinIII/D* null mutations, NGF-responsive afferent axons extend past dorsal laminae I and II, terminating near the central canal and medial ventral cord (figure 1.3; Behar et al., 1996; Taniguchi et al., 1997).

![Diagram](image)

**Figure 1.3 Semaphorin III repels NGF, but not NT3-responsive afferents**
An illustration of a transverse view of an embryonic rat day E16 spinal cord. *R-semalIII* RNA (blue) is expressed in the ventral (V) half of the cord as dorsal root ganglia (DRG) afferents are migrating into the spinal cord. SemalIII is required to repel NGF (red cell body), but not NT3-responsive (black cell body) DRG afferents (Messersmith et al., 1995; Behar et al., 1996; He and Tessier-Lavigne, 1997). The floor plate is shown in brown. D, dorsal. This figure is adapted from Messersmith et al. (1995).
The ability of the Semaphorins to repel axons during development, however, depends on more than just correct expression patterns. Indeed, class III Semaphorins including SemaD, A, E, IV, and Collapsin-1 and 2, but not class I or II Semaphorins, are post-translationally regulated by furin-like endoproteases (Adams et al., 1997). SemaIII homologues have three or more basic amino acid consensus sequences of the RXK/RR, R/KR or RXXR type that are cleaved by an endoprotease that is inhibited by decanoyl-RVKR-chloromethylketone, a furin antagonist (Vey, 1995). By mutating furin recognition sites, Adams et al. (1997) demonstrate that endoproteolytic cleavage at the fourth protease consensus sequence (PCS4), located at the extreme C-terminus, is essential for processing SemaD from an inactive form to a mature form that repels DRG axons at relatively low concentrations. In addition, cleavage at the first PCS (PCS1), located at the C-terminus of the semaphorin domain, abolishes the ability of SemaD to repel DRG axons.

Interestingly, SemaIII activity is also dependent on dimerization (Koppel et al., 1997) which is mediated by cysteine 723 (C723) on Collapsin-1 (Koppel and Raper, 1998). C723 is positioned between the Ig domain and the basic carboxy-tail of Collapsin-1, suggesting that furin-like endoproteolytic cleavage at PCS1 can release the semaphorin domains from the dimer. Furin-like proteins can therefore regulate both the activation and inactivation of SemaIII homologues. Western blot analysis revealed that furin-like processing is indeed developmentally relevant.
since the proportion of PCS1 cleaved versus non-PCS1 cleaved chick Collapsin-1 dramatically increases from E12.5 to E15.5, a period of global SemaIII down-regulation (Adams et al., 1997). Together these results suggest that furin-like endoproteases may regulate the maturation of SemaIII homologues, and inactivate SemaIII after it is no longer required.

How does the mature Semaphorin dimer communicate with a growth cone to induce localized collapse? Using an alkaline-phosphatase (AP)-tagged version of SemaIII, He and Tessier-Lavigne (1997) and Kolodkin et al. (1997) screened cDNA expression libraries in an effort to identify a surface protein that bound SemaIII. Both screens resulted in the isolation of Neuropilin-1, a highly conserved neuronal surface protein previously identified in Xenopus (Takagi et al., 1987; Fujisawa et al., 1989), chick (Takagi et al., 1995), and mouse (Kawakami et al., 1996). When ubiquitously over-expressed in mice, Neuropilin-1 can cause axon defasciculation and sprouting, among a variety of other phenotypes (Kitsukawa et al., 1995). In addition to Neuropilin-1, Kolodkin et al. (1997) cloned a second homologue called Neuropilin-2 by obtaining sequence information from a virtual human EST library that was then used to design a probe to screen a rat cDNA library. Sequence analysis predicts that following the signal sequence, Neuropilin has homology to two complement components called C1r and C1s, otherwise known as CUB domains, followed by two regions that have homology to coagulation factors V and VIII, a MAM domain, and a putative transmembrane domain (figure 1.5; Takagi et al., 1991). Although the cytoplasmic domain is highly conserved, its small size of 40 amino acids suggests that Neuropilin may function in a complex with other surface receptors.

In addition to the finding that Neuropilin-1 can bind SemaIII (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997) several lines of evidence support the idea that Neuropilin is a Semaphorin receptor. First, COS cells expressing Neuropilin-1 or 2 can specifically bind to a different spectrum of Semaphorins (Chen et al., 1997). For example, Neuropilin-1 can bind two
Figure 1.5 Semaphorin signaling
A schematic of the molecules involved in Semaphorin signaling. Most of the information presented here comes from studies of SemaIII homologues only. Before SemaIII can signal, it must be first processed from a pre-protein by furin-like proteases (Adams et al., 1997) and dimerize (Koppel et al., 1997; Koppel and Raper, 1998). Mature SemaIII dimers can bind to Neuropilin receptors (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997) and viral Semaphorins have been shown to bind to Plexin receptors (Comeau et al., 1998). Interestingly, Neuropilin-1 can bind VEGF<sup>trim</sup> (Soker et al., 1998), while Plexin can bind to itself (Ohta et al., 1995). How the Neuropilins and Plexins signal to modify the actin (●) cytoskeleton is unclear, although it is known that rac 1 (●) is required to mediate SemaIII-induced collapse (Jin and Srittmter, 1997). Plexins have a domain that shares significant homology to the sema domain (●), a TM domain (●), and a large intracellular domain of unknown function (●, Ohta et al., 1995). The Neuropilins have two CUB domains (●), two FV/VIII domains (●), a MAM domain (●), a TM domain (●) and a very short cytoplasmic tail (Takagi et al., 1991). The yellow slab represents the cytoplasmic membrane.
AP-tagged SemaIII homologues, called SemaE and Sema IV, but not *Drosophila* SemaII-AP, a class II Semaphorin, or the ectodomain of SemaVIa-AP, a class I Semaphorin. COS cells expressing Neuropilin-2 have similar affinities to the same spectrum of Semaphorins as Neuropilin-1, except that it cannot bind SemaIII-AP. In a similar set of experiments, AP-tagged versions of four SemaIII homologues found in chick, called Collapsin-1, 2, 3, and 5 can bind to Neuropilin-1 expressing COS cells with high affinity (Feiner et al., 1997). Second, as DRG afferents invade the spinal cord, *neuropilin-1* mRNA and protein is expressed in the DRG and the dorsal horn (Kawakami et al., 1995; Kolodkin et al., 1997; He and Tessier-Lavigne, 1997; Chen et al., 1997) and AP-tagged versions of Collapsin-1 and 3 can bind the dorsal columns (Feiner et al., 1997). Moreover, AP versions of the four chick SemaIII homologues bind to distinct, but overlapping regions of the developing chick spinal cord and tectum, which for the most part, express Neuropilin-1 (Feiner et al., 1997; Takagi et al., 1995). These results suggest that the ability of the Neuropilins to bind different Semaphorins depends on additional components, possibly including Neuropilin co-receptors. A fourth piece of evidence that suggests that Neuropilin is a Semaphorin receptor is that antibodies against Neuropilin-1 block both SemaIII-dependent DRG repulsion and growth cone collapse (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Finally, SemaIII was able to collapse growth cones from wild type 12.5 days post-coitus (dpc) DRG explants, but not that from *neuropilin-1* null mutant mice (Kitsukawa et al., 1997). Since *neuropilin-1* mutant embryos do not live past 12.5 dpc, it cannot be ascertained if NGF-responsive DRG afferents project abnormally past the dorsal laminae I and II, as in the *semaIII* mutants.

With numerous Semaphorins and Neuropilins expressed throughout development, how is the specificity of interaction maintained? As described above, Feiner et al. (1997) demonstrated that AP-tagged versions of Collapsin-1,2,3 and 5 bind to different, but overlapping domains of the spinal cord and optic tectum. In addition, both an AP-tagged semaphorin domain and an AP-
tagged Ig/basic carboxy tail of both Collapsin-1 and Collapsin-5 maintained the binding specificity of their respective molecules. This result is in concordance with the finding that Neuropilin can independently bind to the semaphorin domain, and the basic carboxy tail of SemaIII (He and Tessier-Lavigne, 1997). By swapping the semaphorin domains of AP-tagged Collapsin-1 and 5, Feiner et al. (1997) showed that the specificity of binding to regions of the tectum resides in the semaphorin domain, and not in the C-terminal half of the protein. In a related study, Koppel et al. (1997) narrowed down the region of the semaphorin domain responsible for binding specificity. Swapping regions of the semaphorin domain identified a 70 amino acid region at the amino (N)-terminus, from residue 166 to 235, that endows Semaphorin with specificity. Presumably, the different receptor complexes that include the Neuropilins contain components that recognize the different 70 amino acid motif of the various SemaIII homologues.

The aforementioned investigations into the role of SemaIII and Neuropilin in the guidance of spinal cord axons reveal a simple paradigm for how a repulsive guidance cue can modulate the direction of axon migration. Upon binding a mature Semaphorin dimer that may be secreted from a distant cell, a Neuropilin-receptor complex may initiate a signal cascade that ultimately reorganizes the cytoskeleton. The redistribution of cytoskeletal elements work to decrease the likelihood of further Semaphorin contact by essentially turning the growth cone away from the source of Semaphorin expression.

**UNC-6 Mediates Long-Range Repulsion from the Nematode Ventral Nerve Cord**

As mentioned above, C. elegans UNC-6 was the first characterized molecule implicated in long-range axon guidance. Numerous cells and axons in worms homozygous for null mutations in the unc-6 gene have incompletely penetrant migratory defects in both directions along the dorsoventral/circumferential axis (Hedgecock et al., 1987 & 1990). Cloning of the
unc-6 gene revealed that the predicted protein is secreted and highly related to the N-terminus of the B2 subunit of laminin, containing laminin domain VI, three EGF repeats similar to laminin domain V, and a unique carboxy (C)-terminal "netrin" domain (Ishii et al., 1992). The laminin-like structure of UNC-6 suggests that it could be incorporated into the basal lamina, upon which many cells and axons migrate circumferentially (Hedgecock et al., 1987). Indeed, studies using a functional hemaglutinin (HA)-tagged version of UNC-6 suggests that it may be incorporated into the ventral basal lamina (figure 1.6). Not only is UNC-6::HA produced by the ventral neuroectodermal cells during their lateral to ventral midline movement, which serves to cover exposed neuroblasts on the ventral surface with hypodermis, but as the worm increases in length, the ventral source of UNC-6 is supplemented by expression from the ventral nerve cords (Wadsworth et al., 1996). The embryonic migrations of several axons and ectodermal cells, together with post-embryonic migrations of additional motor axons and two mesodermal distal tip cells (DTCs) that lead the somatic gonad migration, all require unc-6 to complete their ventral to dorsal migration (Hedgecock et al., 1990). Since UNC-6 is not expressed in the dorsal half of the worm, but is expressed ventrally, it has the potential to provide polarity information to guide migrations away from the ventral midline.

Putative receptors for guidance cues have also been first described using C. elegans. Null mutations in another C. elegans mutant called unc-5 have trajectory defects only in axons and cells that make a ventral to dorsal circumferential migration (Hedgecock et al., 1990). Cloning the unc-5 gene revealed that it encodes a transmembrane protein that contains two Ig domains, two TSP type I repeats, a predicted transmembrane stretch, followed by intracellular, ZO-1, and death domains (Leung-Hagesteijn et al., 1992; M. Killeen, T. Pawson, C. Hogue, and J. Culotti, personal communication). A mosaic analysis of unc-5 revealed that the wildtype gene product is both necessary and sufficient for the ventral to dorsal guidance of the excretory canal and DTC migration (Leung-Hagesteijn et al., 1992). As expected, various unc-5 transcriptional reporters
and tagged versions of UNC-5 show expression in cells that migrate along a ventral to dorsal trajectory (M-W. Su, M. Killeen, D. Merz, and J. Culotti, personal communication). A simple model that accounts for the ventral to dorsal migratory defects of unc-5 and unc-6 mutants is that unc-5 and unc-6 are in the same genetic pathway. In other words, motile cells use the UNC-5 surface receptor to interpret the ventral UNC-6 guidance cue as repulsive, in turn orienting migrations along the ventral to dorsal axis. Two lines of evidence support this model: First, the penetrance of the unc-5(e53) and unc-6(ev400) putative null mutant phenotypes are no greater than the unc-5(e53); unc-6(ev400) double mutant (Hedgecock et al., 1990). Second, ectopic expression of UNC-5 in mechanosensory neurons forces the reorientation of migrations along the ventral to dorsal axis (Hamelin et al., 1993). The reorientation of the migratory paths taken by axons that ectopically express UNC-5 is dependent on UNC-6, without which axons extend laterally. Clearly, the ventrally expressed UNC-6 guidance cue acts in conjunction with the autonomously expressed UNC-5 transmembrane protein to steer axons dorsally and away from the ventral midline.

A third C. elegans mutant called unc-40 also has ventral to dorsal circumferential guidance defects, and encodes a putative UNC-6 receptor (Chan et al., 1996). The predicted UNC-40 protein encodes four Ig repeats, and six fibronectin type III (FNIII) repeats followed by a transmembrane domain and a 308 amino acid cytoplasmic domain of unknown homology. Like unc-5, unc-40 acts autonomously within the motile cells to steer migrations along the circumferential axis. The penetrance of the ventral to dorsal defects in unc-40 null mutants, however, is less than that in either unc-5(e53) or unc-6(ev400) mutant backgrounds, suggesting it may play only a supporting role in dorsal circumferential migration. Double mutants created between unc-40 (rh66), and unc-5(e53) or unc-6(ev400), do not significantly enhance the defects of unc-5(e53) or unc-6(ev400), respectively. Three additional lines of evidence in C. elegans suggest that UNC-40 may act in the unc-5/unc-6 pathway in a complex with UNC-5 to interpret
the UNC-6 cue as repulsive. First, functional green fluorescent protein (GFP)-tagged versions of UNC-40 are expressed in the same dorsally migrating cells that require and express unc-5 (Chan et al., 1996; M.-W. Su, M. Killeen, D. Merz, and J. Culotti, personal communication). Second, an unc-5 hypomorphic allele (e152) is predicted to generate a truncated version of UNC-5 that requires UNC-40 for its ability to partially mediate UNC-6 repulsion (Dave Merz and Joe Culotti, personal communication). Interestingly, without UNC-40, the truncated version of UNC-5 acts in a dominant negative fashion. That is, the unc-40(e1430); unc-5(e152)/+ double mutant has a similar penetrance of ventral to dorsal distal tip cell migratory defects as the unc-40(e1430); unc-5(e53) double mutant (D. Merz and J. Culotti, personal communication).

Finally, Colavita and Culotti (1998) found that the reorientation of the migratory paths taken by axons that ectopically express UNC-5 is not only dependent on unc-6, but also unc-40. Together, these results strongly imply that UNC-5 and UNC-40 may act in a receptor complex that instructs cell to migrate away from the ventrally expressed UNC-6 guidance cue.

Intriguingly, the unc-5, unc-6, and unc-40 triple mutant enhances the penetrance of the anterior DTC migratory defect of unc-6(ev400) by 14% (+/-2%), suggesting that molecules other than UNC-6 may influence ventral to dorsal guidance through UNC-5 and UNC-40 (Hedgecock et al., 1990). Besides guiding dorsally directed migrations however, UNC-40, but not UNC-5, also plays a major role in guiding migrations towards the ventral midline through attraction to UNC-6 (see the section below).

### 1.4.2 Long-Range Attractive Cues

As mentioned above, Ramon y Cajal and R.W. Sperry have long since established the idea that cell and axon guidance might require chemotactic gradients. Presumably, these hypothetical gradients would increase the likelihood that the next step of a migrating cell or axon would be closer to the source of the graded molecule, in turn, attracting the process to the target destination.
Included in the list of long-range chemoattractants are UNC-6, Semaphorin E and the neurotrophins. Homologues of UNC-6 called the Netrins also mediate both long-range attraction and repulsion. For the sake of continuity, both the long-range attractive and repulsive functions of the Netrins will be discussed in this section. In addition, a TGF-β homologue called UNC-129 may act as a long-range attractant and will be reviewed here. Although members of the neurotrophins can act as long-range chemotropic factors, including NGF, BDNF, NT-3, and NT4/5 (Song et al., 1998; Paves and Saarma, 1997), in vivo significance of this ability is not clear and will not be discussed further.

**UNC-6 Mediates Long-Range Attraction to the Nematode Ventral Nerve Cord**

Not only does UNC-6 act repulsively to drive ventral to dorsal migrations, but mutations in *C. elegans* unc-6 also confer defects in migrations towards the ventral midline (Hedgecock *et al.*, 1987 & 1990). The ventrally restricted expression pattern of UNC-6::HA (Wadsworth *et al.*, 1996) suggests that in addition to repelling migrations dorsally, UNC-6 also acts as a long-range attractive cue needed for the guidance of sensory and motor axons and other mesodermal cells during embryogenesis and larval development (figure 1.6). By ectopically expressing UNC-6 dorsally in an unc-6 mutant background, Gitai *et al.* (1998) dorsally redirected certain axons that normally migrate ventrally. Arguably, this preliminary evidence suggests that UNC-6 may act instructively. A permissive role for circumferential guidance in general, however, cannot be excluded from this experiment.

Although *unc-40* mutants have defects in ventral to dorsal migrations, the most penetrant phenotypes conferred by *unc-40* mutations are errant axon and cell trajectories that are normally directed towards the ventral midline. Most of these axons and cells are those that also have ventrally directed axon guidance defects in *unc-6* mutants. In addition, the *unc-6(ev400); unc-40(rh66)* double mutant has slightly enhanced errors in the ventral axon guidance of the
hermaphrodite specific neurons (HSNs) which control egg-laying, compared to either mutant alone (Hedgecock et al., 1990). Together with the molecular and genetic characterization of \textit{unc-40} described above, these results imply that UNC-40 is a surface receptor on cells and axons that migrate toward the ventral midline and interprets ventral UNC-6 and possibly other cues as attractive. However, since the penetrance of the ventral migratory defects in the \textit{unc-40(e1430)} null is lower that those of the \textit{unc-6(ev400)} null, it is likely that cells express additional receptors that mediate attraction to UNC-6.

Intriguingly, the ventral and dorsal guidance functions of UNC-6 are genetically separable; \textit{unc-6(ev437)}, a revertant of \textit{unc-6(ev400)} in domain VI, mainly has ventral guidance defects, while four other mutations in the second EGF repeat of domain V cause mainly dorsal guidance defects (Wadsworth et al., 1996). These observations suggest a paradigm whereby domain VI of UNC-6 interacts with UNC-40 to mediate ventral to dorsal trajectories, while domain V interacts with UNC-5 and possibly UNC-40 to mediate dorsal to ventral guidance.

The studies of \textit{unc-5}, \textit{unc-6}, and \textit{unc-40} in \textit{C. elegans} have first demonstrated how a distinct molecular cue can guide migrations \textit{in vivo}. However, there are still many questions unanswered. For example, does UNC-6 provide instructive information or is it only permissive to circumferential guidance? Is there an UNC-6 gradient along the ventral to dorsal axis? Does UNC-6 directly guide migrating axons and interact with their putative receptors, UNC-5 and UNC-40? Surprisingly, the \textit{unc-5}, \textit{unc-6} and \textit{unc-40} paradigm is evolutionarily conserved from worms to humans, enabling studies in other systems to address questions raised by guidance studies with \textit{C. elegans} (figure 1.6).
The Netrins can Mediate Long-Range Attraction of Commissural Neurons to the Vertebrate Floor-Plate

It was noted long ago that the commissural neurons of the dorsal vertebrate spinal cord extend processes along a stereotypic trajectory to a unique locale at the ventral midline of the spinal cord called the floor plate (Ramon y Cajal, 1909). Experiments whereby floor plate explants were co-cultured with dorsal spinal cord explants revealed that the floor plate secreted a chemoattractive factor(s) that may play a role in the stereotypical migrations of the commissural axons initially described by Ramon y Cajal (Tessier-Lavigne et al., 1988). Surprisingly, two
isolated proteins that mimic the chemoattractive characteristics of the floor plate in explant assays are both homologues of UNC-6 (Serafini et al., 1994; Kennedy et al., 1994)! They are named Netrin-1 and Netrin-2 after the Sanskrit word "netr", meaning one who guides. The explant assays not only demonstrate that the COS-secreted Netrins can diffuse several hundred micrometers to induce commissural axon outgrowth, but that they can also reorient the trajectories of these axons. These results suggest that the Netrins can act as instructive migratory cues. In support of this conclusion, pan-neuronal and pan-muscle ectopic expression of Drosophila NetrinA and NetrinB in a wildtype background results in numerous migration defects of CNS and PNS axons (Mitchell et al., 1996; Harris et al., 1996). In wild type flies, these neurons express Frazzled, an UNC-40 homologue that is required to guide axons towards a source of Netrin (Kolodziej et al., 1996). If the Netrins were only permissive to migration and not instructive, then the trajectories of these axons would not be affected by ectopic Netrin expression.

The expression pattern of the netrins in the chick spinal cord is consistent with their role in commissural axon attraction to the floor plate (Kennedy et al., 1994). During commissural axon migration in the chick, netrin-1 RNA is highly expressed in the floor plate cells, while netrin-2 RNA is expressed at a lower levels in the ventral two thirds of the spinal cord, except for the floor plate. Although not conclusive, these results suggest that Netrin protein can act at a distance from the source of expression in vivo and that it may be graded along the dorsoventral axis of the chick spinal cord.

In the mouse spinal cord, Netrin-1 is expressed in a pattern that is equivalent to the combined expression pattern of both chick netrins (Serafini et al., 1996). As expected, hypomorphic mutations in mouse netrin-1 result in spinal cord guidance defects that are specific to the commissural axons (Serafini et al., 1996). Mutants also have guidance defects in other brain commissures resulting in the lack of the corpus callosum, the hippocampal commissure, the
anterior commissure, and the presence of an ectopic commissure near the roof of the fourth ventricle at the hindbrain-midbrain junction. The netrin-1 mutants also lack pontine nuclei that are comprised of cells that migrate early in embryogenesis, demonstrating that Netrin-1, like UNC-6, may also be able to guide cell migrations in addition to axons.

All of the genetic and culture experiments so far described only infer that Netrin/UNC-6 is a guidance cue that directs cell and axon migrations. It is possible that Netrin/UNC-6 simply changes the fate of cells that then secrete an unknown circumferential guidance cue. That Netrins directly guide individual axons was shown by de la Torre et al. (1997) using pipettes filled with Netrin-1 as a point source to change the trajectory of Xenopus retinal ganglion cell (RGC) axons by as much as 20 degrees in culture. The turning of RGC axons towards Netrin-1 could be abolished through the addition of antibodies against Netrin-1 to the culture.

In addition to UNC-6, the UNC-40 and UNC-5 receptors are also evolutionarily conserved. deleted in colorectal cancer (dcc), a gene previously characterized for putative tumor suppressor activity (Fearon et al., 1990), is a vertebrate UNC-40 homologue (Chan et al., 1996). While dcc mutants have no preponderance towards tumor formation or gut malformations (Fazeli et al., 1997), four lines of evidence suggest that Dcc mediates axon attraction to the Netrins. First, Netrin-1 binds to COS cells that express Dcc (Keino-Masu et al., 1996). Second, Dcc is expressed on axon tracts that have migratory defects in murine netrin-1 hypomorphs, including the commissural spinal cord neurons, the anterior commissure, and the optic chiasm (see section on short range attraction below for further details; Keino-Masu et al., 1996). Third, mice homozygous for a dcc null mutation have nearly identical defects as netrin-1 hypomorphs including errant commissural axon projections to the floor plate, absent corpus callosum, hippocampal commissure, and pontine nuclei, and a severely reduce anterior commissure (Fazeli et al., 1997). Finally, antibodies against Dcc block floor-plate and Netrin-1 induced outgrowth of commissural neurons from spinal cord explants (Keino-Masu et al., 1996). In addition, the
antibodies also block RGC axon chemoattraction towards Netrin-1 (de la Torre et al., 1997). Together these results strongly suggest that Dcc mediates an attractive response of axon and cell migrations towards a source of Netrin.

Besides Dcc, a second vertebrate UNC-40 homologue called Neogenin exists (Chan et al., 1996). Neogenin is widely expressed in both neural and non-neuronal tissues during the time of axon outgrowth (Vielmetter et al., 1994). If and how Neogenin functions in neural development, however, is not known and will not be discussed further.

Along with the two homologues of UNC-40, vertebrates have at least four homologues of *C. elegans* UNC-5 called UNC5H1, UNC5H2, UNC5H3, formerly known as Rcm (Leonardo et al., 1997), and UNC5H4 (Stein et al., 1998). Mice mutant for UNC5H3 have rostral cerebellar malformations that result from ectopic rostral migratory defects of granule cells (Ackerman et al., 1997; Przyborski et al., 1998). Netrin-1 expression delineates a rostral-caudal barrier that is consistent with a role in repelling UNC5H3-expressing granule cells that would otherwise migrate past the barrier (Przyborski et al., 1998). In support of this model, Netrin-1 can bind to COS cells that express UNC-5 homologues, including UNC5H3 (Leonardo et al., 1997). In addition, Hong et al. (1998) demonstrate that *Xenopus* spinal cord axons transgenic for *C. elegans* UNC-5, or vertebrate UNC5H1 or UNC5H2 are repelled from a Netrin-1 point source. Moreover, axons expressing a chimeric receptor consisting of the NGF ectodomain and the UNC5H2 intracellular domain are repelled by Netrin. Interestingly, this repulsion is Dcc-dependent, since the Netrin-1 induced repulsion is blocked by Dcc antibodies. This result is in concordance with the preliminary finding that the cytoplasmic domain of Dcc can directly bind to the cytoplasmic domain of UNC5H4 as demonstrated in the yeast two hybrid system and by immuno-precipitation experiments (Stein et al., 1998). *In vivo*, however, it is unlikely that Netrin-1 is the sole cue guiding cerebellar granule cells nor is it likely that Dcc is essential for granule cell response to Netrin-1 since neither netrin-1 hypomorphs nor dcc nulls have cerebellar
malformations (Serafini et al., 1996; Fazeli et al., 1997). Likewise, *in vitro* studies have demonstrated that Netrin-1 can repel spinal trochlear motor axons over a long-range (Colamarino and Tessier-Lavigne, 1995) but netrin-1 mutants have no trochlear axon guidance defects (Serafini et al., 1996). These results, together with the incomplete penetrance of some mutant phenotypes of *unc-5, unc-6*, and *unc-40* and that of the *Drosophila netrinA* and *netrinB* suggest additional cues that guide dorsoventral and CNS migrations.

**UNC-129 may act as a Long-Range Attractant to Guide Motoraxons to the Nematode Dorsal Nerve Cord**

In an experiment designed to identify new molecules required for circumferential axon guidance, Colavita and Culotti (1998) screened for mutant genes that suppress ectopic axon migration towards the dorsal midline. As previously discussed, mechanosensory (touch) neurons reorient lateral and ventral migratory trajectories dorsally upon ectopically expressing UNC-5 (Hamelin et al., 1993). The ectopic migration depends not only on the presence, but also on the amount of UNC-6. For example, animals heterozygous for *unc-6* mutations suppress or prevent ectopic, but not wildtype migration towards the dorsal midline (Colavita and Culotti, 1998). The ectopic dorsal migration of the touch neurons is therefore more sensitive to the perturbation of guidance molecules than are ventral to dorsal migrations in the wildtype. Thus, screening for mutants that suppress ectopic dorsal migration may reveal genes that are partially redundant or that only have weak effects on migration, in addition to genes essential for circumferential guidance. In this way Colavita and Culotti (1998) identified eight genes required for ectopic dorsal migration of the touch neurons, including *unc-6, unc-40*, and a novel homologue of TGF-β, called *unc-129* (Colavita et al., 1998). While an *unc-129* putative null mutation nearly completely suppresses ectopic migration towards the dorsal midline, motor axon trajectories are less, albeit significantly affected. Incomplete penetrance of motor axon guidance to the dorsal
midline is the only phenotype of unc-129 null mutants in a wildtype background so far described. These results suggest that unc-129 may have a specific, but non-essential or redundant role in circumferential guidance. Whether or not unc-129 acts in the unc-6 or a parallel pathway cannot be genetically ascertained, since unc-5 and unc-6 null mutations have nearly complete penetrance of motor axon migratory defects towards the dorsal midline. Double mutants between unc-129 and unc-6 are therefore uninformative.

GFP reporters under the control of the 5’ regulatory region of unc-129 are expressed in the body wall muscles of both dorsal quadrants, the DA and DB motor neurons that have migratory defects in unc-129 mutants, and other cells (Colavita et al., 1998). Expression in only the dorsal body wall muscles is sufficient to rescue the axon guidance defects of the DAs and DBs, suggesting that UNC-129 may act as a diffusible chemoattractant for motoraxon guidance toward the dorsal side. Ectopic expression of UNC-129 in the ventral muscle quadrants, however, results in errors not only in the DA and DB motor axon trajectories, but also in migrations not affected in unc-129 mutants, such as the touch axons and the distal tip cells. Since migrations are reoriented towards the source of ectopic UNC-129 in a wildtype background, UNC-129 must act instructively to orient circumferential migrations. It is unknown if UNC-129 acts directly on axons to modulate the direction of migration, a function that until recently was unprecedented for the TGF-βs. Preliminary evidence suggests that BMP-7 and BMP-4, two TGF-β homologues, are expressed by the roof plate of the vertebrate spinal cord and can collapse commissural axons in culture, suggesting that they may contribute to the initial Netrin-independent ventral orientation of commissural migrations (Augsburger et al., 1998; Butler et al., 1998). Alternatively, UNC-129 may indirectly regulate the expression of a guidance molecule(s) by controlling the fate of neighboring cells, a role that is more consistent with the well characterized functions of TGF-βs (Padgett et al., 1998). The wildtype expression
patterns of *unc-5*, *unc-6*, and *unc-40*, however, are not observed to change in an *unc-129* mutant background (Colavita *et al.*, 1998).

**SemE, a Semaphorin III Homologue, is a Bifunctional Guidance Cue**

Similar to the bifunctional guidance properties of UNC-6 and the Netrins, Semaphorin family members may also be able to repel and attract growth cones depending on the axon type. For example, both SemaIII homologues SemD and SemE have been demonstrated to repel sympathetic ganglion axons from chick explants when secreted from 293 cells (Adams *et al.*, 1997). In a separate study, suspended SemD, but not SemE, was able to collapse cortical axons (Bagnard *et al.*, 1998). The authors went on to show that 293-secreted SemE attracts cortical axons from explants, while SemD repels them. In addition, when given the choice of migrating on membrane bound SemE, SemD, or control 293 cell membranes, cortical axons prefer SemE at least 3.5 times as much as the other substrates. Together these results suggest that SemE is repulsive to sympathetic ganglion cells, but attractive to cortical axons. What is the *in vivo* significance of these results if any? Corticofugal axons extend from the cortical plate deep into the intermediate zone of the cortex, and terminate before they reach the sub-ventricular zone. Intriguingly, *semD* mRNA is expressed in the sub-ventricular zone and the deeper ventricular zone, while *semE* mRNA is only expressed in the former (De Carlos and O'Leary, 1992; Bagnard *et al.*, 1998). The localization of AP-tagged versions of SemD and SemE is coincident with the cortical axons in the intermediate zone and the mRNAs of neuropilin-1 and neuropilin-2 (Bagnard *et al.*, 1998). It is plausible that SemE attracts the cortical axons ventrally into the terminal zone while SemD prevents their further advancement into the ventricular zone and beyond. To add another layer of complexity, preliminary evidence from Takahashi *et al.* (1998) indicates that SemD is an agonist of Neuropilin-1, while SemE is an agonist of Neuropilin-2, but an antagonist of the SemD interaction with Neuropilin-1. Together these results suggest the
interesting paradigm whereby SemE may attract corticalfugal axons via Neuropilin-2 and by antagonizing the Neuropilin-1 mediated SemD repulsion. However, as the cortical axons approach increasing concentrations of SemD emanating from the two striata, SemE attraction can no longer overcome SemD repulsion, resulting in the migration of the axons terminating in the intermediate zone. Investigation of this hypothesis will require a detailed study of the corticalfugal migrations in mice mutant for neuropilin-1 (Kitsukawa et al., 1997), semD (Behar et al., 1996; Taniguchi et al., 1997), and semE (Feiner et al., 1998).

1.4.3 Short-Range Attraction

Contrary to long-range cues that may diffuse over tens of cell diameters and provide a polar axis upon which migrations may be oriented, short-range cues are thought to remain cell-associated. The function of a short-range guidance molecule is to thereby grant or refute further contact in an instructive manner. As will be discussed, a short-range cue may function as an attractant and a repellent, not unlike guidance molecules that direct migrations from afar. The only difference between the nature of a short or long-range cue may therefore be the ability of the molecule to diffuse. While it is difficult to assess whether or not a molecule has diffused in vivo, genetic analysis substantiates the spatially restricted action of numerous attractive cues over a short range. These cues can be divided into three groups based on function, including those considered traditional axon guidance molecules, such as UNC-6, NetrinA/B, Netrin-1, and Semaphorin I, and those considered instrumental in axon fasciculation and defasciculation such as Fasciclin II, Connectin, Beat and Side. The third group consists of molecules whose involvement in axon guidance is probably limited to discrete cell contact and target selection, such as Fasciclin III (Chiba et al., 1995; Kose et al., 1997), and Neuromusculin (Kania et al., 1993), and reiterate concepts that will be described for the two previous groups and will therefore not be discussed further.
**UNC-6 and UNC-6 Homologues may Direct Short-Range Migrations**

The detailed description of the expression pattern of HA-tagged UNC-6 in *C. elegans* implicates the cue in the short-range guidance of numerous migrations (Wadsworth *et al.*, 1996). For example, the pioneering migrations of AVA and AVB around the ventral and lateral sides of the presumptive nerve ring and the migration of labial axons into the nerve ring occur on inner labial and ventral cephalic sheaths. These cells probably function like glia in providing neural support (Perkins *et al.*, 1986) and transiently express UNC-6 during these migrations. Transient expression of UNC-6 is also observed in the PVT neuron from birth until maturation. The posterior-ventral midline position of the PVT and the timing of UNC-6 expression coincide with the midline decussation of the left and right PVP axons, whose cell bodies flank PVT. In both *unc-6* and *unc-40* mutants, PVP axons sometimes fail to migrate contralaterally (Wadsworth *et al.*, 1996). While not providing evidence of a direct causative relationship, these observations imply that UNC-6 expression may function as an attractive guidepost at several migratory choice points.

Preliminary evidence from *Drosophila* provides a clue as to how UNC-6 may provide local guidance information. Several workers have shown that while the *netrins* are expressed by midline glia, the proteins localize to both the midline cells and cells that flank commissural neurons along the distal longitudinal axis (Harris *et al.*, 1996; Mitchell *et al.*, 1996; Hiramoto and Hotta, 1998). Using antibodies against NetrinA and B, and the *Drosophila* UNC-40 homologue Frazzled, Hiramoto and Hotta (1998) demonstrate that Netrin localization to the distal cells depends on Frazzled expression by those cells. In *frazzled* mutants, the Netrins are no longer distally localized. Conversely, when Frazzled is ectopically expressed, the Netrin distribution is coincident with Frazzled-expressing cells. The *Drosophila* CNS has two primary defects in mutants that are homozygous for a single small deletion that removes both *netrinA* and *netrinB*, which physically map side by side and will be referred to as the *netrinA/B* locus: First,
commissures often fail to cross the midline, and second, there are several segments in which the longitudinal axon tracts are discontinuous (Harris et al., 1996; Mitchell et al., 1996; Hiramoto and Hotta, 1998). Hiramoto and Hotta (1998) observed that in contrast to wildtype animals, where specific pioneer axons remain in the longitudinal tracts and do not cross the midline, the netrinA/B mutation results in misrouting of pioneer axons in regions not necessarily coincident with the breaks in longitudinal tracts. Together, these results suggest that Frazzled may localize the Netrins to cells that are distal to the midline where they act as attractive guideposts for the longitudinal guidance of pioneer neurons.

The Drosophila Netrins also function as short-range guidance cues during the pioneer migrations of motor axons (Winberg et al., 1998a). Since the functions of numerous other molecules that are discussed later also have a role in the guidance of these same axons, a brief review of the development of the Drosophila embryonic motor nervous system is given. In every trunk segment of the Drosophila embryo, three fascicles of motor neuron axons called the intersegmental nerve (ISN), the segmental nerve (SN), and the transverse nerve (TN) exit the ventrally located CNS. As the fascicles travel circumferentially, in a ventral to dorsal direction, the ISNb and ISNd fascicles branch from the ISN near the ventral oblique muscles, numbered 15, 16, and 17, all of which are innervated by ISNd. Innervation is here defined as the migration of an axon into peri-muscular space and the subsequent arborization of the growth cone into a neuromuscular synapse. ISN and ISNb then continue dorsally, and upon encountering the ventral lateral muscles (VLM), the ISNb defasciculates into RP3, RP1 and RP4, and RP5, while the ISN extends dorsally to innervate the dorsal muscles. RP3 innervates VLM 6 and 7, RP1 and RP4 innervate VLM 13, and RP5 innervates VLM 12. Similar to the ISN, the SN also undergoes a series of bifurcations. As the SN exits the CNS, it branches into the SNa, which innervates the ventral external muscles, and SNa, which continues dorsally. Upon encountering the lateral muscles, SNa bifurcates. The SNa lateral branch innervates muscles 5 and 8, while
the dorsal branch further bifurcates into two branches, one of which innervates lateral muscles 22 and 23, and the other innervates 23 and 24. The third fascicle emanating from the CNS is the TN, which unlike the ISN and the SN, derives from cell bodies located in both the CNS and the PNS. The lateral bipolar dendritic (LBD) cell is situated laterally and between each muscle segment. The LBD sends out a process dorsally and ventrally. The ventral LBD process fasciculates with the two TMN axons emanating from the CNS, thereby forming the TN.

Obviously, during the ventral to dorsal circumferential migration, the major fascicles of the CNS make highly stereotyped defasciculation decisions at stereotypical choice points. Without the netrinA/B or the frazzled genes, RP3 stalls just before bifurcating and innervating VLM 7 and 6, from which Netrin B is normally expressed (Winberg et al., 1998a). This phenotype is rescued by pan-muscle and midline cell expression of either Netrin cue, but not by midline expression alone. Moreover, pan-muscle expression of high levels of either Netrin results in ectopic neuronal bifurcations and innervation of various muscles by SNa. Together, these observations strongly support the idea that NetrinB functions as a short-range guidance cue in the innervation of muscle by CNS neurons.

The ability of the Netrins to act as short-range guidance molecules is not limited to invertebrates (Deiner et al., 1997). In mice, Netrin-1 is expressed by the neuroepithilium that surrounds the RGC axons exiting the optic disc, while Dcc is expressed by the RGC axons during their entire migration through and from the retina. Although RGC axons extend toward the optic disc in both netrin-1 and dcc mutants, they fail to migrate through the few cell layers of the disc and enter the optic stalk. It is therefore likely that Netrin-1 functions as a short-range attractant that is used to guide RGC axons through the optic disc via the Dcc receptor. Since Netrin-1 acts at a distance to guide RGC axon migration in culture in a Dcc-dependent fashion (Deiner et al., 1997; de la Torre et al., 1997), how Netrin remains localized to the
neuroepithilium is unknown. As in *Drosophila*, it is plausible that Dcc or another receptor tethers Netrin to the neuroepithilium of the optic disc.

*Grasshopper Semaphorin I can Act over a Short Range to Promote Neurite Outgrowth and Migration*

In addition to the long-range repulsive abilities of the Semaphorins, at least one member of the family has a role in short-range attraction. Grasshopper Semaphorin I (G-SemaI) was the first member of the Semaphorin family to be discovered and was originally called Fasciclin IV, because of its apparent role in fasciculation (Kolodkin *et al.*, 1992). The G-SemaI monoclonal antibody, mAb 6F8, reveals G-SemaI expression in several circumferential bands of limb bud epithelium beginning at about 34.5% of embryonic development (figure 1.7). Not only are these bands coincident with segment boundaries, but the trochanter band is precisely coincident with a portion of a highly stereotyped migration of a pair of fasciculated pioneer axons, called Ti1s. The Ti1 cell bodies are situated just proximal to the tibia segment boundary. At about 30% of development, the Ti1 neurons send their axons proximally until they encounter the epithelium of the trochanter boundary that expresses the G-SemaI, whereby the axons turn and migrate ventrally along the boundary. At about 35% of development, the Ti1 axons turn again and migrate proximally towards a guidepost cell of the coxa segment boundary (Cx1). Kolodkin *et al.* (1992) specifically disrupted the function of G-SemaI *in vivo* by bathing limb fillets in mAb 6F8, resulting in severe Ti1 defasciculation and branching upon encountering the trochanter segment boundary (figure 1.7). Although this result demonstrates that G-SemaI may have a role in fasciculation, exactly how it functions in guidance was not made clear by this experiment.
The guidance capability of G-Semal was elucidated through the study of the axon migrations of the subgenual organ (SGO). The SGO is situated just distal to the tibia segment boundary that separates the SGO from the Ti1 cell bodies (figure 1.7). A little after the Ti1 axons reach the Cx1 guideposts, about three neurons of the SGO extend axons proximally over the epithelium of the tibia segment boundary that expresses G-Semal. In doing so, the SGO can establish contact with the Ti1 cell bodies. The axons of the SGO continue to migrate proximally via fasciculation with the Ti1 axon track. Heat-shock elimination of the Ti1 neurons, or their mechanical removal, resulted in the stalling of the SGO axons at the proximal boundary of the epithelial band that expresses G-Semal at the tibia segment boundary (Wong et al., 1997). This result suggests that G-Semal is at least permissive to SGO axon migration. If G-Semal function is disrupted with mAb 6F8 before SGO axon outgrowth, no SGO axons grow. In addition, if the fillets are bathed in mAb 6F8 during the period when SGO axons traverse the band of G-Semal,
SGO axons migrate no further. These results suggest that the transmembrane G-SemaI promotes SGO axon outgrowth through short-range attraction and allows migration across G-SemaI expressing cells so that SGO axons can make filopodial contact to the T1 neurons. These results also provide an explanation for T1 defasciculation and branching at the trochanter segment boundary upon mAb 6F8 exposure: G-SemaI may provide a relatively attractive substratum, without which growth cones may seek an equally permissive substrate, in turn resulting in environmental exploration via branching. Alternatively, like other axon guidance molecules, G-SemaI may be bifunctional, attracting SGO growth cones while partially collapsing T1 growth cones, thereby preventing their defasciculation and branching and causing them to stall while other guidance molecules attract the axons ventrally. The latter model is consistent with the short-range repulsive effects of the homologue of grasshopper SemaI in Drosophila, to which it is 60% identical (see the section on short-range repulsion below; Kolodkin et al., 1993).

**Fasciculation as a Result of Short-Range Attraction**

A discussion of short-range guidance cues would be incomplete without mentioning axon bundling/fasciculation. Short-range guidance cues are essential for instructing axons when and where to fasciculate with existing axon tracts and defasciculate near prospective targets. As fascicles travel through targeted regions, axons transiently send out processes to sample the environment. Whether or not these processes defasciculate depends upon the balance of force mediated by molecules that regulate fasciculation and defasciculation. Indeed, by varying the relative levels of fasciculation and axon guidance molecules on muscle, Winberg et al. (1998a) demonstrate that the two groups can precisely mimic each other or mutually suppress one another with respect to muscle innervation. For example, ectopically increasing NetrinA, NetrinB, or Fasciclin II expression on muscle or eliminating the short-range repulsive cue D-SemaII (see below) from muscle results in a discontinuous TN that ectopically innervates the
ventral lateral muscles. However, by simultaneously increasing the levels of D-SemaII and Fasciclin II, the TN remains continuous and without ectopic contacts. Moreover, the loss-of-function of NetrinA, NetrinB or Fasciclin II does not significantly disrupt normal TN target selection or fasciculation, arguing that there are multiple redundant cues that regulate these processes.

A number of homophilic molecules in *Drosophila* have been implicated in mediating fasciculation including Connectin (Con) and Fasciclin II. Con is a glycosylphosphatidylinositol (GPI)-membrane linked surface molecule and contains several leucine-rich repeats. It is expressed on lateral muscles 5, 18, and 21 to 24, ventral muscles 27 and 29, and the SNa and SNc motorneurons that innervate them (Nose *et al.*, 1992 & 1994). Pan-muscle expression of Con results in SNa misrouting from muscle 5 to muscle 12. The phenotype elicited by pan-muscle expression of Con can be eliminated if Con expression is nearly abolished in the SNa via a *connectin* hypomorphic mutation. This result suggests that Con expression on both migrating and target cells is required during the final leg of SNa target acquisition (Nose *et al.*, 1994). The endogenous function of Con, however, is probably redundant since con mutants have no axon trajectory defects. That Connectin is also redundantly required for SNa and SNc fasciculation is suggested by the ability of *connectin* mutants to suppress the abnormal fasciculation of SNa and SNc, but not ISNb and ISN, that is induced by *beaten path* mutations (see below; Fambrough and Goodman, 1996). Together these results suggest that a homophilic Connectin interaction is redundantly required in motorneuron fasciculation and discrete target selection.

*Drosophila* Fasciclin II (Fas II), a homologue of N-CAM, is a member of the transmembrane Ig superfamily and is thought to mediate cell contact in a homophilic fashion (Grenningloh *et al.*, 1991). During the period of axon outgrowth and migration, Fas II is expressed on all motor neurons, the vMP2, MP1, and FN3 longitudinal axon tracts of the CNS (VanVactor *et al.*, 1993), and on the surface of all muscles at lower levels (Schuster *et al.*, 1996).
Later, the expression of Fas II is limited to pre- and post-synaptic terminals where it is required for synapse stabilization and growth (Schuster et al., 1996). Without Fas II expressed from both sides of the terminal, the synapse can still form, but thereafter is retracted, suggesting that it is needed to fortify contacts. Fasciclin II may have a similar role during axon tract fasciculation in that it is required to maintain contacts between selected axons during outgrowth and migration. That Fas II acts as a short-range attractant that promotes fasciculation in a homophilic manner is supported by several lines of evidence. First, the longitudinal axon tracts of the midline on which Fas II is expressed are defasciculated in fas II mutants (Grenningloh et al., 1991; Lin et al., 1994). Second, pan-neuronal expression of Fas II can rescue the defasciculation defects of fas II mutants (Lin et al., 1994). In addition, Fas II over-expression often results in a gain-of-function phenotype whereby nerve tracts that initiate together, including that of MP1 and FN3, fail to defasciculate at the normal choice point (Lin et al., 1994). Moreover, Fas II pan-neuronal expression also results in the fasciculation of the SN motoneuron tract to the ISN in a dose dependent manner (Lin and Goodman, 1994). Third, ectopic expression of Fas II on a subset of ventral muscles results in their specific ectopic innervation by ISN, SN or TN motoneuron branches (Davis et al., 1997). As predicted, the ectopic innervation is dependent on the endogenous Fas II motoneuron expression, since the phenotype is abolished in the fas II null background. Together, these results strongly support that Fasciclin II is required in axon tracts to mediate their fasciculation in a homophilic fashion.

Since Fas II is apparently uniformly distributed along axons how can axons disengage from fascicles at discrete choice points? There are at least five Drosophila genes that when mutated, have a strikingly similar phenotype to that of pan-neuronal over-expression of Fas II, suggesting that they may direct choice point defasciculation. They include beaten path, which encodes a secreted Ig superfamily member called Beat (Fambrough and Goodman, 1996; Mushegian, 1997; Bazan and Goodman, 1997), side step, which encodes another Ig superfamily
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member called Side (Sink and Goodman, 1998), and three receptor protein tyrosine phosphatases (RPTPs) called DPTP69D, DPTP99A and DLAR that have weak Fas II over-expression-like phenotypes (Desai et al., 1996; Krueger et al., 1996). Areas along fascicles that express concentrated amounts of Beat enables axons to defasciculate from the bundle (Fambrough and Goodman, 1996). In beaten path mutants, the ISN and SN motorneuron pathways often do not defasciculate at normal choice points. This phenotype is at least partially suppressed by fasII and connectin mutations, suggesting that Beat is an anti-adhesive that works to overcome the fasciculative effects of Fas II and Connectin. Beat probably does not directly destabilize Fas II or repel axons however, since pan-neuronal over-expression results in no fasciculation or axon guidance defects. Instead, the action of Beat is probably regulated by the specificity of Beat receptors, plausibly including the RPTPs (Desai et al., 1996; Krueger et al., 1996; Desai et al., 1997). In contrast to the autonomous regulation of defasciculation within fascicles by Beat, expression of Side on muscle beds promotes defasciculation in a cell non-autonomous fashion (Sink and Goodman, 1998). How Side interacts with other fasciculation factors, however, is unknown.

The study of short-range attractive cues has revealed their use in both fasciculation and discrete target guidance. In addition, these molecules, homophilic and otherwise, have proven to play multiple redundant roles in several steps of axon guidance.

1.4.4 Short-range Repulsion

While critical experiments are needed to distinguish between a permissive substrate component versus an instructive guidance molecule, it is difficult to conceive how a repulsive cue might not be instructive. But how can a repulsive cue guide an axon to its target destination with any specificity? It is somewhat ironic that Sperry's hypothesized chemoattractive cues that guide RGC axons to specific locations in the tectum are probably chemorepulsive. In a permissive
background, a graded repulsive cue could theoretically steer axons to the area with the least repulsive activity. Such candidate molecules are the Ephrins. Other short-range repulsive cues however, are more limited in their guidance capabilities, such as *Drosophila* Netrin B, and Semaphorin I and II, which instruct axons within fascicles to defasciculate at specific choice points, and prevent ectopic synapse formation. Finally, how axons cross a midline was one of the greater mysteries of developmental neurobiology until the characterization of Round about, Commissureless, and Slit. These three molecules act as gatekeepers of the midline and work to selectively repel axons via cell-cell contact.

*The Invertebrate Semaphorins play a major role in Short-Range Repulsion*

While the invertebrate UNC-6 and Netrin homologues guide migrations via cell-cell contact and from a far, the function of the invertebrate Semaphorins may be limited to short-range guidance. For example, several results strongly support the idea that D-Semal is required to repel axons within fascicles so that they may begin pioneer migrations at specific choice points. First, *Drosophila* Semaphorin I (D-Semal) expression begins just before axonogenesis in the CNS (Kolodkin *et al.*, 1993; Yu *et al.*, 1998). At later stages during axon outgrowth, high levels of expression continue throughout the longitudinal and commissural tracts of the CNS and in all five motor axon branches of the PNS. Second, in wildtype flies, there are three major longitudinal CNS tracts that express the Fasciclin II marker, the proximal pCC/MP2 tract, the MP1 tract, and the distal third longitudinal connective. In animals homozygous for a null or hypomorphic *D-semal* mutation, the distal tract is often discontinuous and is observed to fuse with the medial MP1 tract (Yu *et al.*, 1998). Third, as described in detail above, PNS fascicles make highly stereotyped defasciculation decisions at stereotypical choice points. In *D-semal* mutants, nerve and axon branches often cannot defasciculate at these choice points (Yu *et al.*, 1998). For example, ISNb often failed to completely extend on the ventral muscle bed and
sometimes did not defasciculate from the ISN. Moreover, axons of ISNd, and ISNd itself also failed to separate from ISN at the defasciculation choice point in many segments. Like the fascicles of the ISN, SNa often failed to bifurcate into the branches that innervate the lateral muscle 22, 23, and 24. Instead SNa stalled in 69% of hemisegments. A fourth line of evidence which suggests that D-SemaI is a short-range repellent is that both the aforementioned CNS and PNS defects are completely rescued by pan-neuronal expression of D-SemaI (Yu et al., 1998).

In addition, pan-neuronal expression of a version of D-SemaI that lacks the cytoplasmic domain resulted in a significant partial rescue of guidance defects, suggesting that D-SemaI does function as a ligand to regulate defasciculation at choice points. Finally, ectopic muscle expression of D-SemaI results in qualitatively similar neuronal defects as the loss-of-function (Yu et al., 1998), a phenomenon termed phenocopy. For example, both ISNb and SNa often fail to defasciculate from nerves at choice points that required entry into a muscle bed. When the branches do enter the muscle beds, the axons frequently stall or fail to produce synaptic arborizations. Intriguingly, the ability of the transgene to induce phenocopy was dependent on the expression levels of D-SemaI. Unlike the semal mutant phenocopy that is induced by two copies of the transgene that drives ectopic muscle expression of D-SemaI, one copy of the transgene has no phenotypic defects. Moreover, the phenocopy is significantly enhanced when two copies of the transgene drive D-SemaI muscle expression in a semal null background. In addition to phenocopy, wildtype ISN-branch innervation of dorsal muscles is often prevented upon D-SemaI expression in muscles. This phenotype is only generated, however, if the relative amount of D-SemaI is increased by ectopic muscle expression from two copies of the transgene in the background of the null semal mutation. Together, these results suggest that the transmembrane D-SemaI functions as a ligand to repel axons and fascicles from nerve tracts at specific choice points. In addition, migrating growth cones have a response threshold to
guidance cues and are therefore sensitive to the relative levels of the numerous cues in their environment.

While the Neuropilin receptors have high affinity for class III Semaphorins, they do not bind to Semaphorins of class I or II (see the section on long-range repulsion), suggesting that other Semaphorin receptors exist. During the recent characterization of the immune modulating role of two viral Semaphorins from vaccinia virus (A39R) and alcelaphine herpesvirus I (AHVsema), a viral encoded Semaphorin protein receptor (VESPR) was identified (Comeau et al., 1998). Amazingly, VESPR belongs to a family of previously characterized homophilic neuronal surface proteins called the Plexins (Ohta et al., 1995) that were originally identified with the Neuropilins (Takagi et al., 1987; Satoda et al., 1995)!

Plexin is a large 220 kDa protein, the cDNA of which encodes a predicted signal sequence, followed by a cryptic semaphorin domain, three cysteine-rich repeats, a transmembrane domain and a large ~650 amino acid cytoplasmic domain that has no known homologies except a putative tyrosine phosphorylation site (Comeau et al., 1998; Ohta et al., 1995). The presence of a semaphorin domain in Plexin suggests that the ligand and receptor are evolutionarily related, the significance of which is unknown. Although A39R can bind to human monocytes, up-regulate ICAM-1 expression, and induce cytokine production from bound monocytes (Comeau et al., 1998), Semaphorin’s role in viral infection is also not clear.

Knowing that members of the Semaphorin family can bind to a Plexin homologue, Winberg et al. (1998b) screened for and found two Plexins in Drosophila, called Plexin I and II. RNAs for both Plexins are localized to the cells of CNS during the period of axon outgrowth and migration. Embryos homozygous for a small deficiency that includes Plexin I have penetrant axon migration defects that are strikingly similar to the phenotypes of D-semal mutants. This suggests that Plexin I interacts with or is the receptor for D-Semal. In support of this hypothesis, transheterozygotes of D-semal and the small deficiency that includes Plexin I have phenotypes
that resemble those described in mutants homozygous for either lesion, albeit at a lower penetrance. Moreover, Plexin I deficient heterozygotes can partially suppress the pan-muscle over-expression phenotypes of D-SemaI. Together with the fact that VESPR binds A39R and AHVsema, these preliminary data strongly suggest that Plexin I mediates D-SemaI short-range repulsion. Although the function of Plexin II is unknown, it is plausible that it may interact with the second *Drosophila* Semaphorin, D-SemaII.

Whereas D-SemaI repels axons from axons, D-SemaII seems to repel axons from muscles. In contrast to the pan-neuronal expression pattern of D-SemaI (Yu et al., 1998), D-SemaII is expressed in a much smaller subset of neurons (Kolodkin et al., 1993). In addition, β-galactosidase (β-gal) reporter expression from enhancer-trap insertions into the *D-semaII* locus, together with RNA in situ, suggests that high levels of *D-semaII* muscle expression was limited to a single ventral thoracic muscle in segment T3 (Kolodkin et al., 1993). However, a D-SemaII monoclonal antibody later resolved low levels of pan-muscle expression (Winberg et al., 1998). Several additional experiments demonstrate the role of D-SemaII in repelling certain axons from migrating on muscle. First, in flies mutant for *D-semaII* the TN often ectopically innervates the ventral longitudinal muscles 6, 7, and 13, but not 12 (Winberg et al., 1998). Moreover, RP4 of the ISNb, which normally innervates muscle 12, innervates muscle 12 and muscle 8 of the adjacent segment. Likewise, the SNa lateral branch, which bifurcates to innervate lateral muscles 5 and 8, sometimes only innervates muscle 5 with two branches, as if the second branch cannot leave muscle 5 to reach muscle 8. Second, upon over-expression of D-SemaII with a pan-muscle enhancer, the LBD and TMN axons fail to enter the muscle bed and therefore do not form the TN (Winberg et al., 1998). In addition, RP3 of ISNb stalls early upon D-SemaII pan-muscle expression, failing to innervate muscles 6 and 7. This is in concordance with Matthes et al. (1995) observation that RP3 fails to innervate muscle 6 and 7 when D-SemaII is over-expressed from the *toll* enhancer in a subset of muscles, including 6 and 7. Intriguingly, when
D-SemaII is ectopically expressed in a different subset of cells, including muscle 5, the SNa lateral branch fails to innervate muscles 5 and 8 (Winberg et al., 1998). SNa innervation of muscle 5 and 8, however, is unaffected by pan-muscle expression of D-SemaII. This suggests that like the response of growth cones to D-SemaI, the lateral branch of SNa, but not RP3 of ISNb, is sensitive to the relative levels of D-SemaII expression. Together, these results strongly support a model whereby a low level of D-SemaII muscle expression repels innervation by a selected set of axons, but not others. The difference in axon response to D-SemaII may reflect a variation in the composition of D-SemaII receptor complexes on the different growth cones. Alternatively, the varied response could be due to different expression levels of the D-SemaII receptor, or unknown regulators of D-SemaII action. Resolving these possibilities, or combinations thereof, will have to wait until the nature of the D-SemaII receptor is known.

_Drosophila Netrin B Exhibits Short-Range Repulsive Characteristics_

The detailed genetic study of the PNS innervation of the ventral muscles by Winberg et al. (1998) also reveals that NetrinB can act as short-range chemorepellent in addition to the three other chemotactic functions described above. Normally NetrinA has no ventral muscle expression while NetrinB is expressed in the ventral longitudinal muscles 6 and 7 at moderate levels. The only defect in _netrinA/B_ deficient flies is that RP3 fails to innervate muscle 6 and 7, suggesting that NetrinB can act as a short-range attractant. When over-expressed from a pan-muscle enhancer however, Netrin B enables TN innervation of muscles 6 and 13, but prevents SNa entry on to the ventral longitudinal muscle bed. Mutations in _frazzled_ suppress the effect of NetrinB over-expression on the TN tract, but not on the SNa, suggesting that NetrinB repulsion of SNa is mediated by another receptor. These results are consistent with a model whereby Netrin B acts as a bifunctional short-range chemotactic cue that not only attracts TN and RP3 axons, but also plays an auxiliary role in specifying muscle innervation through repulsion.
The Ephrins are Versatile Cues Required to Repel Cell Contacts and Migrations over a Short-Range

Next to the Semaphorin superfamily, the Ephrin ligands and their respective Eph receptor protein tyrosine kinases are the largest family of known axon guidance molecules. The Ephrins, recently renamed to unify the nomenclature (Eph Nomenclature Committee, 1997), fall into two classes. The Ephrin type A molecules have a globular domain that is required for receptor interaction and a GPI-membrane linkage domain. The Ephrin B molecules are like the A-type, except that they are transmembrane and have a cytoplasmic tail with a conserved C-terminus (as reviewed by Holland et al., 1998; Drescher, 1997). The respective receptors of the Ephrin A and B type molecules are the type A and type B Eph receptor protein tyrosine kinases (RPTKs). The Eph receptors are predicted to have a globular domain required for ligand interaction, followed by a cysteine rich region, two FNIII domains, a transmembrane domain, two conserved membrane proximal tyrosine phosphorylation sites, a kinase domain, and C-terminal tyrosine required for Grb-10 and LMW-PTP engagement (Holland et al., 1998; Drescher, 1997).

Although each receptor type is defined by their respective binding preference for Ephrin type A and B molecules, promiscuous binding between non-cognate types occur. Various Ephrin-Eph binding partners are involved in wide spectrum of developmental processes that include short-range axon repulsion and cell guidance, fasciculation, and cell sorting. Except for one example (Pandey et al., 1996), overwhelming evidence suggests that the Ephrins generally function to mediate contact-dependent repulsion. Without being membrane-bound on the surface of the signaling cell, the Ephrins cannot cluster and, in turn, cannot initiate a signal cascade via the Eph RPTKs on the migrating cell (Davis et al., 1994). Although a wealth of Ephrin and Eph data have been generated, I will only discuss these guidance molecules as they relate to novel or unique concepts in development, such as the multiple functions of individual Ephrins, retrograde signaling, and topographic mapping.
In vivo evidence for Eph receptor-mediated axon guidance first came from the murine knockout of EphB2/Nuk (Henkemeyer et al., 1996). Eph B2 protein null mutants have anterior commissural guidance defects within the lateral tract that connects the two temporal lobes, called acP. The anterior commissural tract called acA that connects the two olfactory bulbs is normal in ephB2 mutants. Intriguingly, the Eph B2 receptor is expressed ventrally along the path taken by the acP axons while the Ephrin B1 ligand is expressed in the anterior commissural axons. This expression pattern suggests that the Eph B2 “receptor” provides a repulsive barrier and may bind to the Ephrin B1 “ligand” on acP growth cones and prevent them from straying from the commissural route. Two additional lines of evidence support this model of retrograde signaling. First, mice homozygous for an allele of Eph B2 whereby the intracellular region was replaced by β-galactosidase are phenotypically wildtype (Henkemeyer et al., 1996), suggesting that Eph B2 plays a non-autonomous role in anterior commissural guidance. Second, Holland et al. (1996) demonstrated that the tyrosines of the conserved C-terminus of Ephrin B1 and B2 are phosphorylated upon exposure to either Eph B2-expressing cells or pre-clustered Eph B2 in solution. In addition, these ligands contain phosphorylated tyrosines in vivo. Together, these results suggest a model in which Eph B2 can repel acP axons via Ephrin B1 to prevent their aberrant trajectory. Another example of retrograde signaling includes glial-expressed RPTPβ inducing neuronal cell adhesion and outgrowth via the GPI membrane-linked Contactin (Peles et al., 1995).

Ephrin B1 and B2 may also play a more traditional role in guiding migrations of cells from vertebrate somites. Both neural crest cell migration and motor axon extension is limited to the rostral halves of each somite in the trunk of vertebrates. Not only are Ephrin B1 and B2 expressed in the caudal halves of the somites, but they can repel neural crest cells and motor axons in culture (Wang and Anderson, 1997; Krull et al., 1997). This suggests that the ligands
may act to segregate migrating cells and axons to the rostral halve of somites and perhaps repel them away from the midline.

In more rostral positions near the hindbrain, the neural crest cells migrate from rhombomeres (r) 2, 4 and 6 to respectively generate the first, second, and third plus fourth branchial arches. Presumptive neural crest cells of the second, third and fourth arches initially converge and then separate to form the mature branchial arches. Ephrin B2, a ligand that can bind both Eph A4 and B1 receptors, is expressed in r2, r4, r6, and in the second branchial arch. Eph A4 is first expressed by r3 and r5 and is thereafter limited to the neural crest cells that migrate into the third arch (Smith et al., 1997). The expression pattern of another receptor, Eph B1, is limited to those neural crest cells of both the third and forth branchial arches. RNAs encoding truncated Eph A4 and B1 receptors injected into Xenopus embryos either together or alone result in the mixing of neural crest cells of branchial arch 3 with neighboring arches (Smith et al., 1997). The same result is obtained upon widespread ectopic expression of Ephrin B2. These findings suggests that Ephrin B2 creates domains that are repulsive to migrating neural crest cells that express the Eph A4 and B1 receptors, thereby preventing the mixing of determined neural crest cells.

The Ephrins may also be involved in cell sorting by minimizing the contact area between receptor-expressing cells and ligand-expressing cells. For example, Wang et al. (1998) demonstrated that Ephrin B2 and its cognate receptor, Eph B4 are the earliest known markers of arterial and venous endothelial cells. After vasculogenesis, which generates the rudimentary vascular network, but before angiogenesis, which remodels and expands the vasculature, Ephrin B2 and Eph B4 are expressed on presumptive arterial and venous cells of the primary capillary plexus, respectively. ephrin B2 mutant mice have severe defects in angiogenesis, if it occurs at all. Interestingly, both venous and arterial vessel remodeling is affected in the mutant, suggesting that signaling to and from Ephrin B2 expressing cells is required for continued
capillary development. Exactly how Ephrin functions in angiogenesis is unclear. Ephrin could minimize contacts between presumptive arterial and venous cells in the capillary plexus via repulsion. Alternatively, Ephrin B2 could initiate or propagate an angiogenic signal. In support of the former hypothesis, preliminary analysis suggests that complementary expression of the VAB-1 Eph receptor and the VAB-2 and LIG-1 GPI-linked Ephrin ligands on the ventral posterior hypodermal cells of C. elegans may minimize contacts between cells (X.W., P.J.R., J.G.C., and T.P., unpublished results). Regardless, these results highlight the multiple uses of the Ephrin ligands throughout development. Ephrin B1 and B2 may not only autonomously guide anterior commissural axons, but also guide the migrations of motor axons, neural crest cells, corpus callosum axons, habenular commissural axons, and the cells of the palatal shelves to prevent cleft palate via contact-mediated repulsion (Orioli et al., 1997). The finding that Ephrin B2 is also required in branchial arch formation and angiogenesis reiterates how a guidance cue can have alternative functions depending on the receptor context.

Finally, topographic maps may use the Ephrins to generate spatial coordinates to guide axon projections. Topographic maps were first proposed by Sperry (1942 & 1950, as cited in Sperry, 1963) to explain the consistency of the spatial relationships between axons that project from one brain structure to another. These maps were originally conceived as gradients of as little as two molecules in a perpendicular orientation. The concentration of the two molecules at any one coordinate could serve as a marker for that coordinate. This hypothesis was used to explain the remarkable specificity of axon projections from the retina to the optic tectum in wild type fish and upon experimental manipulations (Sperry, 1963). Although the existence of such molecules had been inferred for years (Walter et al., 1987), the cues themselves were first identified serendipitously (Cheng and Flanagan, 1994) and then later by a directed approach (Drescher et al., 1995). Chick RNA in situ revealed that Ephrin A2 is increasingly expressed from the anterior tectum to the posterior tectum, and its cognate receptor Eph A3 is increasingly
expressed from the nasal RGCs to the temporal RGCs (Cheng et al., 1995). Antibodies corroborate that Ephrin A2 is expressed in an A/P gradient along the tectum (Monschau et al., 1997). In addition, the binding pattern of an AP-tagged version of Eph A3 on the tectum reflects the Ephrin A2 gradient, and is dependent on a surface protein(s) that is GPI-linked. Conversely, AP-tagged Ephrin A2 has a greater affinity for temporal RGC axons in culture and in situ than that from nasal origin. These results suggest a model whereby upon arrival of the RGC axons from the retina to the tectum, the Eph A3-expressing temporal axons avoid the posterior tectum because it expresses high levels of Ephrin A2 ligand, while nasal axons are permitted posterior access. Two lines of evidence further support this model. First, when presented in alternating stripes of 293T cell membranes, temporal axons avoid membranes prepared with Ephrin A2-transfected cells in culture, while nasal axons show no preference (Nakamoto et al., 1996). Second, upon infection of chick tectal cells with a retrovirus transgenic for Ephrin A2, temporal axons avoided the infected areas, while nasal axons again showed no avoidance of Ephrin A2 expressing cells (Nakamoto et al., 1996).

Since nasal RGC axons have low levels of the Eph A3 receptor, they can invade the posterior tectal regions that express the repulsive Ephrin A2 ligand. Why the nasal axons avoid residence within the anterior tectum is not understood, since no preference of nasal axons for either half of the tectum has ever been demonstrated in vitro. Why those axons are prevented from leaving the tectum, on the other hand, is better-understood (Monschau et al., 1997). ephrin A5 RNA and protein is highly expressed at the extreme posterior of the tectum. Like Ephrin A2, Ephrin A5 binds to the Eph A3 receptor, but also to the Eph A4 and A5 receptors with slightly lower affinities. The latter receptors may be uniformly expressed by all RGC axons or of slightly higher expression in nasal RGCs. As expected from the binding profiles and expression patterns, slightly less Ephrin A5 is required to collapse temporal RGC axons than nasal ones. These data suggest that Ephrin A5 may form a repulsive barrier that prevent axons from leaving
the tectum, a notion that is supported by observations in Zebrafish (Brennan et al., 1997). Expression patterns and mutations of mouse Ephrin A5, however, point to a function that complements that of Ephrin A2 in that it may further repel temporal axons (Frisen et al., 1998).

Clearly, retinal-tectal topographic mapping heavily relies on the Ephrins to label tectal coordinates from which Eph receptor-expressing RGC axons are repelled. Why temporal axons project where they do is becoming clearer, but why nasal axons project where they do still remains a mystery. While our understanding of topographic mapping is far from complete, it is inspiring that evidence has supported the underlying concepts of Sperry's chemoaffinity hypothesis in that we know that a small number of molecules can determine the spatial relationships of cells that project processes over a relatively large distance.

A Conditional Response of Axons to a Short-Range Repulsive Molecule Solves the Mystery of How Axons Decussate at the Midline

The nervous system of most animals is nearly bilaterally symmetrical along a midline. As discussed above, both short and long-range cues attract and guide axons along longitudinal tracts that flank either side of the midline. Since the left and right tracts remain distinct, the midline is also thought to be the source of a barrier that is repulsive to most migrating cells. Of course, animals need to coordinate their left and right sides and do so with commissural axons that break through the midline barrier. Herein lies the paradox. How can the midline prevent some axons from crossing, while simultaneously attracting others to cross, and once on the other side, repel them from lingering or recrossing? Apparently, the paradox is resolved through the regulation of a receptor that mediates repulsion from a short-range midline cue called Slit.

The first clue that addressed how axons are selected to cross or not cross the midline came from an extensive screen to identify mutants defective in Drosophila CNS midline patterning (Seeger et al., 1993). Two complementation groups were isolated that have opposite
phenotypes. The first gene is called *commissurless* (*comm*) and is required for axons to cross the midline. Commissural axons that normally traverse the midline in wildtype flies almost always do not in *comm* mutants, hence the name. The second gene, called *round about 1* (*robo1*) is required to prevent longitudinal axons from crossing the midline and commissural axons from recrossing the midline upon reaching the other side (Seeger et al., 1993; Kidd et al., 1998b). In *robo1* mutants, these axon tracts run in circles around midline glia, hence the name. Interestingly, the *comm; robo1* double mutant has the same phenotype as *robo*, suggesting that without *robo1*, *comm* is no longer required. *comm* may therefore function upstream of *robo1*. These data suggest two alternative models as to how *comm* may allow midline crossing in the presence of *robo1*-mediated repulsion: *comm* may overcome *robo1* by attracting commissural axons to the midline. Alternatively, *comm* may be required to suppress *robo1*-mediated repulsion of commissural axons. Several lines of evidence support the latter model. First, *round about* encodes D-Robo1, which is predicted to have a signal sequence, five Ig repeats followed by three fibronectin type III (FNIII) repeats, a transmembrane domain and a large 457 amino acid cytoplasmic domain (Kidd et al., 1998a). D-Robo1 is highly expressed on longitudinal midline axon tracts, but is barely detectable on commissural growth cones before crossing the midline. After crossing however, a dramatic increase in D-Robo1 expression on the commissural growth cones is observed (Kidd et al., 1998a). Moreover, expression of D-Robo1 in a subset of longitudinal axons can rescue their *robo1* phenotype in the background of a *robo1* mutation, suggesting that D-Robo1 functions cell-autonomously. Together these results are consistent with D-Robo1 being a receptor that interprets a midline cue(s) as repulsive.

In addition to *robo1*, a second D-Robo1 homologue, designated D-Robo2 has been cloned in *Drosophila*, along with Robo1 and Robo2 homologues in rat, human, and a single Robo-1 homologue in *C. elegans* called SAX-3 (Kidd et al., 1998a; Zallen et al., 1998). Studies of rat *robo1* RNA and GFP-tagged SAX-3 reveal an expression pattern that is consistent with the
known function of D-Robo1 (Kidd et al., 1998a; Zallen et al., 1998). In addition to the robo-like phenotypes whereby longitudinal axons fail to remain on the left or right ventral cords and instead make numerous midline crossovers, sax-3 mutants also have longitudinal axon guidance defects (Zallen et al., 1998). It is plausible that the two functions of SAX-3, namely longitudinal and midline axon guidance, are divided amongst the two Robo homologues found in other organisms.

The second line of evidence that suggests that Comm suppresses Robo-1-mediated repulsion comes from the characterization of slit, a previously characterized gene in Drosophila that is now thought to be a D-Robo1 ligand. The predicted protein product of slit is called dSlit and encodes a signal sequence, several leucine-rich repeats, and seven EGF-like repeats (Rothberg et al., 1988 & 1990). Not only is it expressed from the midline glia, but dSlit adheres to the growth cones of commissural axons as they traverse the midline. dSlit can also bind to cells expressing D-Robo1 in culture, but not control cells, and can directly bind to AP-tagged D-Robo1 in a cell-free environment (Bland et al., 1998). Moreover, rat homologues called rSlit-1 and rSlit-2 are expressed in the vertebrate midline floor plate cells and can bind to their respective Robo counterparts (Brose et al., 1998). As expected, Drosophila slit mutants have robo1-like defects such that the CNS collapses inward due to the axon tracts repeatedly migrating around midline glia (Kidd et al., 1998c). However, slit mutants have a second phenotype that is manifested early in the development of the midline glial cells whereby they often fail to cluster and fully differentiate (Rothberg et al., 1990; Battye et al., 1998). This second function of slit is speculated to be mediated via a second receptor, plausibly D-Robo2 (Kidd et al., 1998c). Together, all of these results strongly suggest that Slit is secreted by the midline glial cells and repels longitudinal axons and commissural axons that have crossed the midline by directly binding the Robo receptor. The spatial relationship between the longitudinal tracts and the midline glia suggests that Slit may not be a long-range repellent and may instead
act through contact-mediated repulsion. This hypothesis is supported by the observation that Slit can localize to the surface of cells in culture (Rothberg et al., 1988).

Finally, comm encodes a novel protein that is secreted by the midline glia (Tear et al., 1996). As the commissural axons cross the midline, the growth cones appear to pick up and absorb Comm from the midline. In comm hypomorphs, levels of Robo expression on both longitudinal and commissural axons and growth cones increases and is coincidental with a severe reduction in commissure formation (Kidd et al., 1998b). In addition, pan-neuronal over-expression of Comm results in a robo phenotype and the virtual depletion of Robo protein. Together these results strongly suggest that Comm drastically down-regulates the expression of Robo on the surface of axons and growth cones enabling commissural axons to overcome the repulsion of Slit.

The characterization of Robo, Slit and Comm helps to resolve the paradox associated with crossing a midline and suggests the following model. CNS and other axons are probably attracted to the midline by long-range attractive cues such as the Netrins. In response to the local repulsion of Slit at the midline, however, longitudinal axons are prevented from crossing since they express high levels of Robo, the Slit receptor. Commissural neurons, on-the-other-hand, have low levels of Robo expression and can therefore initiate migration over the midline, where low Robo expression is further reinforced by Comm. Commissural axons are prevented from loitering at the midline since expression of Robo is up-regulated on the commissural axons after crossing the midline. How Robo expression is increased after traversing the midline is a problem in need of investigation. Perhaps timing autonomously regulates the increase in Robo expression. Alternatively, there could be a second post-translational modulator of Robo that is more tightly associated with the midline than Comm. Upon nearing the precise midline, Robo expression on the surface of the commissural growth cones could somehow be triggered to
increase. In order to understand the fundamentals of crossing a midline, this issue needs to be addressed.

1.5 A Summary of Axon Guidance Cues

In reviewing how the major classes of cues guide migrations, several themes emerge. First, a single cue can guide multiple migrations. For example, UNC-6 can guide sensory and motor axons ventrally, motor axons dorsally, ectodermal and mesodermal cells ventrally, and mesodermal cells dorsally. Similarly, Ephrin B2 may repel neural crest cells, corpus callosum axons, habenular commissural axons, the cells of the palatal shelves, and endothelial cells of the primary capillary plexus.

A second theme that comes from the study of multiple guidance cues is that any one migration may be guided by multiple cues. For example, the DA and DB motor neurons send their axons from the ventral cord to the dorsal cord. Not only is this migration partially dependent on UNC-6 ventral expression, but it also partially requires the dorsal expression of UNC-129. In Drosophila, several guidance and fasciculation cues are required for the axons of the ISNb to reach discrete targets in the ventral muscle bed from the midline, including that of Slit, Semal, SemaII, and NetrinB.

Third, individual molecules can have multiple functions. Whether or not a molecule acts as a long or short-range attractive or repulsive guidance cue depends on the localization of the cue and the receptor complex on the migrating cell. For example, Netrin-1 is required to attract commissural axons over a long-range, but can function as a short-range attractive molecule to guide RGC axons from the retina into the optic stalk. Molecular cues of the same family can also have diverse roles in guidance. For example, vertebrate SemaIII selectively repels sensory afferents over a long range from the ventral half of the spinal cord, while Drosophila SemaII is required to inhibit ectopic innervation of muscle beds over a short-range. In contrast,
Grasshopper Semal attracts SGO axons over a very short-range. Whether or not axons respond in an attractive or repulsive manner probably reflects the spectrum of surface receptors displayed, or the state of intracellular signaling components (Song et al., 1998). It appears as though any cue can influence the direction of migration of any cell. Indeed, axons that normally ignore the UNC-6 guidance cue or are attracted to it can be induced to respond repulsively to UNC-6 by simply ectopically expressing the UNC-5 receptor from those cells. Whether or not cells in general are competent to respond to a plethora of guidance cues, but do not since they lack the appropriate receptor complex remains to be seen.

The multiple roles of individual types of cues probably enables the development of a complex nervous system without the use of several billion distinct molecules. Indeed, the human genome is estimated to contain about 100,000 genes. It is implausible that for each of tens of billions of neurons there would be multiple cues that guide them to their target destinations. Instead, diverse cell types in various temporal and spatial locales can use similar cues to make similar or dissimilar guidance choices.

Finally, evolutionarily disparate animal species have conserved the use of guidance molecules to solve analogous developmental problems. The most striking example is the use of Netrin-1, a mammalian homologue of UNC-6, to attract commissural axons to the ventral floor plate via the Dcc receptor, a homologue of UNC-40. Of course, in C. elegans, UNC-6 is required to attract UNC-40 expressing neurons to the ventral cord. Other examples of developmental and genetic evolutionary conservation include the role of the Robo/SAX-3 receptor family in responding to repulsion from the midline in worms, flies, mice and humans. Also, the repulsive action of cell-bound Ephrins on cells expressing their cognate Eph receptors is thought to be able to minimize the contact area between adjacent groups of cells that respectively express each molecule. This function is apparently conserved from the worm
Ephrins, VAB-2 and LIG-1, and Eph receptor, VAB-1, to the mammalian Ephrin B2 and Eph B1, A4 and B4.

Given that the difference in the overall complexity of *C. elegans* and humans is mainly one of a quantitative character, and not qualitative, it follows that many of the homologous genes used throughout development will be applied to analogous programs. It is apparent that a significant part of the difference in the number of *C. elegans* genes (14,000) versus human genes (100,000) is due to gene duplication and/or modification. For every *C. elegans* gene discussed above, there are at least two vertebrate homologues. For example, *C. elegans* has a single UNC-6 and UNC-40, while mammals have at least two of each; *C. elegans* has a single UNC-5, while mammals have at least four; *C. elegans* probably has four Ephrins, while vertebrates have at least eight and so on. In some cases, the role of homologues in certain migrations may be completely redundant and only revealed upon double mutant analysis as in Eph B2 and Eph A4 (Orioli et al., 1996). In other cases, such as the functional relationship between *C. elegans* SAX-3 and *Drosophila* Robo-1 and Robo-2, the role of the single receptor in *C. elegans* may have been distributed amongst the two homologues in *Drosophila*. In other cases still, such as the UNC-6/Netrin-1 ligand and the UNC-40/Dcc receptor, the functional relationship between the homologues is readily apparent. Conversely, how the involvement of Neogenin in terminal neuronal differentiation relates to the function of its worm homologue, UNC-40, is not known. When directly comparing homologues to *C. elegans*, it is therefore important to make several considerations. First, the homologue may have no obvious function with respect to a specific phenotype due to redundancy with multiple homologues. Second, the homologue may have only a subset of phenotypes that relate to the worm gene due to an allocation of function amongst multiple homologues. Finally, the homologue may have a function that is unrelated to what is known about the worm gene either because the homologue has been allowed to take on divergent functions, or because of incomplete characterization of either gene.
1.6 The Roles of Semaphorin Outside of Axon Guidance

Unlike other guidance molecules such as the Netrins and the Ephrins, the Semaphorins have not been described to function as cues that guide the migration of cells. To date, the characterization of the guidance functions of the Semaphorins has only revealed a role in axon migration. However, mutational and expression analysis of various Semaphorin family members suggest that they function in many different developmental and pathological processes.

*Semaphorins as Tumor Suppressors*

The correlation of chromosomal deletions with tumor progression implies the existence of tumor suppressors within the deleted locus. Region p21.3 of human chromosome 3 is deleted in small cell lung cancers (SCLCs) and cell lines. Interestingly, three independent research groups have found two *semaphorin* genes, namely *H-semA-V* and *H-semalV* that reside within the 3p21.3 locus (Sekido *et al.*, 1996; Roche *et al.*, 1996; and Xiang *et al.*, 1996). Sekido *et al.* (1996) have also found that the two *semallII* homologues undergo a loss-of-heterozygosity in SCLC cell lines. The workers went on to show that H-Sema-A(V) was expressed in only 1 of 23 SCLCs and 7 of 16 non-SCLCs, compared to 19 of 23 SCLCs and 13 of 16 SCLCs for H-SEMAIV. Both Roche *et al.* (1996) and Sekido *et al.* (1996) have shown that not only are the *semaphorins* deleted in immortalized cell lines, but these genes are also disrupted in some real lung cancers. These results suggest that the Semaphorins may have a role in tumor suppression.

5q21-22 is a second locus that is also deleted in many SCLCs. In contrast to locus 3p21.3, which is deleted in the early stages of carcinogenesis, 5q21-22 is deleted in the late stages of tumor development. Ueno *et al.* (1998) have cloned an 8 to 10 megabase (Mb) region that encodes at least 14 RNAs, including one for CD100, a class IV Semaphorin (figure 1.2; Hall *et al.*, 1996). Whether CD100 is involved in the etiology of SCLC is not known, but two results
suggest that it possible. First, CD100-transfected cells can improve the viability and induce the aggregation and differentiation of B-cells (Hall et al., 1996). An enhancement of this phenotype is observed when the cells are incubated with a third group of cells that are transfected with CD40L, a B-cell crosslinker (Clark and Ledbetter, 1994). Normally CD40L induces the B-cell expression of CD23, an IgE receptor that induces B cell aggregation only upon autoproteolysis (Gordon, 1992). Co-culture with CD100 positive cells results in the proteolysis of CD23 on B-cells, whose expression was induced by CD40-transfected cells (Hall et al., 1996), suggesting that CD23 mediates the effects of CD100 on B-cells. It is conceivable that CD100 acts as a tumor suppressor by reinforcing cell adhesion and in turn provides a barrier against metastasis.

Second, although CD100 is normally expressed by germinal center B-cells, CD100 expression is not observed in 93% of follicular non-Hodgkin's lymphomas (Dorfman et al., 1998). Together these results suggest a model whereby Semaphorin may be required to prevent metastasis through increased cell affinity.

Finally, it should be noted that the SemalII receptor Neuropilin-1 is also a receptor for a major splice variant of vascular endothelial growth factor 165 (VEGF, Soker et al., 1998). Not only is VEGF required for angiogenesis and vasculogenesis during normal development, but it is also highly expressed by tumors (Dvorak et al., 1991). That VEGF is required for the vascularization of tumors is demonstrated by halting further tumor growth by interfering with its function (Kim et al., 1993; Kendell and Thomas, 1993). Two lines of evidence suggest that Neuropilin may play a role in VEGF-mediated vascularization. First, Neuropilin-1 probably acts in a complex with the tyrosine kinase-insert domain-containing receptor (KDR) to mediate VEGF activity (Soker et al., 1998). Second, pan-over-expression of Neuropilin-1 results in excess capillaries and blood vessels (Kitsukawa et al., 1995). Although purely speculative, it is plausible that Semaphorin expression by tumors can block VEGF-Neuropilin interaction and thereby prevent the vascularization of tumors. If this were so, the early disruption of semaphorin
genes would be essential to tumor progression. Further investigation into the roles of Semaphorin, VEGF, and Neuropilin during oncogenesis may lead to the discovery of drug targets and drugs that starve cancerous cells to death.

Unexplained Semaphorin Mutant Phenotypes

Mutation of either Drosophila semaphorin I or II results in a highly penetrant lethal phenotype where only 10 to 25% of the flies survive to adulthood (Yu et al., 1998; Kolodkin et al., 1993). In addition, escapers of the D-semaII mutant lethal phenotype have several behavioral defects such as visual disorientation, and the inability to drink or fly. Although the behavioral abnormalities can probably be explained by the requirement of D-SemaII for discrete target recognition of CNS neurons (Winberg et al., 1998), why D-semaI and II mutant flies die is a mystery. The expression patterns of the two Drosophila semaphorins outside of the CNS also cannot readily explain the lethality. While maternal contributions of Drosophila semaphorin is not observed, non-neuronal zygotic expression of D-SemaI is apparently limited to epithelial segment boundaries near the site of ventral muscle attachment at stage 14 (Yu et al., 1998). Non-neuronal expression of D-semaII RNA expression begins at stage 10 in weak epidermal stripes (Kolodkin et al., 1993), and is later observed in all muscles (Winberg et al., 1998). Although a post-mortem analysis of mutant semaphorin larvae has not been reported, flies that do eclose have no morphological defects.

Aside from the axon guidance defects previously discussed, mice homozygous for sema III mutations also have unexplained phenotypes. Two independent groups generated knockouts of murine sema III in different genetic backgrounds. Behar and colleagues (1996) used a 129 background, while Taniguchi et al. (1997) used both C57BL/6 and CD-1 mice. 129 and CD-1 strains homozygous for semaIII mutations die in utero, post-natally, and at weaning (Behar et al., 1996; Taniguchi et al., 1997), while the mutation in C57BL/6 strains confers no lethality. This
difference suggests that either C57BL/6 carries a suppressor mutation(s) of the semaphorin mutant phenotype or that the 129 or CD-1 strains carry an enhancer(s). Regardless, semalII mutants of the 129 and CD-1 genetic background probably die of heart failure as post-mortem analysis reveals that the right atrium is grossly dilated and the right ventricle is hypertrophied. The 12% of the semalII-129 or CD-1 mutants that survive to adulthood are small and are uncoordinated, but can still breed and rear progeny. These latter phenotypes are shared with semalII-C57BL/6 mutants. Interestingly, fusions of cervical bones and partial duplication of ribs is observed in semalII mutants of the 129 genetic background, but not that of C57BL/6. Details of this phenotype have not been reported for the semalII-CD-1 strain. No dermal, muscle, or limb irregularities were found in any murine semalII mutant. Although it is not understood how Sema111 is involved in these processes, it is speculated that it could restrain the growth of heart, bone and cartilage tissue.

1.7 General Summary

From the above survey of molecular axon guidance cues it is obvious that we are beginning to have a firm grasp on how a nervous system can establish a network of billions of connections in a stereotypical fashion. While there are undoubtedly groups of cues that are yet to be discovered, the large families that have been described have elucidated many general principles of axon guidance and have justified a reductionist genetic approach. Various combinations of different types of molecules guide an axon from its point of origin to its target destination in a step-wise manner. It is the combinatorial nature of guidance that allows a small number of cues to direct the precise connection of billions of cells. In many cases, these molecules are repeatedly used in different temporal and spatial locales. In addition, the very cues that guide axons can also be used in other developmental processes to direct cell migrations and instruct appropriate contact formation.
I have used the simple and well understood model organism, *Caenorhabditis elegans*, to further our understanding of how the Semaphorins are used in axon guidance and to investigate its unknown role in other areas of development. Based on what was known about the Semaphorins, I hypothesized that *semaphorin* mutations in *C. elegans* would result in axon guidance defects. In addition, because of the poorly understood roles of the Semaphorins outside of axon guidance, I hypothesized that *C. elegans semaphorins* may play a broad role in other developmental programs. The relatively simple and well-characterized anatomy of *C. elegans* might enable a better understanding of the roles of the Semaphorins throughout development. Furthermore, like UNC-6, LIG-1, and SAX-3, the findings may be directly applicable to analogous developmental programs in all animals.

Using reverse genetic techniques, I have cloned and characterized two *C. elegans semaphorins*. While *C. elegans* Semaphorin I may be required in the development of the neuromusculature that controls defecation, *C. elegans* Semaphorin II is required for axon guidance and plays a broad role in the regulation of cell contacts between epidermal cells. Semaphorin II regulates cell-cell contacts in a manner that is highly reminiscent of the its characterized function in growth cone repulsion and filopodial collapse. These results suggest that the Semaphorins may be a general tool of animal biology used to regulate contacts between a cell and its environment.
Chapter Two

The Cloning and Characterization of

C. elegans semaphorin I
2.1 Introduction

The stereotypical connections and paths taken by migrating cells and axons depend on a relatively small number of guidance cues that are used repeatedly throughout development for multiple tasks. Many of these guidance molecules are conserved from worms to humans. For example, the UNC-6 ligand is both a repellant and attractant to distinct sets of neurons that travel in a circumferential path in the nematode, *C. elegans* (Hedgecock *et al.*, 1990). The response taken by an axon upon encountering UNC-6 depends upon the receptor(s) expressed on the surface of the growth cone. A repulsive response to UNC-6 is mediated by the transmembrane receptors UNC-5 (Leung-Hagesteijn *et al.*, 1992, Hamelin *et al.* 1993) and UNC-40 (Colavita and Culotti 1998), while attraction is mediated by UNC-40 (Chan *et al.* 1996). The vertebrate UNC-6 homologues called the Netrins, function in an analogous manner. In the ventral floor plate of the spinal cord, the Netrins are thought to repel UNC-5-expressing trochlear motor axons (Serafini *et al.*, 1994; Leonardo *et al.*, 1997) and attract commissural neurons that express the UNC-40 homologue Dcc (Keino-Masu *et al.* 1996). Not only do the UNC-6/Netrins have similar effects on the filopodia of growth cones of highly divergent species, but they also direct the migrations of analogous growth cones and axons.

In contrast to the striking orthology of the UNC-5, -6, and -40 homologues between organisms as diverse as worms, flies, mice, and humans, functional homologies between the Semaphorin family members are not as readily apparent. For example, *Drosophila* Semaphorin I (D-SemaI) is required to repel fascicles at specific choice points in the PNS in a dose dependent manner (Yu *et al.*, 1998), while grasshopper (G)-SemaI promotes axon outgrowth and probably attracts axons over a short distance (Wong *et al.*, 1997). Similarly, the primary function of SemaII in *Drosophila* may be in discrete target guidance (Matthes *et al.*, 1995; Kolodkin *et al.*, 1993) while in *C. elegans*, SemaII has a major role in the morphogenesis of the ectoderm (chapter 4). Also, unlike the bifunctional nature of the UNC-6 and the Netrin molecules, only
SemE may act to both repel some growth cones and attract others (Bagnard et al., 1998), while most other Semaphorins either repel growth cones, such as D-SemaI (Yu et al., 1998), D-SemaII (Matthes et al., 1995; Winberg et al., 1998a), Ce-SemaII (chapter 4), SemA, SemD (Adams et al., 1997; Bagnard et al., 1998), M/R/H-SemaIII (Messersmith et al., 1995; Behar et al., 1996; Taniguchi et al., 1997), Sema Z1a (Shoji et al., 1998), Collapsin-1, Collapsin-2, Collapsin-3 (Luo et al., 1993; Fan and Raper, 1995; Koppel et al., 1997), or attract growth cones, such as G-SemaI (Wong et al., 1997), but not both.

Do Semaphorin family members from disparate species have orthology? Functional descriptions of the Semaphorins are incomplete and a better understanding of the role of the Semaphorins throughout the development of an organism may yet lead to cross-phylum similarities akin to the UNC-5, -6, and -40 paradigm. For example, an understanding of the functions of *C. elegans* SemaI within the nervous system and throughout development may reveal analogies comparable to the functions of SemaI in other model systems. For these reasons, I have undertaken the characterization of *C. elegans* Semaphorin 1 (els-1).

We have cloned and sequenced the els-1 cDNA, called evCe-SemaI, and have found that it is the second least conserved SemaI sub-family member with respect to predicted amino acid sequence. In contrast to the expression of G-SemaI and D-SemaI throughout the nervous system, els-1 reporter constructs are consistently expressed in only one neuron. Functional characterization shows that els-1 may be involved in the neuromusculature system required in defecation. My results show that disrupting the function of Ce-SemaI causes defecation defects. Furthermore, els-1 reporters show expression in the four enteric muscles and possibly one of the two neurons that control the expulsion step during defecation. It may be that Ce-SemaI expression in the enteric muscles is required to attract the proper neural connections, similar to the function of G-SemaI attracting axons from the subgenual organ. Alternatively, Ce-SemaI may function to repel axons that would otherwise form ectopic synapses to the axon or the
enteric muscles that control defecation, akin to the function of D-SemaII. These results suggest that Ce-SemaI may have functions analogous to either G-SemaI or D-SemaI.

2.2 Results

2.2.1 The Cloning of *C. elegans* semaphorin I

Since the Semaphorin family of genes was known to exist in viruses, grasshoppers, moths, flies, and humans, a degenerate PCR approach was used to find a *C. elegans* semaphorin homologue. Degenerate primers were designed from conserved regions of the semaphorin domain by Kolodkin *et al.* (1993), and by me, and used in polymerase chain reactions (PCRs) at non-stringent cycling conditions. Various PCR primer combinations and PCR techniques were used as described in the materials and methods. PCR amplicons of the predicted size were cloned and sequenced. The amplicon sequence was translated into three reading frames and compared to all sequences reported to GenBank using the BLASTp program (Altschul *et al.*, 1990). One amplicon out of approximately 60 had high matching scores to Semaphorin family members.

The primers used to generate this fragment, called *evceseml.2*, from *C. elegans* cDNA template were *sem* 105, and *sem* 515 (see materials and methods). In retrospect, *sem* 105 had incorrect pairing in only 3 of the 21 bases, all of which are at the 5' end, and *sem* 515 had only one incorrect pairing with the *C. elegans* semaphorin sequence.

The 1.2 kb *evceseml.2* PCR product was used to probe a Northern blot and hybridized to a single 2.7 kb band (figure 2.1). The DNA sequence of *evceseml.2* was compared to GenBank sequences using BLASTn (Altschul *et al.*, 1990) and identified *cm20b4* as an exact match. *cm20b4* is a *C. elegans* cDNA generated in a cDNA sequencing project (A. Coulson, personal communication). The portion of *cm20b4* that overlaps *evceseml.2* was sequenced and found to correspond exactly to *evceseml.2*. *cm20b4* however, identified two bands on Northern blots of
Fiye 2.1 Nortbern blots probed with serrmjplrorin I cDNA hgments. Both autoradiographs show a probed Northern blot of 5 µg of C. elegans poly(A)' RNA isolated from a wildtype (N2) population of mixed developmental stages. The probed blots were exposed to Kodak X-ray film with a radioactive reflector screen for over 2 days at -70°C. A. An autoradiograph of a blot probed with the 1.2 kb degenerate PCR fragment evcesemI.2 labelled with 32P. The resulting band is approximately 2.7 kilobases. B. An autoradiograph of the same type of blot as in (A) probed with the 3.0 kb cm20b4 cDNA clone labelled with 32P. The resulting two bands are approximately 2.4 and 2.7 kilobases. Markers labelled on either side of the autoradiographs are in RNA kilobase size units.

Figure 2.1 Northern blots probed with semaphorin I cDNA fragments. Both autoradiographs show a probed Northern blot of 5 µg of C. elegans poly(A)' RNA isolated from a wildtype (N2) population of mixed developmental stages. The probed blots were exposed to Kodak X-ray film with a radioactive reflector screen for over 2 days at -70°C. A. An autoradiograph of a blot probed with the 1.2 kb degenerate PCR fragment evcesemI.2 labelled with 32P. The resulting band is approximately 2.7 kilobases. B. An autoradiograph of the same type of blot as in (A) probed with the 3.0 kb cm20b4 cDNA clone labelled with 32P. The resulting two bands are approximately 2.4 and 2.7 kilobases. Markers labelled on either side of the autoradiographs are in RNA kilobase size units.
2.4 and 2.7 kb (figure 2.1). It was not known if the additional band of 2.4 kb was an alternative transcript. Screening a *C. elegans* cDNA library (a gift from S. Kim) with *cm20b4* revealed two populations of cDNAs; four clones hybridized to *evcesem1.2*, and more than 6 did not (data not shown). Sequence analysis of the portions of *cm20b4* that did not overlap with *evcesem1.2* revealed that *cm20b4* was a chimera of two clones.

One clone isolated from the library screen, called *evcesem2.35*, is missing only 350 bases of 5' sequence relative to *cm20b4* (figure 2.2). The two cDNAs were spliced together to create the full length *C. elegans* semaphorin cDNA called *evcesemfl*. The size of *evcesemfl* corresponds to the size found on Northerns probed with *evcesem1.2*. The predicted protein product of *evcesemfl* has all of the hallmarks of a Semaphorin I-type molecule, including a signal sequence, a 490 amino acid semaphorin domain, a transmembrane domain and an intracellular domain of no known homology (figure 2.2). I therefore named the gene *C. elegans* semaphorin I or *els-1*, and the predicted protein product Ce-Semal according to the nomenclature of invertebrate Semaphorins.

Fourteen out of a possible seventeen cysteines conserved within the Semaphorin family are present in the *els-1* predicted protein product (figure 2.3). As shown in table 2.1, of the four other members of the Semai family, the semaphorin domain of Ce-Semal is most like that of G-Semai; of the non-Semai family members considered, it is most like Collapsin-1, followed by Ce-SemalII and D-SemalII.

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<td>41</td>
<td>42</td>
<td>35</td>
</tr>
<tr>
<td>G-Semal</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>60</td>
<td>39</td>
<td>47</td>
<td>36</td>
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<tr>
<td>D-Semal</td>
<td>-</td>
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<td>38</td>
<td>49</td>
<td>37</td>
</tr>
<tr>
<td>Ce-SemalII</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>36</td>
<td>-</td>
<td>32</td>
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<td>D-SemalII</td>
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<td>-</td>
<td>36</td>
</tr>
</tbody>
</table>

The table gives the percentage of amino acid identity between the predicted Semaphorin domains of selected Semaphorin family members.
Figure 2.2 Construction of the *els*-1 cDNA and the predicted domain structure of Ce-SemaI

A. A schematic of the clones used to construct the full length *C. elegans semaphorin I* cDNA clone, called *evcesemfl*. The degenerate-PCR product *evceseml.2* was used in a virtual screen of GenBank to find *cm20b4*. A cDNA library was then screened using 32P labelled *cm20b4* as a probe, resulting in the isolation of *evcesem2.35*. It was then discovered that *cm20b4* was chimeric. To build the full length *els*-1 cDNA, the 5' 297 bp of *cm20b4* was spliced on to *evcesem2.35* using the restriction site Pst I (P) to aid in cloning. B, Bam HI; E, Eco RI; S, Sma I; Xh, Xba I; Xh, Xho I. The asterisks represent the stop codon. The black boxes represent the predicted coding sequence of *els*-1 and the white boxes represent non-coding sequence. The blue box represents a chimeric portion of *cm20b4* that is not part of *els*-1. B. A Kyte-Doolittle hydrophilicity plot of the 730 amino acid predicted Semaphorin I protein sequence. The extremes of the ten unit hydrophilicity scale is shown to the left of the plot. The relatively long stretches of hydrophobic residues are highlighted in yellow, for the predicted signal sequence (SS) and blue, for the predicted transmembrane domain (TM). C. A schematic of the predicted domains of *C. elegans* Semaphorin I. The predicted signal sequence is yellow, the semaphorin domain is black with yellow stripes, the transmembrane domain is blue and the cytoplasmic domain is green. Note that the indicated start and stop codons in (A) are aligned with the beginning and end of the sequence of Ce-SemaI in (B) and (C).
Figure 2.3 A multiple sequence alignment showing conserved residues in Ce-Semal
The alignment includes G-Semal (accession # L00709), Tribolium Semaphorin I (T-Semal, accession # L26080), D-Semal and II (accession # L26082 and L26083, respectively), C. elegans Semaphorin I and II (no accession numbers yet) and chick Collapsin-I (accession # U02528).

The predicted CeSemal signal sequence is underlined, and the transmembrane domain is boxed.

Sequences were aligned using the Maligned program (Clark, 1992) and the alignment of Kolodkin et al. (1993) as a guide.
A third *C. elegans semaphorin* homologue has recently been sequenced by the Sanger Center *C. elegans* sequencing consortium (Cambridge, UK). The predicted protein product has the same domain configuration as Ce-Sema1 and shares 25% amino acid identity with Ce-Sema1 over the Semaphorin domain (table 2.1). I call the predicted protein of the third *C. elegans* semaphorin Ce-Sema1b. For more information on Ce-Sema1b see chapter 5.

Yuji Kohara’s *C. elegans* cDNA sequencing project isolated a third *els-1* cDNA fragment called *yk12h7* from the EST group number CELK00656 (Y. Kohara, personal communication). Y. Kohara’s group physically mapped *yk12h7* through hybridization to a yeast artificial chromosome (YACs) grid that contains most of the *C. elegans* genome. YACs Y54E5 and Y53C6 contain two overlapping genomic fragments that specifically hybridize to *yk12h7*. Seven contiguous cosmids that were defined by the overlap between the two YACs were digested with various restriction enzymes and probed with evcesemfl.2 (figure 2.4). Two overlapping cosmids called K09A10 and F14B11 hybridized to the *els-1* probe, but because K09A10 was prone to rearrangements, only F14B11 was further characterized. Restriction mapping of a 13 kb sub-clone of F14B11 called evg5 revealed a similar pattern of restriction sites as evcesemfl cDNA. This result suggested that evg5 contained at least the entire coding sequence of the *els-1* predicted product. Analysis of several small fragments of evg5 showed 100% sequence identity to evcesemfl, further supporting the conclusion that evg5 encodes Semaphorin I. The *C. elegans* sequencing consortium recently sequenced most of Y54E5, including the *els-1* locus, confirming the accuracy of my restriction map and the conclusion that evg5 encodes the *els-1* gene. However, without a loss-of-function mutation to test the rescue of evg5, this supposition cannot be tested genetically. Figure 2.5 shows 13 kb of the *els-1* gene sequence defined by two Xba I
Figure 2.4 *els-l* is on cosmid F14B11. A. At the time of experimentation, the physical map of the region defined by the overlap of YACs Y54E5 and Y53C6 was covered by about seven cosmids and two gaps. Presently there is only one gap. The cosmid carrying the *unc-54* gene is indicated. K11B4 (as indicated by an asterisk) was not obtained, prepped, digested or blotted. The drawing is not to scale. B. The DNA from cosmids W05D9, W07D8, F14B11, K05C4, F39B2, and K09A10 was digested with Hind III. C03C9 was digested with Eco RI. The digested DNA was separated in a 1% agarose gel containing ethidium bromide and photographed using UV trans-illumination. The isolate carrying the cosmid K09A10 had lost the cosmid before or early during propagation. Size markers are indicated. C. A Southern blot of the gel shown in (B). The blot was probed with a fragment of *cm20b4* which hybridized to a 4 kb and a 1.8 kb Hind III fragment of F14B11 (arrowheads), corresponding to the Hind III sites at -1267, 3028, and 4938 relative to the initiator methionine of *els-l*. D. The same blot as in (C) but stripped and probed with the PCR fragment *evceseml.2*. As expected, only the 1.8 kb Hind III band of F14B11 hybridizes to the probe (arrowhead). M indicates the marker lane. Faint bands can be seen at the 1.6 kb maker band to which all probes in my hands non-specifically hybridize to. E. The restriction map of evg5, the subclone of F14B11. The double vertical line represents the breakpoint between K11B4 sequence homology to the left, and Y54E5 sequence to the right. Xb, Xba I; E, Eco RI; B, Bam HI; H, Hind III; S, Sac I; Xh, Xho I; C, Cla I; P, Pst I; ATG, the predicted initiator methionine; *, the predicted stop codon. The scale bar represents 1 kb.
Figure 2.5 The els-1 gene
A 10039 bp fragment of the Y54E5 YAC clone containing the els-1 gene. Shown is the nucleotide sequence of els-1 beginning at a Hind III site at -1267 and ending at the Xba I site at 8772. Numbers are relative to the adenosine of the predicted initiator methionine (+1). The candidate TATA box at nucleotide -344 and the poly-adenylation signal at nucleotide position +8242 is underlined twice. The poly-adenylation tract begins at +8256. The line on which each exon begins is numbered in the second column. Coding sequences are underlined.

sites, which is contained within the evg5 sub-clone. Figure 2.4e illustrates the intron-exon structure of the els-1 locus. There are 14 predicted exons spaced over 8000 base pairs (bp).

Typical of many C. elegans genes, the largest intron of els-1 is the first, which is 2859 bp. The smallest intron is the tenth, with only 47 bp.

2.2.2 Disrupting the function of semaphorin I

The cosmid that carries els-1, F14B11 is less than 40 kb to the left of the cosmid that carries unc-54. Based on the very small distance from unc-54, a gene whose genetic and physical position are known, the genetic position of els-1 is approximately 27.21 map units relative to dpy-5 at 0.00 on the right arm of linkage group I. Several mutants for which the gene has not yet been identified genetically map to the left of unc-54, and are therefore candidate els-1 mutants.

Arrays containing F14B11 or the els-1 sub-clone evg5 cannot complement or rescue the mutant phenotypes of let-49, which dies during larval development, unc-122, which coils when the worm moves backwards or aex-5, which is constipated. This result suggests that let-49, unc-122, and aex-5 do not contain lesions in the els-1 locus. Alternatively, F14B11 or evg5 may not contain all elements required to transgenically rescue animals with a semaphorin I loss-of-function mutation.

Pre-complementation screens were used as a second approach to isolate an els-1 mutant.

A pre-complementation screen requires a stable genetic line that contains an extra-chromosomal
array containing the gene of interest that is transmitted to approximately half of the offspring. In many ways extra-chromosomal arrays behave like chromosomes except that they can be lost in both somatic and germ-line tissues at certain frequencies that vary depending on the genetic line. After a population carrying the array is mutagenized, individuals are cloned. F1s or F2s from a clone are screened for candidate mutations in the gene of interest. Only clones whose non-transgenic offspring are mutant, and whose transgenic offspring are rescued for that mutant phenotype, are candidates that may contain a mutation in the gene of interest.

The screen makes two assumptions. First, it must be assumed a priori that extra-chromosomal arrays carrying the transgene of interest can rescue the mutant phenotype(s) that result from the chromosomal mutation of the respective gene. Second, success depends on a mutant phenotype that is detectable at the level of the dissection microscope.

The progeny of 3796 mutagenized transgenic F2s that contained the extra-chromosomal array Ex[els-l(+)(evg5); rol-6(su1006)] were screened for phenotypes that were rescued by the array. 43 candidates that were either dependent on the array for viability or for rescue of a mutant phenotype were isolated. None of these mutants however, were linked to the unc-54 locus, and hence to els-l. A hemizygous variation of this screen was done to increase the efficiency of obtaining mutants in the region uncovered by a large chromosomal deficiency. 3653 F1s were cloned from a mutagenized parent population containing either the extra-chromosomal array Ex[els-l(+)(evg5); rol-6(su1006)] and the deficiency eDf11 which was genetically balanced by let-r202, or the extra-chromosomal array Ex[els-l(+)(F14B11); rol-6(su1006)] and the deficiency eDf15 balanced by let-r202. The non-transgenic progeny of each F1 were screened for mutant phenotypes that were rescued in transgenic siblings. Of the 12 candidates isolated in this screen, none were linked to the els-l locus.

Since forward genetic screens did not yield a semaphorin mutant, a transgenic approach was used in hopes of defining a function(s) of the els-l gene. Like the Ephrins (Davis et al.,
1994), it was hypothesized that Ce-SemaI must first associate with itself in the membrane in order to signal or function. Based on this assumption, a construct was built that was predicted to act in a dominant-negative fashion: *evpDNTMI* includes most of *els-1*, including the signal sequence, the transmembrane domain and the intracellular domain, but lacks most of the semaphorin domain (figure 2.6). If Ce-SemaI does indeed act within a complex, the non-functional *evpDNTMI* product is predicted to sequester endogenous Ce-SemaI binding partners, thereby reducing the efficiency of the pathway.

A second type of construct that was built in anticipation of disrupting Ce-SemaI function was a mini-gene version of *els-1* that had 1.8 kb of coding sequence inverted, called *evpAnti-MGS* (figure 2.6). It was reasoned that the "anti-sense" message transcribed from *evpAnti-MGS* would interfere with the endogenous function of *els-1* in much the same way that RNAi disrupts gene function (A. Fire and S. Harrison, personal communication, WBG, 10 (2):89). Observation at the level of the dissection microscope showed that animals transgenic for integrated versions of extra-chromosomal arrays containing *evpDNTMI* or *evpAnti-MGS* were constipated.

Detailed descriptions of the defecation cycle of *C. elegans* are given by Thomas (1990) and will be summarized here. Wildtype *C. elegans* defecates every 40 to 50 seconds. Three steps of coordinated muscle contraction called the defecation motor program (DMP) forces fecal matter out through the anus. First, the posterior body wall muscles contract, a step called pboc (posterior body contraction). This forces intestinal contents towards the anterior end. A negative pressure caused by the relaxation of the posterior body wall muscles then drives the intestinal contents towards the posterior end. A second after pboc, the anterior body wall muscles or head muscles contract, resulting in an anterior body contraction (aboc) which in turn causes the pharynx to further compress intestinal contents. Finally, the enteric muscles co-ordinate contractions at the apex of aboc to expel (exp) the feces from the posterior intestine and rectum.
Construct

A. *els-I*(+) (*evg5*), a wildtype *els-I* genomic fragment

![Diagram of construct A]

B. *evpDNTMI*, an in-frame deletion of the *els-I*(+) semaphorin domain

![Diagram of construct B]

C. *evpMGS*, an *els-I* minigene

![Diagram of construct C]

D. *evpAnti-MGS*, the *evpMGS* minigene with 1.8 kb of coding sequence inverted

![Diagram of construct D]

E. *evpXS.GFP75*, the 5' regulatory elements of *els-I*(+) driving GFP expression

![Diagram of construct E]

Phenotype

not tested

constipated

constipated

constipated

wildtype

Figure 2.6. A schematic of *evg5*-related constructs and their phenotypic consequences.

A. The *evg5* sub-clone of the F14B11 cosmid that is predicted to contain most of the *els-I*(+) gene. B. The *evpDNTMI* construct that is predicted to act in a dominant-negative fashion since it encodes the signal sequence, the transmembrane domain and the intracellular domain of *els-I*(+), but not the majority of the semaphorin domain. *evpDNTMI* contains all of *evg5* up to the Cla I site, which is ligated to the Eco RV - Xho I fragment of the F14B11 cosmid. C. The *evpMGS* minigene includes all of *evg5* up to the Sac I site, followed by the *semaphorin I* cDNA. *evpMGS* includes the genomic 3' end of *els-I*(+) from Hind III to Eco RI. D. The *evpAnti-MGS* construct has the 1.8 kb Cla I fragment inverted, and 27 bases from the second Cla I fragment missing. E. *evpXS.GFP75*, an *els-I*:GFP reporter construct that has the putative 5' regulatory elements of *els-I*(+) driving GFP expression (see figure 2.9 and the text for greater details). The phenotypes of animals that are transgenic for these constructs are reported in the right hand column (see figure 2.7 and the test for greater details). The black boxes are known exons of *els-I*, and the green boxes are the exons of the GFP vector *pPD95.75*. The scale is indicated, and the restriction site abbreviations are as reported in figure 2.4.
The enteric muscles are a group of four muscles that are interconnected via gap junctions, consisting of two intestinal muscles, an anal sphincter and an anal depressor (White, 1988). The anal sphincter muscle wraps around the intestinal-rectal junction and opens only upon muscle relaxation. As the anal depressor muscle opens the anus and the anal sphincter relaxes, the intestinal muscles contract and expel the feces. If any one of pboc, aboc or exp is eliminated, defecation cannot occur and results in worms that are filled with intestinal contents, otherwise known as a constipation phenotype.

Figure 2.7 shows that compared to wildtype worms that have an expulsion step every defecation cycle, worms transgenic for evpAnti-MGS or evpDNTMI expulse in less than 65% of the observed defecation cycles, resulting in constipated worms. The phenotype is not dependent on the co-transformational marker used to follow the transgenic arrays since expulsion-defective transgenic animals were observed when either dyp-20(+) or the dominant rol-6(su1006) markers were used. Interestingly, animals transgenic for a construct encoding a minigene version of els-1 called evpMGS (figure 2.6), have defective expulsions as well (figure 2.7). Animals transgenic for an els-1 reporter construct that contains the putative 5' regulatory elements of els-1 (figure 2.6), however, are not constipated (data not shown).

Since the genetic map position of aex-5(sa23) roughly correlates with the physical map position of els-1 relative to unc-54, and the phenotype of aex-5 is similar to that of worms carrying evpDNTMI or evpAnti-MGS, the ability of F14B11 to rescue aex-5 was re-examined. Worms that are homozygous for the aex-5(sa23) mutation are defective in both the aboc and exp steps of the defecation cycle (Thomas, 1990). Since it is difficult to objectively evaluate defects in the aboc step of the defecation cycle, it is uncertain if the transgenic strains that contain evpDNTMI, evpAnti-MGS, or evpMGS are aboc defective. However, it is clear that F14B11 does not rescue the defective expulsion step of aex-5 (figure 2.7). Although these studies may have hinted at the function of els-1, a detailed evaluation of the cellular defects caused by these
transgenic arrays were not further investigated without first having a genetic loss-of-function of *els-1* in hand. That is, since the mechanism behind the transgene-induced phenotypes is not understood, in depth analysis of the phenotype was delayed until an *els-1* mutant was obtained.

A more direct approach was finally taken to isolate a genetic mutation within *els-1*. Zwaal et al. (1993) had previously shown that a transposon (Tc1) mediated reverse genetic approach to targeted mutagenesis in *C. elegans* was feasible. The authors obtained Tc1 alleles and Tc1-derived deletions from approximately 50% of the targeted genes. A detailed description of the methodology of Tc1 mediated reverse genetics is given in chapter 3 but will be summarized here for continuity. A Tc1 insertion into the gene of interest is obtained by using Tc1 specific primers in combination with gene specific primers. A PCR amplicon will only be

![Graph showing the percentage of cycles in which expulsion occurs for various strains. Four dpy-20(e1282°) (WT) animals had a total of twenty fecal expulsions in twenty defecation cycles. Six dpy20(e1282°); evls[els-1(+)] (evpMGS); dpy-20(+)) (MGS) animals had a total of twenty eight expulsions out of forty six defecation cycles. Thirty two dpy20(e1282°); evls[evpAnti-MGS; dpy-20(+)] (Anti-MGS) animals had a total of one hundred and twelve expulsions out of one hundred and seventy eight observed defecation cycles. Five evls[evpAnti-MGS; rol-6(su1006)] (Anti-MGS*) animals had a total of four expulsion out of ten observed cycles. Eleven dpy20(e1282°); evls[evpDNTMI; dpy-20(+)] (DNTMI) animals had a total of sixteen expulsions out of fifty seven defecation cycles. Four evln[evpDNTMI; rol-6(su1006)] (DNTMI*) animals had a total of eleven expulsion out of twenty nine observed defecation cycles. Finally, twenty four aex-5(sa23); evls[els-1(+)](F14B11); rol-6(su1006)] (aex-5;F14B11) animals had a total of three expulsions out of one hundred and twenty six observed defecation cycles. Percentages are marked on top of each bar.]

Figure 2.7 Animals transgenic for dominant-negative *els-1*-related constructs are constipated. A graph showing the percentage of cycles in which expulsion occurs for various strains. Four dpy-20(e1282°) (WT) animals had a total of twenty fecal expulsions in twenty defecation cycles. Six dpy20(e1282°); evls[els-1(+)] (evpMGS); dpy-20(+)) (MGS) animals had a total of twenty eight expulsions out of forty six defecation cycles. Thirty two dpy20(e1282°); evls[evpAnti-MGS; dpy-20(+)] (Anti-MGS) animals had a total of one hundred and twelve expulsions out of one hundred and seventy eight observed defecation cycles. Five evls[evpAnti-MGS; rol-6(su1006)] (Anti-MGS*) animals had a total of four expulsion out of ten observed cycles. Eleven dpy20(e1282°); evls[evpDNTMI; dpy-20(+)] (DNTMI) animals had a total of sixteen expulsions out of fifty seven defecation cycles. Four evln[evpDNTMI; rol-6(su1006)] (DNTMI*) animals had a total of eleven expulsion out of twenty nine observed defecation cycles. Finally, twenty four aex-5(sa23); evls[els-1(+)](F14B11); rol-6(su1006)] (aex-5;F14B11) animals had a total of three expulsions out of one hundred and twenty six observed defecation cycles. Percentages are marked on top of each bar.
generated if the transposon inserts relatively close to the recognition site of the gene specific primer (figure 3.1). Genomic DNA aliquots representing a frozen library of worms derived from a strain that has a moderate frequency of Tc1 transposition are screened in this fashion. The recovered Tc1 allele, however, does not necessarily confer a loss of function since the transposition target sites are adenine and thymine rich and therefore predisposed to inserting into introns (van Luenen and Plasterk, 1994). In addition, the Tc1 element is often efficiently spliced out during RNA processing (Rushforth and Anderson, 1996). To obtain a loss-of-function allele, a Tc1-derived deletion must be isolated. Tc1 excision results in a double-stranded break and leaves the DNA liable to exonucleases or imprecise repair (Carr and Anderson, 1994), occasionally deleting flanking DNA. To isolate Tc1-derived deletions, the strain carrying the Tc1 allele of interest is used to seed a second frozen library of worms. The DNA from this "sub-library" is then screened using only gene specific primers that flank the Tc1 insertion site. A deletion is detected by virtue of smaller PCR amplicon than that generated from the template of the parental strain (figure 3.1).

Figure 2.8 shows the primers used to screen the Tc1 library for a Tc1 insertion allele of els-1. Exhaustive screens of the Tc1 library with most combinations of els-1 specific primers and Tc1 specific primers resulted in the isolation of NW1033 [mut-2(r459)i, els-1(ev586::Tc1)i; dpy-19(n1347)III] using primers I.A. and R1 and the nested primer set I.B and R2. Worms homozygous for ev586::Tc1 have no phenotypes relative to heterozygous ev586::Tc1 or homozygous wildtype siblings at the level of the dissection microscope. A sub-library constructed from 150 six centimeter plates confluent with NW 1033 worms was screened for deletions in els-1 without success. The NW1033 sub-library was screened again for a second cis-Tc1 insertion in els-1(ev586::Tc1) (see figure 3.6, 3.7 and 3.8 of chapter 3 for more details), resulting in the isolation of els-1(ev587::cis-Tc1) (figure 2.8). Preliminary analysis suggests that cis-Tc1 elements have a greater somatic excision frequency (data not shown). To investigate if
the enhanced somatic excision is an indicator of a corresponding enhancement of germline Tcl-derived deletions, a sub-library is being constructed with the strain NW1244 (mab-20(ev600::cis-Tcl), mut-2(r459), els-1(ev587::cis-Tcl); dpy-19(n1347)III). The double was created to improve the fecundity and health of the strains carrying both alleles without losing the mutator mut-2(r459). Not only will this sub-library be screened for Tcl-derived deletions, but if it is observed that the DNA situated between the two cis-Tcl is frequently deleted, gene replacement will be attempted (see future directions in chapter 5).

A. The wildtype els-1 sub-clone, evg5

B. PCR Primers

C. els-1(ev586::Tcl)

D. els-1(ev587::cis-Tcl)

Figure 2.8 A schematic of an els-1 genomic restriction map, PCR primers, and Tcl alleles. A. The F14B11 cosmid-derived sub-clone evg5. B. els-1 PCR primer positions. The few restriction sites that are shown are for reference only. C. The position of the Tcl element in els-1(ev586::Tcl). The insertion was detected using the els-1 primers I.A, I.B, and the Tcl primers R1 and R2. D. The position of the Tcl elements in els-1(ev587::cis-Tcl), which was derived from a sub-library seeded with the NW 1033 (mut-2(r459), els-1(ev586::Tcl); dpy-19(n1347)III) strain. The second Tcl element was detected using els-1 primers I.D, I.C, and Tcl primers R1 and R2. The restriction sites are abbreviated as in figure 2.4. The order of the labelled nested primers corresponds to their order with respect to the 5' to 3' order of the gene. The scale bar is as indicated.
2.2.3 *semaphorin 1* Expression Patterns

A mixed population of worms was fixed according to the method of Finney and Ruvkin (1990) and stained with a monoclonal antibody mAb 6F8 that was used to clone the first Semaphorin, G-SemaI (Kolodkin et al., 1992). Unfortunately, the grasshopper monoclonal antibody did not recognize any antigen in *C. elegans*. Instead, three SemaI reporter constructs (figure 2.9) were built by inserting approximately 3.8 kb of DNA 5' to the predicted initiator methionine of *els-1* into β-galactosidase and green fluorescent protein (GFP) reporter vectors provided by A. Fire (Fire et al., personal communication; Heim et al., 1995). Transgenic animals containing arrays of *els-1*:lacZ reporter constructs (figure 2.9) exhibited a graded pattern of β-galactosidase expression (figure 2.10). Hypodermal and body wall muscle cells express the reporter greater in the posterior half than in the anterior half of larvae and adult animals. Hypodermal expression is observed early in mid embryogenesis, at the beginning of morphogenesis (200 minutes). β-galactosidase is also expressed from head nuclei tentatively identified as inner labial socket and sheath cells (data not shown).

**GFP** expressed from *evpXS.GFP75* is observed early in embryogenesis at about the 50-cell stage in the E lineage (figure 2.11). In larvae and adults, expression is observed in all body wall muscles, the vulva and uterine muscles, the enteric muscles, including the two intestinal muscles, the anal depressor and the anal sphincter muscles (figure 2.12). Reporter expression is also observed in the inner labial socket and sheath cells. **GFP** is consistently expressed in only one neuron and is tentatively identified as the AVL (White et al., 1988). The cell body of the neuron that expresses the **GFP** is situated near the nerve ring and the axon runs in a left to right direction around the nerve ring, and the axon process terminates near the pre-anal ganglion, all of which are features of interneuron AVL.
**A. els-1**

![Diagram A](image_a)

**B. evpXSC.LZ (els-1:pPD16.51-nls)**

![Diagram B](image_b)

**C. evpXSN.LZ (els-1:pPD16.51+nls)**

![Diagram C](image_c)

**D. evpXS.GFP75 (els-1:pPD95.75)**

![Diagram D](image_d)

**Figure 2.9. els-1 reporter constructs.** Transcriptional fusion reporter constructs were built from a PCR-generated els-1 fragment containing promoter and enhancer elements from the evg5 template. A. The evg5 sub-clone of F14B11. els-1 known exons are represented with black boxes. B. evpXSC.LZ is a reporter construct consisting of 4 kb of els-1 5' regulatory sequence driving β-galactosidase expression from pPD16.51 with the nuclear localization sequence (nls) removed. C. evpXSN.LZ is a reporter construct consisting of 4 kb of els-1 5' regulatory sequence driving β-galactosidase expression from pPD16.51 with an nls. The β-galactosidase coding sequence in both (A) and (B) is represented with blue boxes. D. evpXS.GFP75 is an els-1:GFP transcriptional fusion reporter construct. GFP coding sequences are represented by green boxes. The scale bar is 1 kb.

In two out of over 200 evls[els-1:GFP (evpXS.GFP75); rol-6(su1006)] animals examined however, a second unidentified neuron of similar conformation to AVL was also observed to express GFP, the significance of which is not understood. Other cells that express GFP under the control of evpXS.GFP75 include sperm (figure 2.12), and the distal tip cells throughout development (figure 2.13).
Figure 2.10 An els-1:LacZ expression pattern. Animals transgenic for extra-chromosomal arrays containing els-1:LacZ reporter constructs result in a graded anterior to posterior pattern. A. A Nomarski micrograph of an L1 larva of the genotype dpy-20(n1282): evEx[els-1:LacZ (evpXSC.LZ75)]; dpy-20(+)]. B. A Nomarski micrograph composite of a mid-stage larva of the genotype dpy-20(n1282): evEx[els-1:LacZ (evpXSN.LZ75); dpy-20(+)]. Anterior is to the left.

Figure 2.11. The els-1:GFP embryonic expression pattern. Embryos transgenic for evls[els-1:GFP (evpXSC.GFP75); rol-6(su1006)], containing 4 kb of els-1(+) DNA 5' to the initiator methionine, show early reporter expression before the 100 cell stage within the E cell lineage. A. An epifluorescent micrograph showing GFP expression in four cells of the E lineage. One cell is below the plane of focus. B. A DIC micrograph of the 50 cell-stage embryo (approximately) shown in (A). C. An epifluorescent micrograph showing GFP expression in the eight presumptive gut cells of the E lineage. D. A DIC micrograph of the 100 cell-stage embryo (approximately) shown in (C). Bar is 10 μm.
Figure 2.13 Expression of els-1::GFP throughout the migration of the DTCs. *evls*/*els-1::GFP (evpXSC.GFP); rol-6(*su1006*)* transgenic animals express GFP in both distal tip cells (DTC) throughout their migration (see figure 4.6 for a schematic). A. GFP is expressed in both the anterior and posterior DTCs (arrows) during the early L2 stage. C. Shown here is GFP expressed in the posterior DTC (arrow) during the L3 to L4 larval transition. GFP is also expressed in the anterior DTC (not shown) and posterior DTC (arrow) during the early (E) and late (G) L4 stage. DIC micrographs on the right are of those animals depicted in the epifluorescent micrographs on the left. Bar is 10 μm.
2.3 Discussion

In this chapter I have described the cloning of a *C. elegans* Semaphorin I homologue called *els-1*. Transgenic assays have demonstrated that *els-1* may be involved in the function or the development of the neuromuscular system involved in regulating defecation, a supposition supported by *els-1* transcriptional fusions that report expression in the enteric muscles and possibly one of the two neurons that regulate expulsion (Thomas, 1990).

The *els-1* gene has recently been sequenced to completion by the *C. elegans* sequencing consortium at the Sanger Center (Cambridge, U.K.). When comparing the restriction map and sequence tags from the F14B11 sub-clone evg5, to the sequence of *els-1* residing on Y54E5, I discovered a discrepancy between the two pieces of data. Approximately 600 bp at the 5' end of evg5 contained sequence that is homologous to K11B4 but not the released sequence of Y54E5, which begins where the sequence identical to K11B4 ends. K11B4 is a cosmid that resides approximately 40 kb to the left of F14 B11 on the current physical map. The simplest explanation for this discrepancy is that the physical map of the region spanning cosmid K11B4 to K05C4 is inverted (figure 2.4) and the sequence of K11B4 overlaps with the left end of F14B11. Alternatively, F14B11 could be a chimeric clone produced during shotgun cloning of the genome. It seems unlikely, however, that out of 100 MB of genomic DNA, F14B11 would be chimeric with a neighboring piece of the genome.

Since *els-1* sub-clones could not rescue candidate *els-1* mutants that were mapped just to the left of *unc-54* by others, a pre-complementation screen for *els-1* loss-of-function mutants was done. Although over 30 *els-1* mutant candidates resulted from screening over 7000 mutagenized genomes, none mapped to the *els-1* locus. It is plausible that those candidate *els-1* mutants could be suppressed by *els-1* over-expression or over-expression of an *els-1* variant (Stinchcomb *et al.*, 1985) generated during the formation of the extra-chromosomal arrays. Since extra-chromosomal arrays typically contain many concatemerized copies of the injected DNA,
transgene expression levels are generally higher than the endogenous expression level (see chapter 4 for more details). If mutants could be suppressed by els-1 over-expression, the respective mutant phenotype would be dependent on the extra-chromosomal array. The resulting mutants from the pre-complementation screen were not investigated further, since obtaining an els-1 loss-of-function mutation was the primary goal.

Why were no els-1 mutations isolated in the pre-complementation screen? First, as mentioned above, pre-complementation screen depends on a transgene that will rescue the desired loss-of-function mutation. Neither F14B11 nor the evg5 sub-clone was sequenced to completion; it is possible that the clones contain one or more mutations resulting from clone propagation. Arrays carrying such clones may generate a Ce-SemaI product that would be incapable of rescuing an els-1 loss-of-function mutation. Second, the relative position of evg5 within F14B11 is unknown; it is possible that either F14B11 and/or evg5 does not contain enhancer elements that are sufficient to rescue a loss-of-function mutation. Although 3.6 kb of DNA 5' to the predicted initiator methionine is sufficient to drive reporter expression in a number of cells, the resulting pattern of gene activity may not reflect that of the endogenous gene because of a loss of necessary DNA elements. Both of these explanations can also be applied to the failure of els-1 transgenes to rescue putative els-1 mutants, namely let-49, unc-122, and aex-5. Paradoxically, questions regarding the ability of the F14B11 or evg5 transgenes to generate functional products that are expressed in the correct cells at the correct time cannot be readily addressed without an els-1 mutant.

Without a mutant in hand, however, the function of Ce-SemaI was investigated using dominant-negative transgenes. Two lines of evidence suggest that els-1 has a role in C. elegans defecation. First, animals transgenic for the evpMGS, evpAnti-MGS, and evpDNTMI constructs were constipated probably because of lacking an expulsion event in every defecation cycle. The observation that a sense transgene (evpMGS) has a similar phenotype to anti-sense transgene
(evpAnti-MGS) is not unprecedented. A. Fire and S. Harrison (personal communication, WBG, 10 (2):89) observed that sense unc-54 transgenes over-express UNC-54 (myosin), which in turn resulted in a phenotype that was similar to those animals that carried an unc-54 anti-sense transgene that drastically reduced UNC-54 levels. The authors also reported that the removal of unc-54 fragments from a sense transgene again resulted in a phenotype similar to the unc-54 loss-of-function phenotype.

The second line of evidence that suggests that els-1 has a role in fecal expulsion is the expression patterns generated from GFP or LacZ reporters driven by putative els-1 promoter and enhancer elements. Among other cells and tissues, els-1 reporters are expressed in the four enteric muscles required for the expulsion step of defecation (Thomas, 1990). Expression is also observed in one of two neurons that control the enteric muscles, tentatively identified as the AVL (White et al., 1988).

On average, animals transgenic for evpMGS, evpAnti-MGS or evpDNTMI omitted between 47% and 72% of expulsions. The variability observed between transgenic strains carrying different co-transformation markers and between genetic lines of identical genotype may be explained by a variation of transgenic expression levels from arrays (further discussed in chapter 4). Not only do independent genetic lines of the same genotype carry different transgene copy numbers on arrays, but the level of expression from each copy may vary as well.

Exactly how the els-1 transgenes interfere with expulsion, however, is unknown. Over-expression of els-1 from evpMGS may deregulate the els-1 pathway by surpassing regulatory capabilities. For example, if the sub-cellular localization of Ce-Sema1 on the enteric muscles were important for function and regulated by anchorage to a limiting binding partner, then too much Ce-Sema1 would escape spatial regulation and possibly permit ectopic synapse formation or prevent endogenous synapse formation. Alternatively, the formation of concatemerized arrays may produce and contain rearranged version of the els-1 transgenic arrays that were
unintentionally created. To distinguish between these two alternatives will require an \( els-l \) antibody to examine both the sub-cellular localization and the protein levels of Ce-SemaI. Finally, animals transgenic for \( els-l \) reporter constructs are not constipated (data not shown), suggesting that the promoter and enhancer elements are not squelching limiting transcription factors essential for genes required in defecation.

The \( els-l \) anti-sense messages from the transgenic arrays may result in the down-regulation of the expression of the gene of interest by interfering with transcription or translation (A. Fire, and S. Harrison, personal communication, WBG, 10 (2):89). However, it is also possible that by some abnormal rearrangement the \( evpAnti-MGS \) transgene generates gain-of-function protein products.

Finally, it is unknown how \( evpDNTMI \) interferes with fecal expulsion. High levels of the \( evpDNTMI \) protein product may disrupt endogenous Ce-SemaI homophilic or heterophilic interactions by titrating Ce-SemaI binding partners required for proper signaling, possibly including Ce-SemaI cis-interaction. Alternatively, the \( evpDNTMI \) protein product may have gain-of-function properties that disrupt the development of cells and/or tissues in which it is expressed. However, the tissues in which the \( els-l \) promoter is active such as the hypodermis and body wall muscles are apparently normal in animals transgenic for \( evpDNTMI \), lending less support for the latter alternative.

While I am still searching for a chromosomal mutation that will cause a loss-of-function phenotype in \( els-l \) via \( Tc1 \)-mediated mutagenesis, speculation can only be made about the exact role of \( els-l \). Like \textit{Drosophila} SemaI, does Ce-SemaI function to repel axons at discrete choice points? It is easily imagined that Ce-SemaI expression from the AVL is required to repel selected post-embryonic axons such as the VDs from the AVL or the ventral cord. Since transgenic animals carrying \( evpMGS \), \( evpAnti-MGS \) or \( evpDNTMI \) are not obviously uncoordinated, however, the latter scenario is unlikely. It is plausible that Ce-SemaI expression
from the enteric muscles repel axons that would otherwise make ectopic contacts and disrupt the control of expulsion. On the other hand, Ce-SemaI may have a role in promoting axon outgrowth and attracting growth cones akin to grasshopper SemaI. The anterior to posterior graded expression observed in worms transgenic for els-1:LacZ reporter constructs could serve to promote the extension of the AVL and other axons in a posterior direction. In addition, expression of Ce-SemaI from the enteric muscles could attract axons and promote synapse formation. Finally, the discovery of a second semaphorin type I gene in *C. elegans* whose predicted product I call Ce-SemaIb brings redundancy into question. It is possible that the pre-complementation screens yield no els-1 mutants because the major functions of Ce-SemaI are redundant with Ce-SemaIb. Chromosomal mutation of all semaphorin genes in *C. elegans* will directly address these questions.

2.4 Materials and Methods

2.4.1 Molecular Biology

Molecular biology protocols not listed here were carried out using standard methodology described in Sambrook *et al.* (1989). Northern blots were prepared and probed as described in Spence *et al.* (1990).

2.4.2 Degenerate PCR

To find a semaphorin gene in *C. elegans*, a degenerate PCR approach was used. The following degenerate PCR primers were designed by Kolodkin, Matthes and Goodman (1993) from the grasshopper semaphorin I sequence (Kolodkin *et al.*, 1992): sem 105 was designed from amino acids DDCQNYI or 5'-GA(T/C)-GA(T/C)-TG(T/C)-CA(A/G)-AA(T/C)-TA(T/C)-AT(T/C)-3'; sem 230 was designed from amino acids ETAVEY(I/M) or 5'-AT-(A/G)TA-(C/T)TC-NAC-
NGC-NGT-(T/C)TC-3'; sem 353 was designed from amino acids PRPGQCV or 5'-CCN-(A/C)GN-CCN-GGN-CA(A/G)-TG(T/C)-GTN-3'; and sem 518 was designed from amino acids YCAWDNVE or 5'-(T/C)TC-NAC-(A/G)TT-(G/A)TC-(C/T)CA-NGC-(A/G)CA-(A/G)TA-3'.

In addition, I designed a nested set of degenerate PCR primers using the Semaphorin family consensus sequence provided in Kolodkin et al. (1993) as a guide. The primer sequences are as follows: sem 220 was designed for amino acids FFFRETA or 5'-TT(T/C)-TT(T/C)-TT(T/C)-(C/A)GN-GA(A/G)-CAN-GC-3'; sem 227 was designed for amino acids VEYINCG or 5'-GTN-GA(A/G)-TA(T/C)-ATN-AA(T/C)-TG(T/C)-GG-3'; sem 356 was designed for amino acids VPEPRPG or 5'-CC-NGG-NC(G/T)-NGG-(T/C)TC-NGG-NAC-3'; sem 515 as designed for amino acids DPYCAWD or 5'-TC-CCA-N(C/G)C-(A/G)CA-(A/G)TA-NGG-(A/G)TC-3'. The nested set of primers, sem 227 and sem 356 had restriction sites added to their 5' ends to facilitate cloning of the nested product into the Cla I and Xba I digested cloning vector pKS(-) Bluescript.

For typical concentrations of PCR components used, see the materials and methods of chapter 3. The polymerase chain reaction cycles were as follows: 5 minute hot start at 94°C, followed by three cycles of 94°C for 15 seconds, 49°C for 30 seconds, a ramp from 49°C to 72°C for 2 minutes, and 72°C for 1.5 minutes. The reactions then cycled for 36 more cycles of 94°C for 15 seconds, 52°C for 1 minute and 72°C for 1.5 minutes. After the cycles were complete, a 72°C extension period of 10 minutes allowed incompletely extended products to be completed. Reactions could be stored at 4°C indefinitely. All combinations of primers designed by Kolodkin et al., (1993) and me were used.

Reaction templates included C. elegans genomic DNA (a gift from Michel Hamelin), a C. elegans cDNA library (a gift from Stuart Kim), and cDNA that I made from C. elegans RNA (isolated by Shirley Chan, Marie Killeen or Rob Steven). The cDNA was made in batches using 1 to 4 µl of RNA. The RNA was incubated with 0.8 µg of random primer, 2 µl RNase Guard
(Pharmacia™) and 44 µl of ddH2O for 3 minutes at 94°C, and then cooled on ice for 10 minutes to prevent intramolecular annealing. To this mixture, 8 µl of 0.1M DTT, 2 µl of RNase Guard, 4.0 µl of 25mM dNTPs, 4.0 µl of Superscript reverse transcriptase, and 16.8 µl of 5X Superscript buffer were added. This mixture was incubated for 10 minutes at 22°C, then 45 minutes at 42°C, and 5 minutes at 94°C. The cDNA was then purified and isolated by recovering the eluate when the mixture was forced through Centricon™ tubes at 2750 rmps.

A fraction of each reaction was separated on a 1% agarose gel. Reactions that produced bands of the expected size were then separated on 1% low melting point gels. The candidate semaphorin fragments were manually cut out from the gel, and the DNA was isolated.

To clone the PCR amplicons, products containing Lem 227 and Lem 356 were digested with Cla I and Xba I and cloned into either pKS(-) Bluescript that had been digested with Cla I and Xba I. Alternatively, 5 µg of Eco RV digested vector was incubated at 72°C for two hours with 5 units of Taq enzyme (Boehringer Mannheim™), 1X Taq buffer, and 6 µl of 10mM dTTP in a reaction volume of 30 µl. This procedure, which I call TED, for TTPed treated vector, adds TTP onto the 3’ blunt ends of the Eco RV treated vector. Since Taq adds an ATP to the 3’ ends of PCR products, the amplicon is easily cloned into the TED treated vector.

2.4.3 Tc1 Alleles

The original Tc1 library (see chapter 3) was screened with the following primers for a Tc1 allele(s) of els-1. I.1 begins at 3349 bp relative the predicted initiator methionine of els-1, 5’-ATG CCG TCT ACA ACC TAT CAC-3’; I.2 begins at 3448 bp, 5’-AAC CAC CTG CAG AAC ATA TCG-3’; I.3 begins at 6359 bp, 5’-GGC GAT ACT AGA ATC CCA AGC-3’; I.4 begins at 6386 bp, 5’-TCG CAA TTT TCT GCT TCT CAC-3’; I.6 begins at 7748 bp, 5’-TTC ACA TTG GAT GAT GCG TCC C-3’; I.9 begins at -2085 bp, 5’-CTC ACC TGG TCT TCT GCT CTC G-
3'; I.10 begins at -1988 bp, 5'-GAT CTC TCT TGT TGG ATT CTG G-3'; I.11 begins at 1129, 5'-GGT GAG AAC AAT ACG CC-3'; I.12 begins at 1159, 5'-CCG CCA AGT CGA GAG AAA CAG C-3'; I.13 I.A begins at -235, 5'-CTG TGA GTG ACA CAT ACC CCG-3'; I.13 begins at 237, 5'-CAG GAG TCG TAA TCA TAG CG-3'; I.14 begins at 350, 5'TCG TCA GAG TAT GGA TAG TG-3'; I.C begins at 3371 bp, 5'-AAT TGA TAG GTT GTA GAC GGC-3'; I.D. begins at 3473, 5'-CTC GAT ATG TTC TGC AGG TGG-3'; I.Y begins at -856 bp, 5'-CAC ACT CTT TTC AAC ATC GGG TGC-3'; and I.Z begins at -783, 5'-TCA TTG TCG TGT CTG CC-3'.

PCR recipes and conditions are given in chapter 3. The sequence of the Tc1 specific primers L1, L2, R1 and R2 are given in chapter 3.

2.4.4 Constructs

evDNTMI was constructed using two F14B11 fragments. The 13 kb subclone of F14B11 called evg5 was cut with Cla I, treated with mung bean nuclease to remove the 3' overhangs (Sambrook et al., 1989), then cut with Xba. The 7.5 kb fragment of evg5 from the 5' Xba I site to the Cla I site was isolated. Next, the 6 kb gB11 F14B11 subclone was cut with Eco RV and Xho I, releasing the 3 kb Eco RV7212-Xho I10219 fragment. The two fragments were ligated together into a pKS(-) Bluescript vector cut with Xba I and Xho I.

The anti-sense construct evpAnti-MGS was a derivative of evpMGS, which was constructed in three steps. First, the 1.8 kb Hind III7863 to Eco RI8316 genomic fragment of a F14B11 subclone called evg9 and the SacI901 to Hind III2192 cDNA fragment from cesemfl were ligated into Sac I/Eco RI cut pKS(-). The 3 kb insert was then excised and isolated, as was the 3.8 kb fragment of evg5 generated from the 5' Xba I site to the Sac I43 site. Both fragments were ligated into Xba I/Eco RI cut pUC12 vector. Finally the 0.9 kb Sac I55 to Sac I901 cDNA fragment was cloned into the Sac I49/Sac I901 site of the pUC12 intermediate product to generate
evpMGS. To create the evpAnti-MGS minigene, a 1.8 kb Cla I fragment of evpMGS was simply inverted.

To create evpXSC.LZ and evpXSN.LZ, the reporter vector pPD16.51 was cut with Sma I and Xba I, except for evpXSC.LZ where pPD16.51 was first treated with Kpn I to release the nuclear localization signal and then re-ligated. To create evpXS.GFP75, the reporter vector pPD95.75 was opened with Xba I and Sma I. The els-1 promoter/enhancer DNA fragment was generated using primers T7 and I.M/ClaI which hybridizes to the sequence flanking the initiator methionine and has a 5' Cla I site to facilitate cloning. The sequence of I. M/Cla is 5'-CCA TCG ATG CGC AIG TAT CGC AIG GG-3'. After the PCR product was generated and cloned, it was cut with Cla I and the blunt ends were filled with Klenow (Sambrook et al., 1989), and then cut with Xba I. The 3.8 kb fragment was then cloned into the three reporter vectors.

2.4.5 Transgenics

Extra-chromosomal arrays containing constructs of interest were generated by micro-injection into the syncitial gonad of N2 or dpy-20(e1282a) (Mello and Fire, 1995). Transformants were selected based on the dominant roller phenotype of the rol-6(su1006) or the ability of the dpy-20(+) co-transformation marker to rescue a recessive dpy-20(e1282a) mutation present in the injected animal. Exposure of strains to 3000 rads of a $^{137}$Ce source was used to integrate extra-chromosomal arrays into genomic DNA (a good protocol is prescribed at http://www.dartmouth.edu/artsci/bio/ambros/protocols/ MGH_protocols/koelle_prot/X0007_integrating_arrays.html).

2.4.6 LacZ Staining

This protocol is modified from a protocol developed by Gufeng Xie and Eric Aamodt (personal communication). To prepare the formaldehyde fixative, 200 mg of paraformaldehyde is
dissolved into 900 μl of 5.0 μM of NaOH at 65°C for 15 minutes. Worms are collected into a microcentrifuge tube to which 500 μl of cold 2X MRWB (see appendix), 100 μl of dissolved formaldehyde, and 400 μl of ddH₂O is added. The suspension is mixed before and during a 35 minute incubation at 4°C, after which the worms are washed twice with 1.0 ml of TTB (see appendix). Next, 960 μl of TTB and 10 μl of β-mercaptoethanol are added and incubated for 10 minutes at room temperature while mixing. The tubes are then placed upright, the worms allowed to settle for 5 minutes, and then rinsed once with 1X BO₃ and 20 μl of DTT without mixing. The worms are then rinsed twice in ddH₂O and allowed to settle after each rinse.

To stain the fixed worms for β-galactosidase activity, the worms are incubated overnight at room temperature in a modified version of A. Fire's staining solution (personal communication, see appendix).

2.4.7 Cosmid DNA Mega-Preparation

Transgenic bacteria from a 50 ml culture grown overnight are spun down at 8000 rpm on a tabletop Sorvall™ centrifuge. The pellets are resuspended in 3 mls of ice cold cosmid solution 1 (see appendix A) for 5 minutes at room temperature, after which 6 mls of cosmid solution II (see appendix A) was added. The suspension was mixed well by inversion and stored on ice for 5 minutes. 4.5 mls of ice-cold cosmid solution III (3 M NaOAc, pH 4.8) was added and the suspension was mixed well by inversion and stored on ice for 5 to 60 minutes. The suspension was then spun at 10 000 rpm in a RC-5B Sorvall™ floor model centrifuge. The supernatant was filtered through Kimwipes™ into a clean polypropylene tube. The DNA was then precipitated with two volumes of ethanol (there is enough salt in the preparation to precipitate the DNA). The pellet was then resuspended in 400 μl of ddH₂O and 400 μl of 4M LiCl in a 1.8 ml microcentrifuge tube and spun at maximum speed in a microcentrifuge at 4°C. 60 μl of cosmid
solution IV was added to the supernatant and incubated at 37°C for 30 minutes, after which the DNA was precipitated with two volumes of ethanol and 10% 3M sodium acetate (pH 5.2). The DNA was resuspended in 500 µl of ddH2O and phenol, followed by phenol-chloroform, and chloroform (extracted in equal volumes of the organic solvents). The DNA was again resuspended in 100 µl of TE (pH 8.0) or ddH2O.

2.4.8 Pre-complementation Screens

This protocol has been modified from Supriya Shivakumar (personal communication). Transgenic lines of worms of either evEx[els-1(+)(evg5); rol-6(su1006)] or eDf11/let-r202(e2000); evEx[els-1(+)(F14B11); rol-6(su1006)] or eDf15/let-r202(e2000); evEx[els-1(+)(F14B11); rol-6(su1006)] genotype were generated by microinjection of approximately 100 µg/ml of each DNA into oogonia. Genetic lines were selected that transmitted the extra-chromosomal array, which is marked by the rol-6d transgene, to about 50% of the progeny. A population containing many roller L4 larvae was washed and incubated at room temperature in 0.5% of the mutagen, ethylmethonesulfonate (EMS) in 5 mls of M9 buffer (see appendix). The suspension was continually mixed on a rocker for four hours. Several hundred mutagenized rolling L4s were picked onto large (10 cm) culture plates seeded with the E. coli strain OP50. In screens that used hemizygous strains that carry a large deficiency uncovering the els-1 locus, healthy rolling F1's were cloned onto small (3 cm) culture plates. In non-hemizygous pre-complementation screens, groups of 5 to 10 F1's were picked onto medium (6 cm) culture plates, and the resulting rolling F2's were cloned onto small plates. Progeny of the clones that did not carry the marked extra-chromosomal array were screened for candidate els-1 mutations by screening for phenotypes (including lethality) that were rescued by the array that was present in rolling siblings. Several candidate els-1 mutant rolling F3 were cloned to insure the propagation of the observed phenomenon.
2.4.9 Mapping Candidate $els$-I Mutations from the Pre-Complementation Screen

If mutant candidates ($evXXX$) from the non-hemizygous screen were linked to the $els$-I locus, then the mutation should be linked to the $unc$-54 locus. To address linkage to $unc$-54, the $F_1$ from $+/unc$-54(e1152) males crossed with $evXXX$, $Ex[els$-I$(+) (evg5); rol$-6(su1006)] hermaphrodites were cloned. If $evXXX$ were linked to $unc$-54, then the candidate lethal mutation should be balanced by $unc$-54.

In the case of the hemizygous screens, single non-rolling $F_1$ hermaphrodites from $+/unc$-54(e1152) males crossed with $eDfXX/evXXX$, $let$-r202(e2000); $Ex[els$-I$(+) (FL14B11); rol$-6(su1006)] hermaphrodites were mated to several $eDf1/let$-r202(e2000) males. Only crosses that produced no rollers and some $unc$-54 $F_{1s}$ from the latter cross were considered. If, out of all the cloned $F_{1s}$, some $F_{1s}$ throw only wild type progeny, then the mutation is not linked to the $els$-I locus. In the case of $evXXX$ being a lethal allele of $els$-I, then out of all the cloned $F_{1s}$, no $F_{1s}$ should throw only wild type progeny; all $F_{1s}$ will either throw all or some $unc$-54s. In the case of $evXXX$ being a non-lethal $els$-I allele, then some of the cloned $F_{1s}$ should be balanced for that non-lethal $els$-I phenotype.

\[
\begin{array}{c}
\text{i.e.:} \\
\frac{evXXX, \text{let-r202}}{eDfXX} \varphi \times \frac{unc$-54}{+} \sigma \\
\downarrow \\
\frac{unc$-54}{evXXX, \text{let-r202}} \varphi \times \frac{eDf11/\text{let-r202}}{\text{let-r202}} \\
\end{array}
\]

- $evXXX$ not linked to $els$-I locus
  - $eDf11/unc$-54 phenotype: viable
  - $let$-r202/unc-54 phenotype: throws $unc$s
  - $let$-r202/let$-r202$ phenotype: non-viable
- $evXXX$ linked to $els$-I locus
  - $eDf11/unc$-54 phenotype: non-viable or otherwise
  - $evXXX, \text{let-r202}$ phenotype: non-viable
  - $let$-r202/unc-54 phenotype: throws $unc$s
  - $evXXX, \text{let-r202}$ phenotype: viable
Chapter Three

Tc1 Transposon-Mediated Reverse Genetics in C. elegans:
Improving the Efficiency of Allele Isolation

Although I was the principle investigator of this work, the data presented herein is a large
collaboration between me and many individuals and laboratories. Charles E. Warren
collaborated with me in the design and construction of the library. Additional help in the
construction of the original library was given by two summer students, Carolyn So and Sabeena
Chopra. Others have contributed to the construction of the sub-libraries, including Charles E.
Warren, Xiangmin Wang and Adam Hart, Lijia Zhang the “Tc1-technician”, and me. Lijia
Zhang also isolated many of the Tc1 alleles requested by various laboratories. Xiangmin Wang,
Adam Hart and I generated deletions within lig-1 and lig-3. Guy Tanentzapf and I generated the
deletion in ecr-b. Yo Suzuki and I generated the disruption in dbl-1. Of course, laboratories that
requested Tc1 alleles designed and delivered the appropriate PCR primers to us.
3.1 Introduction

The small genome and expediency of genetic analysis in *C. elegans* make it an excellent model organism for the study of the genetic control of metazoan development. It has been repeatedly shown that the information gained from the biological analysis of *C. elegans* can lead to a greater understanding of eukaryotic biology, including that of mammals (Chan *et al.*, 1996; Sternberg *et al.*, 1994). Evolutionary conservation of genetic pathways has enabled the transfer of information gained from invertebrate biology to the study of vertebrates. Since over 99% of the *C. elegans* genome has been sequenced to date (*C. elegans* Genome Sequencing Consortium, 1998) reverse genetics is now a widely accessible entry point to these genetic pathways. After targeted mutagenesis disrupts a suspected pathway component, enhancer and suppressor screens may identify other components within the biological process of interest.

While classical or forward genetics seeks to provide a molecular explanation for a given phenotype, reverse genetics is concerned with identifying a phenotype for a molecularly characterized gene. Although the approach is different, each strategy can yield similar information upon completion of analysis. Several reverse genetic approaches in *C. elegans* require only small amounts of genomic information, and each have their respective advantages and disadvantages. For example, in pre-complementation screens (Shivakumar *et al.*, 1995), only a functional transgene of interest is required to obtain a putative loss-of-function mutation in the respective chromosomal gene of interest. The screen makes use of the incomplete transmission of extra-chromosomal arrays (Mello and Fire, 1995) that carry the transgene to progeny and is best suited for genetic lines that pass on the array to about 50% of the progeny. F2 clonal pre-complementation screens rely on the transgene of the array to rescue a visible or lethal phenotype induced by chromosomal mutation in the respective gene of interest. Putative chromosomal mutations of interest can be isolated by screening for cloned F2s that throw F3s that are mutant only without the transgene. This approach may yield multiple alleles of the gene
of interest, but has the disadvantage of generating spurious mutations in genes other than those of interest that depend on the array for survival (see chapter 2). A second disadvantage of pre-complementation screens is that the transgene is presumed to be sufficient to rescue a loss-of-function phenotype generated by a chromosomal mutation in the gene of interest. Of course, it is difficult to test the validity of this assumption without the loss-of-function chromosomal mutation in hand. Finally, pre-complementation screens require that the loss-of-function mutation is detectable at the level of the dissection microscope.

A second method of targeted disruption of gene function is RNA interference (RNAi), which has recently been reviewed by Tabara et al., (1998). By injecting double stranded RNA that is identical in sequence to the amino acid coding sequence of a given gene product into C. elegans, the function of the targeted gene can be disrupted in both parental and filial generations (Fire et al., 1998). This technique has the advantage of requiring only a partial cDNA for the production of dsRNA. The functional inhibition of RNAi can last several generations (Tabara et al., 1998), and can interact with other loss-of-function mutations in a predictable manner. The major disadvantage, however, is that the mechanism of the epigenetic effect of RNAi is not understood, leaving questions of specificity and interactions with genetic phenotypes open to argument.

A third approach to targeted functional disruption of known genes relies on the polymerase chain reaction (PCR) to identify genomic polymorphisms in a gene of interest. After mutagenizing and dividing parental populations into “samples”, progeny from each sample are divided into aliquots. Genomic DNA from one aliquot is extracted, while the other worm aliquots from the same sample are frozen. Sample DNA is then screened for polymorphisms within the locus of interest using PCR. A genetic line carrying the predicted loss-of-function polymorphism can then be isolated from the candidate frozen worm samples by thawing the worm aliquot and cloning the reanimated worms. After progeny are generated, the parents are
assayed for the polymorphism using single-worm PCR, in turn resulting in the identification of the genetic line of interest. This technique was originally developed by Rushforth et al. (1993) using the *C. elegans* transposable element Tc1 as the mutagen. Insertion of a Tc1 element into the gene of interest, however, does not necessarily confer a loss of function. Not only does the transposon preferentially insert into AT-rich DNA sequence and therefore into introns (van Luenen and Plasterk, 1994), but Tc1 elements can be efficiently spliced out of the mRNA during message processing (Rushforth & Anderson, 1996). However, the transpositional frequency of excision is nearly as frequent as insertion. Tc1 excision however, creates a double stranded break in the site of insertion (Plasterk and Groenen, 1992) leaving the locus liable to exonucleases and imprecise repair (Plasterk and Groenen, 1992) which occasionally results in a deletion (figure 3.1). In much the same way insertions are isolated, Tc1-derived deletions can be.

The isolation of a Tc1-derived deletion, however, is a laborious task and requires two steps of library construction. First, a Tc-1 allele of the gene of interest must be isolated from the original library. Second, the strain carrying the Tc1 allele must be propagated within a “sub-library” to generate and isolate Tc1-derived deletion allele. Recently, the two-step process of Tc1-targeted mutagenesis has been circumvented through the use of a library made with worms that have been mutagenized with chemicals such as ethyl methanesulfonate (EMS) or trimethylpsoralen (TMP) and ultraviolet (UV) radiation, instead of the Tc1 transposon (Jansen et al., 1997). The chemically mutagenized library can be screened for small deletions by PCR in the same manner that Tc1-derived deletions are identified. However, since the mutagen may directly lead to the loss-of-function in a gene of interest, only one step of library construction is required.
A. Germ Line Insertion

B. Germ line Tc1 Excision and Imprecise Repair

Figure 3.1. An overview of transposon-mediated mutagenesis
A. A Tc1 insertion into the gene of interest is detected in a PCR using the distal gene-specific primer (GSP).1 and the Tc1-specific primer (TSP)-R1, followed by the nested or proximal set, GSP.2 and TSP-R2. Assaying the progeny of the Po confirms that the insertion occurred within germ line, and not somatic tissue. B. By using GSPs in a PCR designed to optimize the amplification of small fragments, Tc1-derived deletions can be detected in favor of the large and more numerous amplicons generated from either wildtype or Tc1-containing templates. The genetic background of the strain of worms used to isolate both the Tc1 insertions and the Tc1-derived deletions must facilitate a moderate rate of Tc1 transposition. In this schematic, a Tc1 derived deletion is detected using PCR with the distal primers GSP1 and GSP4, followed by the nested set GSP2 and GSP3. R1 and R2 are the 5' TSPs; L1 and L2, are the 3' TSPs; Po, the parental generation; F1, the first filial generation.

In this Chapter, I describe the construction and screening of a frozen library of worms containing mobile Tc1 transposable elements. The DNA samples of my Tc1 library are pooled in such a way that a large proportion of the library can be screened for as many as eight Tc1 alleles in a single day. The efficacy of obtaining Tc1-derived deletions is also improved by mating several strains of worms that contain different identified Tc1s to obtain a single strain
that contains multiple Tc1 alleles of interest. This strain is then used to make a "sub-library" that is screened for deletion derivatives of the many Tc1 alleles of interest. In addition, the sub-library can be screened for Tc1 elements in other genes, thus maintaining the library in perpetuity. I also demonstrate that the traditional fashion in which polymorphisms are genotyped within single worms can be improved on. I show that more than eight alleles can be genotyped from a single individual worm. Using these innovative techniques, we have isolated over 40 Tc1 and Tc1-derived deletions in over 30 genes.

3.2 Results

3.2.1 The Isolation of 33 Tc1 Alleles in 27 Different Genes

By using gene specific primers (GSPs) and Tc1 specific primers (TSPs), PCRs easily detect insertions of Tc1 transposable elements into a given locus from a complex template mixture (figure 3.1). Since transposition occurs both somatically and within the germ line (Emmons and Yesner, 1984; Ruan and Emmons, 1987; Zwaal et al., 1993), the efficient recovery of desired heritable insertions is dependent on differentiating between the signals generated from each type of template. Unfortunately, there is no qualitative difference between a PCR fragment, otherwise called an amplicon, which has been generated from a germline template or a somatic template. A germline insertion can however, be quantitatively distinguished from somatic insertions. The number of somatic insertions in a particular locus is, on average, constant within a given strain. A particular germline template can therefore outnumber somatic templates of the same locus if the germline insertion event occurs in one of the founding generations of a given population. For example, based on empirical observations (figures 3.4D, 3.5C, and 3.8A) a gross overestimation of the chance that a particular worm carries a somatic insertion for a given locus is 1/20. If a germ line insertion occurs in the parent generation of a plate seeded with five worms, the chance that any particular individual within future generations harbors the phenotypically silent Tc1
tends toward 1/10. In worms carrying the germ line insertion, every cell harbors at least one Tc1 allele at the locus of interest. Somatic templates, however, are harbored in only the progeny of the cell in which the somatic event occurred. The absolute number of germ line templates is therefore much greater than twice the number of somatic templates for a given locus. A serial dilution of the DNA templates from the population will therefore reveal a dilution whereby somatic templates are less likely to result in a PCR amplicon than germ line templates. Somatic events will therefore not interfere with the detection of germline events.

After processing over 500 independent samples of MT 3126 \{mut-2(r459); dpy-19(n1347)\}-derived populations (figure 3.2), each sample DNA was diluted by combining 15 together, thereby creating an “alpha-pool”. My screens have empirically shown (table 3.1) that an average final alpha-pool DNA concentration of 5 to 20 ng per μl of PCR mixture reduces somatic background to a minimum while retaining the ability to identify germ line events. This DNA concentration translates to a dilution factor of two and five for small and large alpha-pools, respectively. Initially there were not enough alpha-pools to create a useful second tier pooling system, however, once the number of alpha-pools reached 64, they were further pooled with 15 other alpha-pools thereby creating “beta-pools” (figure 3.3). Each alpha-pool belongs to three of twelve beta-pools, which are arranged along three co-ordinates (see appendix C).

Figure 3.4 shows the results of a typical beta-pool PCR screen for a Tc1 insertion into the \textit{C. elegans} homologue of an Eph ligand called \textit{lig-1}. Candidate positive alpha-pool addresses are chosen based on the similarity of amplicons between the three beta-pool co-ordinates. If the beta-pool reactions representing the three co-ordinates have similar amplicons in at least two out of three replicates, then the positive alpha-pool is further investigated. The 15 samples that make up the positive alpha-pool address are assayed for a similar candidate amplicon. If a sample is positive for the candidate amplicon in both replicate reactions, the sample DNA is serially
Table 3.1 Isolated Tc1 alleles are distributed throughout the *C. elegans* genome.

<table>
<thead>
<tr>
<th>Allele*</th>
<th>Strain*</th>
<th>Homologue and Gene Description</th>
<th>Cosmid</th>
<th>Orient Position of Insertion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ahr-1(ev609::Tc1)I</td>
<td>NW 1111</td>
<td>aryl hydrocarbon receptor</td>
<td>UNK⁶</td>
<td>R intron 5</td>
</tr>
<tr>
<td>2 cdh-x(ev576::Tc1)V</td>
<td>NW 1037</td>
<td>non-classical cadherin</td>
<td>F14B11</td>
<td>F -186, 5'</td>
</tr>
<tr>
<td>3 Ce(k-(ev640::Tc1)III</td>
<td>NW 1193</td>
<td>eEF-2 Kinase</td>
<td>F42A10</td>
<td>R -exon 2</td>
</tr>
<tr>
<td>4 Ce(k-(ev613::Tc1)</td>
<td>NW 1115</td>
<td>Rb tumor suppressor</td>
<td>C32F10</td>
<td>F intron 9</td>
</tr>
<tr>
<td>5 c-Knot(ev624::Tc1)V</td>
<td>NW 1133</td>
<td>cerebrus homologue</td>
<td>C35B12</td>
<td>F intron 2</td>
</tr>
<tr>
<td>6 dlb-1(ev580::Tc1)V</td>
<td>NW 1041</td>
<td>a TGF-B homologue</td>
<td>T25F10</td>
<td>F exon 4</td>
</tr>
<tr>
<td>7 dpr-1(ev167::Tc1)X</td>
<td>NW 1124</td>
<td>dopamine receptor</td>
<td>F01E11</td>
<td>F exon 4</td>
</tr>
<tr>
<td>8 dhp-2(ev597::Tc1)X</td>
<td>NW 1091</td>
<td>a da-f-12 homologue</td>
<td>F33D4</td>
<td>R UNK</td>
</tr>
<tr>
<td>9 dhs-ze(ev595::Tc1)X</td>
<td>NW 1089</td>
<td>a 2nd da-f-12 homologue</td>
<td>ZK662</td>
<td>R intron 9</td>
</tr>
<tr>
<td>10 dhs-ze(ev599::Tc1)X</td>
<td>NW 1090</td>
<td>a 2nd da-f-12 homologue</td>
<td>ZK662</td>
<td>R intron 8</td>
</tr>
<tr>
<td>11 ebn-1(ev598::Tc1)X</td>
<td>NW 1092</td>
<td>fork head transcription factor homologue of brain factor 1</td>
<td>T14G2</td>
<td>F intron 1</td>
</tr>
<tr>
<td>12 ecr-b(ev578::Tc1)X</td>
<td>NW 1039</td>
<td>a crumbs homologue</td>
<td>F11C7</td>
<td>R +3143, intron 9</td>
</tr>
<tr>
<td>13 eif-4(ev606::Tc1)</td>
<td>NW 1108</td>
<td>transcription factor eIF-4e</td>
<td>UNK</td>
<td>R intron 1</td>
</tr>
<tr>
<td>14 eif-4(ev607::Tc1)</td>
<td>NW 1109</td>
<td>transcription factor eIF-4e</td>
<td>UNK</td>
<td>F 5' to ATG</td>
</tr>
<tr>
<td>15 els-1(ev586::Tc1)I</td>
<td>NW 1033</td>
<td>a semaphorin I homologue</td>
<td>F14B11</td>
<td>R intron 1</td>
</tr>
<tr>
<td>16 els-1(ev587::Tc1)I</td>
<td>NW 1058</td>
<td>cis-Tc1s in els-I</td>
<td>F14B11</td>
<td>F intron 1</td>
</tr>
<tr>
<td>17 elt-3(ev616::Tc1)III</td>
<td>NW 1122</td>
<td>GATA transcription factor</td>
<td>K02B7</td>
<td>R 3' to Stop</td>
</tr>
<tr>
<td>18 ets-1(ev583::Tc1)X</td>
<td>NW 1044</td>
<td>fli-1 transcription factor</td>
<td>C42D8</td>
<td>F intron 1</td>
</tr>
<tr>
<td>19 ets-2(ev599::Tc1)X</td>
<td>NW 1093</td>
<td>a 2nd fli-1 homologue</td>
<td>T08H4</td>
<td>F UNK</td>
</tr>
<tr>
<td>20 gly-1(ev571::Tc1)I</td>
<td>NW 1032</td>
<td>core 2 GlcNac-T glycosylase</td>
<td>F44F4</td>
<td>F exon 3</td>
</tr>
<tr>
<td>21 gly-2(ev581::Tc1)I</td>
<td>NW 1042</td>
<td>GlcNac-T V glycosylase</td>
<td>C55B7</td>
<td>R intron 7</td>
</tr>
<tr>
<td>22 ipk-1(ev615::Tc1)X</td>
<td>NW 1121</td>
<td>the inhibitor of PKR</td>
<td>C55B6</td>
<td>F intron 3</td>
</tr>
<tr>
<td>23 lig-1(ev584::Tc1)IV</td>
<td>NW 1049</td>
<td>an ephrin type homologue</td>
<td>C43F9</td>
<td>R exon 4</td>
</tr>
<tr>
<td>24 lig-3(ev589::Tc1)X</td>
<td>NW 1073</td>
<td>a 2nd ephrin type homologue</td>
<td>F15A2</td>
<td>F 5' to ATG</td>
</tr>
<tr>
<td>25 mab-20(ev573::Tc1)</td>
<td>NW 1034</td>
<td>a semaphorin II homologue</td>
<td>NA</td>
<td>R +102, intron 1</td>
</tr>
<tr>
<td>26 mab-20(ev606::Tc1)I</td>
<td>NW 1094</td>
<td>cis-Tc1s in mab-20</td>
<td>NA</td>
<td>R intron 3</td>
</tr>
<tr>
<td>27 nid-1(ev608::Tc1)</td>
<td>NW 1110</td>
<td>Nidojen/Entactin</td>
<td>UNK</td>
<td>R -exon 2</td>
</tr>
<tr>
<td>28 pFAT(ev619::Tc1)X</td>
<td>NW 1128</td>
<td>Spectrin</td>
<td>K10B3</td>
<td>F -exon 10</td>
</tr>
<tr>
<td>29 prk-1(ev614::Tc1)X</td>
<td>NW 1120</td>
<td>PKR</td>
<td>F46C3</td>
<td>F -intron 3</td>
</tr>
<tr>
<td>30 unc-52(ev611::Tc1)II</td>
<td>NW 1113</td>
<td>Perlecan</td>
<td>C38C6</td>
<td>R intron 32</td>
</tr>
<tr>
<td>31 unc-52(ev612::Tc1)II</td>
<td>NW 1114</td>
<td>Perlecan</td>
<td>C38C6</td>
<td>R exon 32</td>
</tr>
<tr>
<td>32 was-1(ev604::Tc1)IV</td>
<td>NW 1106</td>
<td>a Wiskott-like gene (WASp)</td>
<td>C07G1</td>
<td>F exon 3</td>
</tr>
<tr>
<td>33 was-1(ev605::Tc1)IV</td>
<td>NW 1107</td>
<td>a Wiskott-like gene (WASp)</td>
<td>C07G1</td>
<td>R intron 4</td>
</tr>
</tbody>
</table>

*The genetic map positions that follow the allele designation are based on nearby mutations that are physically and genetically mapped.

*All strains listed are derivations of MT 3126 and therefore probably contain the alleles *mut-2(r459)I; dpy-19(n1347)III*.

*The orientation refers to that of the transposase coding sequence of the relative to the gene. The transposase and the gene are in the same orientation (F) or not (R).

*The numbers given for insertion position is with respect to the adenosine (+1) of the initiator methionine. Some positions of the transposons are close to the intron/exon boundary and their location is estimated (-) based on GSP location and amplicon size.

*Some of our collaborators have failed to provide information to me (UNK).
Figure 3.2. A schematic of library construction
A. Five MT 3126 [mut-2(r459); dpy-19(n1347)III] (Collins et al., 1989)-worms are placed on each of over 600 OP50 seeded NGM plates. B. Plates are incubated for about 10 to 12 days until plates are confluent with starved L1s. C. The worms are collected and divided into four aliquots, three of which are frozen at -70°C. D. The DNA of the fourth aliquot is extracted, 80% of which is used to create an alpha pool with 14 other samples and is stored at -70°C. The remaining 20% is divided into two and represents the individual sample DNA and can also be stored at -70°C.
Figure 3.3. The organization of individual samples within beta-pools
Eight tenths of the DNA collected from each sample is pooled with 14 other samples to create an alpha pool. A. 15 sample DNAs constitute one alpha-pool, 64 alpha-pools are used in the organization of beta-pools. B. There are 16 alpha-pools within each beta-pool, named X1-X4, Y1-Y4, and Z1-Z4. Each alpha-pool belongs to three beta-pools. For example, alpha-pool 1 would belong to beta-pools X1, Y1, and Z1, while alpha-pool 2 would belong to beta-pools X2, Y1, and Z1, etc. There are a total of 12 beta-pools; four in each of the three coordinates (see Appendix C for library details).
Figure 3.4. A typical screen for a Tc1 allele
A. A beta-pool screen for a lig-1::Tcl allele. Each beta-pool is screened in triplicate. The asterisks(*) shows three beta-pools, X1, Y2, and Z4 which are positive for a putative insertion using the GSPs C & D, and the TSPs L1 & L2 which generate a product of approximately 1 kb. The combination of positive beta –pools indicate that the alpha-pool 945, harbors the putative positive sample. B. A screen of samples 931 to 945 that constitute alpha-pool 945, and the confirmation that alpha-pool 945 is positive. Each sample and the alpha-pool are screened in duplicate. Sample 942 is positive. C. The positive template of sample 942 serially dilutes at least 128 times. D. The isolation of the strain NW 1049 [mut-2(r459); dpy-19(n1347)III; lig-l(ev584::Tcl)IV], showing the result of a single round of single worm PCR using primers D + L1. Hermaphrodite numbers 942.10, .12, .25, and .26 are positive for lig-l(ev584::Tcl). Except for D, gels show the products of the second round of nested PCR. M. 1 kb DNA ladder: A. 5' gene specific primers A+ B; L, Tc1 specific primers L1 + L2; C, 3' gene specific primers D + C; R, Tc1 specific primers R1 + R2; X, x-coordinate beta-pools; Y, y-coordinate beta-pools; Z, z-coordinate beta-pools. Non-specific bands are assumed to be somatic insertions.

diluted to ensure that the putative positive is generated from a germ line template and is not the result of a somatic insertion hot spot. If the template dilutes at least 8 times and still gives a similar amplicon, the sample is thawed and 50 to 300 worms are cloned, depending on the dilution factor of the template and the viability of the frozen sample. Single-worm PCR (SWPCR) is then used to identify which fertile parent carries the Tc1 allele of interest. Since the dilution factor of the template was greater than 128 times in the case of the lig-1::Tcl (figure 3.4, table 3.3), a small number of thawed parents were cloned and assayed; seven out of 48 individuals were positive for the Tc1 allele. The Tc1 insertion was germ line in all individuals tested since their progeny generated similar amplicons. Before there were enough samples to construct beta-pools, alpha-pools were directly screened in a similar fashion as that done for the lig-1::Tcl example above.

Table 3.1 also shows that the transposon orientation relative to the gene into which it is inserted is random, as 48% are in the same orientation and 52% are not. 73% of the transposons have inserted into non-coding regions. The bias Tc1 insertion sites probably reflect the preference of the transposon to insert into AT rich sequence (table 3.2; van Luenen and Plasterk, 1994; Korswagen et al., 1996), of which introns are rich.
Table 3.2. The positions of Tc1 insertion sites are conserved

<table>
<thead>
<tr>
<th>Allele</th>
<th>Insertion Site*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus</td>
<td>CAYAT (AT) ATRTG</td>
</tr>
<tr>
<td>cdh-4(ev576::Tc1)</td>
<td>ACGAGACAT (AT) AAATTAGCTG</td>
</tr>
<tr>
<td>ecr-b(ev578::Tc1)</td>
<td>AAAGTACAT (AT) ATATATACC</td>
</tr>
<tr>
<td>els-1(ev586::Tc1)</td>
<td>AT ATGTTCCTCC</td>
</tr>
<tr>
<td>mab-20(ev573::Tc1)</td>
<td>ACCATAACGAT (AT) CTGTGTACA</td>
</tr>
<tr>
<td>lig-l(ev584::Tc1)</td>
<td>ACCTGTCAT (AT) ATGATAGTG</td>
</tr>
<tr>
<td>gly-1(ev581::Tc1)</td>
<td>ACACAAACAT (AT) ATGCTCTGA</td>
</tr>
<tr>
<td>gly-2(ev581::Tc1)</td>
<td>CAATCAATAT (AT) ATATATAGTC</td>
</tr>
<tr>
<td>ahr-l(ev609::Tc1)</td>
<td>(AT) TAATATAAT</td>
</tr>
<tr>
<td>dbl-l(ev580::Tc1)</td>
<td>ACTGATTCT (AT) AGCTGATT</td>
</tr>
</tbody>
</table>

*The orientation of the reported insertion site is relative to the orientation of the transposon.

3.3.2 Improving the Efficiency of Library Screening

Library Construction

The construction of other frozen mutant worm banks use worms or "samples" that were raised on very large (10 cm) plates that contained more food than normal, thereby enabling the growth of three, four or even five generations of worms (Zwaal et al. 1993). While this method obviously works, we were concerned that the recovery of strains that carry the desired alleles may require a laborious sub-selection procedure (Rushforth et al., 1993). In addition, it might be difficult to determine the difference between somatic background and germ line events that occur in later generations in such large samples. Indeed, Zwaal et al. (1993) show that recovered alleles are derived from samples containing a high frequency (~50%) of positive worms, suggesting that the germ line event occurred in the parental or first filial generation and that culture past the third generation is superfluous. We sought to improve the efficacy of strain recovery by growing smaller sized samples with fewer generations, a technique implicitly suggested by Andachi and Kohara (personal communication, 1995). We hypothesized that samples grown on small (3.5 cm) or large (6 cm) plates containing normal amounts of food would allow easier recovery of candidate alleles. If more samples were required to achieve an adequate success rate, it was reasoned that screening efficiency could be improved through increased sample pooling and
Chapter Three

primer multiplexing techniques. Considering only the original library, which consists of 38 alpha-pools named 15 through 570 (appendix B), 23 pools were made from small samples, 9 were made from large samples and the remaining 6 were a mixture of the two sizes. Each small plate was seeded with two healthy hermaphrodites and each large plate was seeded with five healthy hermaphrodites, which translates to roughly equal numbers of useful genetic lines derived from the small and large samples; 53,040 and 61,200, respectively. Five out of the twelve alleles isolated from the original library came from small samples, while 7 of twelve came from large samples (table 3.3), an anticipated ratio based on the relative number of mutated genomes that constitute the small and large samples. From the 33 Tc1 alleles isolated from 27 requests, an average of 10% of the assayed worms carried the desired allele (table 3.3 and table 3.5). While the recovery rate of Tc1 alleles of interest from our library is approximately 61%, the recovery rate from small and large samples were proportionately the same. In addition, no more effort was required to isolate an allele from a large sample than a small one. However, since 2.4 fold more genomes are represented in each large sample compared to each small sample, less effort and materials are therefore required to generate a library of large samples. Hence, only large samples were used to construct the library after the initial 570 samples.

Primer Multiplexing

Initially, Tc1 alleles were recovered from alpha-pool screens using primers for a single locus. Since most of the candidate amplicons fell into a size range of 500 to 1500 bp, and single reactions were able to generate multiple amplicons of that size range, I hypothesized that the

---

1 Based on the recovery frequencies, recovered alleles were probably generated from germ-line events occurring in the parental or first filial generation. On average, healthy MT 3126 hermaphrodites were observed to generate 33 fertile progeny. Each small sample therefore contained (1 parent + 33 F1s) x 2 parents x 2N = 136 independent genetic lines, and each large sample contains (1 parent + 33 F1s) x 5 parents x 2N = 340 detectable genetic lines. 26 small alpha-pools x 15 samples x 136 lines = 53,040 genetic lines. 12 large alpha-pools x 15 samples x 340 lines = 61,200 genetic lines.
### Table 3.3. The extent of sample dilution correlates with the frequency of positive individuals within the thawed population

<table>
<thead>
<tr>
<th>Allele</th>
<th>Sample Dilution&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Isolation Frequency&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Screening Technique&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Size of Pool&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ahr-1(ev609::Tc1)</td>
<td>3.9</td>
<td>64X</td>
<td>80% (75)</td>
<td>β/multiplex L, 240</td>
</tr>
<tr>
<td>2 cdh-x(ev576::Tc1)</td>
<td>4.8</td>
<td>16X&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2% (119)</td>
<td>α L, 240</td>
</tr>
<tr>
<td>3 Cejk-l(ev640::Tc1)</td>
<td>-1.4</td>
<td>32X</td>
<td>1% (126)</td>
<td>β/multiplex L, 900</td>
</tr>
<tr>
<td>4 Cerb(ev613::Tc1)</td>
<td>-0.0</td>
<td>64X</td>
<td>2% (102)</td>
<td>β/multiplex L, 780</td>
</tr>
<tr>
<td>5 c-Knot(ev624::Tc1)</td>
<td>1.6</td>
<td>128X</td>
<td>40% (20)</td>
<td>β/multiplex L, 585</td>
</tr>
<tr>
<td>6 dbl-1(ev580::Tc1)</td>
<td>0.0</td>
<td>16X</td>
<td>1% (149)</td>
<td>α L, 645</td>
</tr>
<tr>
<td>7 dpr-1(ev617::Tc1)</td>
<td>-2.2</td>
<td>128X</td>
<td>40% (47)</td>
<td>β/multiplex L, 735</td>
</tr>
<tr>
<td>8 dhh-ftev597::Tc1</td>
<td>3.5</td>
<td>128X</td>
<td>29% (17)</td>
<td>β/multiplex L, 555</td>
</tr>
<tr>
<td>9 dhh-z(ev595::Tc1)</td>
<td>22.3</td>
<td>128X</td>
<td>9% (35)</td>
<td>β/multiplex L, 705</td>
</tr>
<tr>
<td>10 dth-z(ev596::Tc1)</td>
<td>22.3</td>
<td>128X</td>
<td>25% (12)</td>
<td>β/multiplex L, 60</td>
</tr>
<tr>
<td>11 ebn-1(ev598::Tc1)</td>
<td>0.0</td>
<td>64X</td>
<td>6% (36)</td>
<td>β/multiplex L, 615</td>
</tr>
<tr>
<td>12 ecr-b(ev578::Tc1)</td>
<td>24.1</td>
<td>16X&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2% (42)</td>
<td>α S, 180</td>
</tr>
<tr>
<td>13 eif-4ev609::Tc1</td>
<td>128X</td>
<td>128X</td>
<td>2% (48)</td>
<td>β/multiplex L, 915</td>
</tr>
<tr>
<td>14 eif-4ev607::Tc1</td>
<td>32X</td>
<td>32X</td>
<td>2% (64)</td>
<td>β/multiplex L, 1105</td>
</tr>
<tr>
<td>15 els-1(ev586::Tc1)</td>
<td>27.2</td>
<td>32X</td>
<td>2% (91)</td>
<td>α L, 555</td>
</tr>
<tr>
<td>16 els-1(ev587::Tc1)</td>
<td>27.2</td>
<td>32X</td>
<td>2% (88)</td>
<td>sub-library/ α L, 825</td>
</tr>
<tr>
<td>17 els-3 (ev516::Tc1)</td>
<td>23.4</td>
<td>128X</td>
<td>21% (33)</td>
<td>β/multiplex S, 360</td>
</tr>
<tr>
<td>18 els-1(ev583::Tc1)</td>
<td>5.9</td>
<td>8X</td>
<td>1% (72)</td>
<td>α L, 615</td>
</tr>
<tr>
<td>19 els-2(ev599::Tc1)</td>
<td>5.25</td>
<td>128X</td>
<td>26% (43)</td>
<td>β L, 645</td>
</tr>
<tr>
<td>20 gly-1(ev571::Tc1)</td>
<td>3.1</td>
<td>UNK</td>
<td>1% (97)</td>
<td>α M, 135</td>
</tr>
<tr>
<td>21 gly-2(ev581::Tc1)</td>
<td>1.1</td>
<td>16X</td>
<td>2% (96)</td>
<td>α L, 720</td>
</tr>
<tr>
<td>22 ipk-1(ev15::Tc1)</td>
<td>128X</td>
<td>128X</td>
<td>100% (3)</td>
<td>β/multiplex M, 120</td>
</tr>
<tr>
<td>23 lig-3(ev584::Tc1)</td>
<td>4.6</td>
<td>128X</td>
<td>15% (48)</td>
<td>β L, 945</td>
</tr>
<tr>
<td>24 lig-3(ev589::Tc1)</td>
<td>11.6</td>
<td>128X</td>
<td>29% (49)</td>
<td>β L, 990</td>
</tr>
<tr>
<td>25 mab-20(ev673::Tc1)</td>
<td>-10.4</td>
<td>16X&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40% (25)</td>
<td>α L, 75</td>
</tr>
<tr>
<td>26 mab-20(ev606::Tc1)</td>
<td>-10.4</td>
<td>32X</td>
<td>4% (48)</td>
<td>sub-library/ α L, 945</td>
</tr>
<tr>
<td>27 nid-1(ev608::Tc1)</td>
<td></td>
<td>128X</td>
<td>6% (52)</td>
<td>β/multiplex L, 900</td>
</tr>
<tr>
<td>28 pFAT(ev619::Tc1)</td>
<td>-11.5</td>
<td>128X</td>
<td>33% (9)</td>
<td>β/multiplex L, 795</td>
</tr>
<tr>
<td>29 pkr-1 (ev614::Tc1)</td>
<td></td>
<td>64X</td>
<td>2% (68)</td>
<td>β/multiplex L, 450</td>
</tr>
<tr>
<td>30 unc-52(ev611::Tc1)</td>
<td>23.3</td>
<td>128X</td>
<td>9% (64)</td>
<td>β/multiplex L, 645</td>
</tr>
<tr>
<td>31 unc-52(ev612::Tc1)</td>
<td>23.3</td>
<td>128X</td>
<td>36% (11)</td>
<td>β/multiplex L, 1030</td>
</tr>
<tr>
<td>32 was-1(ev604::Tc1)</td>
<td>3.5</td>
<td>128X</td>
<td>28% (18)</td>
<td>β/multiplex S, 375</td>
</tr>
<tr>
<td>33 was-1(ev605::Tc1)</td>
<td>3.5</td>
<td>64X</td>
<td>9% (33)</td>
<td>β/multiplex L, 600</td>
</tr>
</tbody>
</table>

<sup>a</sup>The sample dilution refers to greatest two-fold dilution factor of the sample DNA that resulted in the PCR amplicon of interest. In some cases<sup>b</sup>, however, the greatest two fold dilution factor assayed was 16X.

<sup>b</sup>The total number of worms screened is shown in brackets.

<sup>c</sup>The screening technique refers to how the allele was isolated, either through an alpha-pool screen (α), a beta-pool screen (β), or a screen limited to a sub-library. Multiplex refers to using primers specific for several different genes in the same reaction, otherwise known as primer multiplexing.

<sup>d</sup>The size of the pool refers to the composition of the screened pools; they were either derived from small (S) samples, large (L) samples, or a mixture of both (M) (see materials and methods).
Figure 3.5. A 13 kb locus is successfully scanned for insertions in less than 300 reactions. The *semaphorin I* sub-library was screened for a second Tc1 insertion. A. An illustration of *els-l(ev586::Tc1)* and relevant primers. Each arrow represents two primers. B. A multiplex PCR screen of nine alpha-pools using the distal GSPs 1.7, I.D and I.3, and the R1 TSP, and proximal GSPs I.8, I.C, and I.3, and the R2 TSP. A triplicate can be seen in alpha-pool 825. C. A multiplex PCR screen of the same alpha-pools as in B but using the distal GSPs I.1, and I.6, and the R1 TSP, and the proximal GSPs I.2 and I.5, and the R2 TSP. Each reaction in B and C was done in triplicate. D. Simplex nested PCR is used to sort out which primer combination resulted in the triplicate seen in B. Legend: C, primer I.C; 3, primer I.3; 8, primer I.8; R, primer R2. E. Samples constituting alpha-pool 825 were screened for the second insertion using distal primers I.D and R1, and proximal primers I.C and R2. F. The template of sample number 812 can be serially diluted more than 32 times and still generate an amplicon. Not shown are the 88 SWPCRs used to isolate NW1058 (*mut-2(r459)*, *els-l(ev587::cis-Tc1)*; *dpy-19(n1347)III*). M. 1 kb ladder. B through F show amplicons resolved on 1% agarose gels stained with ethidium bromide.

Screening efficiency could be improved by using a multiplex GSP strategy without a loss in specificity. Primer multiplexing refers to the use of many gene specific primers in a single reaction. For an example of multiplex screening efficiency, figure 3.5 illustrates that a total of 279 reactions were used to isolate a cis-Tc1 allele from a NW 1033 sub-library. In this example, 34% fewer reactions were required than if a simplex strategy were used. The multiplex strategy was used for as many as five different sets of GSPs to isolate some of the 33 Tc1 alleles (table 3.3). Since beta-pool multiplexing can be used successfully to screen for many alleles in fewer reactions, our screens are now done using this methodology.

**Sample DNA Dilution**

Experience had shown that samples that do not generate an amplicon upon sample DNA dilution, never yield the candidate allele. It was hypothesized that the further that the sample DNA can be titrated and still generate a candidate amplicon in a PCR, the greater the chance of recovering the desired strain within the frozen aliquot. When the sample DNA dilution factor and the isolation frequency data from the recovered alleles (table 3.3) are plotted (figure 3.6), the slope of the resulting trend-line is positive,
suggesting a positive correlation of the data plotted on the two axes. Therefore, not only does the dilution factor predict the chances of isolating the desired strain, but it also predicts the frequency of animals within the population that carry the allele of interest. Included in the scatter plot of figure 3.6 are the only three samples that did not yield a strain carrying the candidate allele but whose sample DNA dilution factors were greater than 4X (16X, 32X, and 64X, respectively). Conversely, more than three attempts proved that candidate alleles are never isolated from thawed samples if the sample DNA did not dilute more than 4 fold to produce the desired amplicon. The standard dilution factor-threshold for thawing a candidate sample is now eight times.

The Relationship Between Sample Dilution and Allele Recovery

![Graph showing the relationship between sample DNA dilution and allele recovery.](image)

Figure 3.6 An X/Y scatter plot of sample DNA dilution versus the frequency of Tc1 allele recovery

The line through the graph shows the computer-calculated trend. The inclined slope of the trend line indicates a positive correlation between the extent of sample DNA dilution and the frequency of worms within the candidate population that carry the allele of interest. The data points in the graph are derived from Table 3.3, columns three and four.

SWPCR and the Construction of Strains Containing Multiple Tc1 Alleles

Effective single-worm-genotyping obviated the need to know the heterozygous or homozygous state of several alleles in a single individual. The single worm lysate (see materials and
methods) would therefore have to be divided up into numerous aliquots. To test how many aliquots could be derived from a single worm, the lysate of a single worm was diluted serially. Figure 3.7 illustrates that normal PCR conditions (see materials and methods) can generate a visible amplicon using a single worm lysate that is diluted at least 64 times. The PCR band is still very strong at a dilution factor of 32 times, suggesting that the single worm lysate can be divided at least 32 times without the loss of amplicon visibility, enabling at least 32 separate genotyping assays. I call this technique “split-SWPCR”. This result has several applications: First, the number of SWPCRs needed in screens for gross polymorphisms can be reduced by at least three-fold by creating a two dimensional template matrix. Figure 3.8 shows that the lysates from 64 worms thawed from a candidate sample (identified in figure 3.5), were pooled in two dimensions whereby every lysate contributes to two pools and creates internal duplicate controls. The four resulting bands suggested that two out of four possible addresses or worms carried the allele of interest. After repeating the SWPCR using the leftover lysates of worms 812.29, 812.31, 812.37 and 812.39 (data not shown), only 812.29 and 812.39 were positive. In total, the number of PCRs required was reduced from 64 to 20.

![Figure 3.7](image_url)

**Figure 3.7. Titration of a single worm PCR template.** The template of a single NW 1035 [mab-20(ev574)] L4 worm can be diluted to about 64 times and a PCR can still generate a clearly visible amplicon. The GSPs II.Y and II.10 were used to generate the amplicon. 4 µl of each reaction was resolved on an ethidium bromide stained 1% agarose gel. M, 1 kb ladder.
A second use for the split-SWPCR technique is in identifying multiple alleles from a single individual. To isolate deletion derivations of a Tc1 allele, a sub-library is seeded with the strain homozygous for the Tc1 of interest. For multiple alleles, however, this procedure becomes laborious. Instead of generating a separate sub-library for separate alleles, one sub-library can be constructed from a single strain that contains multiple Tc1 alleles of interest. Depending on the map position and the ease of creating recombinants, this can be done for upwards of six or more distinct Tc1 alleles. Figure 3.9 illustrates the use of split-SWPCR in the creation of NW 1087 \( mab-20(ev573::Tc1)I, \) \( mut-2(r459)I; dpy-19(n1347)III; \) \( lig-l(ev584::Tc1)IV; \) \( cdh-x(ev576::Tc1)V; ets-l(ev583::Tc1)XI, \) a single strain that is homozygous for four Tc1 alleles. A sub-library of 195 samples was created with NW 1087, resulting in the isolation of Tc1-deletion derivatives of \( lig-l \) and \( mab-20. \)

### 3.2.3 The Isolation of Tc1-Derived Deletions

Techniques for isolating Tc1-derived deletions have traditionally relied on sub-culturing Tc1 strains of interest and directly screening those cultures for deletions that may predictably lead to a loss of gene function (Zwaal et al., 1993; Rushforth et al., 1993; Maryon et al., 1996; Sengupta et al., 1996). Instead of discarding those samples, I prepare the Tc1-derived sub-cultures like the original library, creating a “sub-library”. The benefits of such a sub-library are multifold: First, the sub-library can be repeatedly screened for Tc1-derived deletions using a variety of different GSP sets. For example, figure 3.10 shows the isolation of two \( mab-20 \) alleles, \( ev574, \) and \( ev575. \) All techniques and strategies developed for the isolation of Tc1 alleles are applied towards the isolation of deletion derivatives. For example, alpha-pools are constructed from 15 sub-library samples and the extent of sample DNA dilution resulting in the desired amplicon is interpreted as the relative probability of a sample containing the germ line polymorphism of interest. Table 3.4 shows the Tc1 deletion derivatives isolated in our lab.
Figure 3.8. Reducing the number of SWPCRs fourfold.
The lysates of fertile hermaphrodites thawed from an aliquot of sample 812 were pooled thereby reducing the number of single worm PCRs from 64 to 16. A. PCR amplicons generated from the systematically pooled lysates of 64 worms and primers I.C + R2. Results indicate that at least two of the isolates 812.29, .31, .37, and .39 must contain the second Tc1 insertion. B. A schematic illustrating the pooling strategy of the 64 single worm lysates, highlighting putative isolates. C. Of the four possible isolates, 812.29 and 812.39 contain both the first (Tc1₁, using primers I.A+R1) and second (Tc1₂, using primers I.C+R2) cis Tc1 elements. D. An illustration of the resultant allele els-I(ev587::cis-Tc1) with the relevant primers shown. Primer arrows represent two primers. M, 1 kb ladder; .29, isolate 812.29; .39, isolate 812.39. All 1% agarose gels are stained with ethidium bromide and show the amplicons generated from a single round of PCR.
Figure 3.9. Dividing a single worm lysate enables the genotyping of several alleles simultaneously.
The 1% agarose gel shows PCR amplicons generated using the lysate of 3 single fertile parents called QHet 1, 2 and 3, respectively, as template. The lysate of each animal was split into four and assayed for the presence of alleles ev583::Tc1, ev584::Tc1, ev576::Tc1, and ev573::Tc1 using primers to identify the Tc1 elements in ets-1, lig-1, cdh-4, and mab-20, respectively. Qhet, quadruple heterozygote; M, 1 kb ladder.

Most of the alleles were isolated in screens of approximately 200 samples, and are less than 3 kb, a size limit imposed by the distance between the GSP pairs used in the deletion screen. No phenotypes could be found in strains homozygous for some of the deletion alleles.

In an experiment designed to test if ultraviolet radiation could enhance the frequency of detectable Tc1 derived deletions, as implicitly reported by Sengupta et al. (1996), half of the NW1034 [mab-20(ev573::Tc1)I, mut-2(r459)I; dpy-19(n1347)III] sub-library was irradiated (see materials and methods and appendix B). Interestingly, of the two mab-20 deletions derived from the NW1034, both came from irradiated samples.

A second benefit of permanently storing Tc1 sub-cultured samples is that, as mentioned above, they can be screened not only for Tc1 derived deletions, but also for Tc1 insertions into
Figure 3.10. A typical screen for a deletion allele.
An alpha-pool screen of the NW 1034 [mab-20(ev573::Tcl)I, mut-2(r459)II; dpy-19(n1347)III] sub-library for a Tcl-derived deletion of mab-20. Alpha-pools 855 and 885 contain two putative independent deletions of approximately 1.6 kb and 1.5 kb, respectively. B. A serial dilution of samples 853 and 878 show that each template dilutes at least 16 times. The alpha-pool amplicons are repeated for a positive control. C. The isolation of parent individuals 853.61 and 878.31 that resulted in strains NW 1035 [mab-20(ev574)II] and NW 1036 [mab-20(ev575)II], respectively. The 1% agarose gels in A and B show the second round products generated from nested PCR with distal GSPs II.Y + II.10, and proximal GSPs II.Z + II.9. The gel in C shows products generated in a single round of SWPCR with primers II.Y and II.10. M, markers.
other loci. Seven Tc1 alleles were also isolated from the NW1034 sub-library. Interestingly, five out of seven alleles came from the UV irradiated alpha-pools. These results suggest that ultraviolet radiation may serve as a Tc1 catalyst, increasing the transpositional activity, and in turn resulting in a greater number of independent Tc1 alleles per generation and consequently, a greater number of Tc1 derived deletions.

When comparing the success of isolating alleles from the various sub-libraries, however, the NW 1037 [mut-2(r459)I; dpy-19(n1347)III; cdh-x(ev576::Tc1)V] sub-library has by far the

Table 3.4. Tc1-derived polymorphisms

<table>
<thead>
<tr>
<th>Allele</th>
<th>Polymorphism</th>
<th>Phenotype</th>
<th>Sample Numbera (size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdh-x(ev577) V</td>
<td>removes EGF domains and throws the transcript out of frame</td>
<td>none found</td>
<td>158 (L)</td>
</tr>
<tr>
<td>dbl-1(ev580::Tc1) V</td>
<td>a Tc1 insertion in an exon that results in a loss-of-function</td>
<td>small bodies and defects in the male tail</td>
<td>NA (L)b</td>
</tr>
<tr>
<td>ecr-b X</td>
<td>removes EGF domains and throws the transcript out of frame</td>
<td>none found</td>
<td>UNK</td>
</tr>
<tr>
<td>lig-1(ev568) IV</td>
<td>a 1.9 kb deletion that removes all exons except 20 amino acids of the last exon</td>
<td>defects in hypodermal morphogenesis</td>
<td>195 (L)</td>
</tr>
<tr>
<td>lig-3(ev684) X</td>
<td>a 1.8 kb deletion that deletes DNA 5' to the ATG, exon 1 and exon 2</td>
<td>none found</td>
<td>200 (L)</td>
</tr>
<tr>
<td>mab-20(ev574) I</td>
<td>a 1.5 kb deletion that removes DNA 5' to the ATG and exon 1</td>
<td>defects in hypodermal morphogenesis</td>
<td>210 (L)</td>
</tr>
<tr>
<td>mab-20(ev575) I</td>
<td>a 1.6 kb deletion similar to ev574 but may be linked to mab-20(+)</td>
<td>NA</td>
<td>210 (L)</td>
</tr>
<tr>
<td>mab-20(ev610) I</td>
<td>a 2.5 kb deletion that removes DNA 5' to the ATG and exon 1</td>
<td>none found</td>
<td>195 (L)</td>
</tr>
</tbody>
</table>

aThe sample number refers to the number of samples that were screened in order to isolate a particular allele. The size of the samples is given in brackets (see experimental procedures).

bdbl-1(ev580::Tc1) contains a Tc1 inserted into an exon that results in a loss-of-function and was therefore isolated in a Tc1 screen, not in a screen for deletions. NA, not applicable.
greatest success rate (table 3.5). On average, one deletion allele was isolated from every 28, 33, 30, and 20 large samples from the original library, the *semaphorin I* sub-library, the *mab-20* sub-library, and the *cdh-x* sub-library, respectively (table 3.5).

**Table 3.5 A comparison of the Tc1 isolation efficacy of the libraries**

<table>
<thead>
<tr>
<th>Library</th>
<th>Strain</th>
<th>Screened Samples per Allele&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Screened Worms per Isolate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Screened Worms per Allele&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Original Library</td>
<td><em>mut-2(r459)l; dpy-19(n1347)III</em></td>
<td>31 (156/5)</td>
<td>11 (193/17)</td>
<td>39 (193/5)</td>
</tr>
<tr>
<td>2 Original Library</td>
<td><em>mut-2(r459)l; dpy-19(n1347)III</em></td>
<td>26 (180/7)</td>
<td>5 (407/84)</td>
<td>58 (407/7)</td>
</tr>
<tr>
<td>- Original library sub-totals</td>
<td></td>
<td>28</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>3 <em>Sema I</em> sub-library</td>
<td><em>mut-2(r459)l, els-1(ev586::Tc1)l; dpy-19(n1347)III</em></td>
<td>33 (165/5)</td>
<td>12 (342/28)</td>
<td>68 (342/5)</td>
</tr>
<tr>
<td>4 <em>Sema II</em> sub-library (+UV)</td>
<td><em>mut-20(ev573::Tc1)l, mut-2(r459)l; dpy-19(n1347)III</em></td>
<td>21 (105/5)</td>
<td>23 (322/14)</td>
<td>64 (322/5)</td>
</tr>
<tr>
<td>5 <em>Sema II</em> sub-library (no UV)</td>
<td><em>mut-20(ev573::Tc1)l, mut-2(r459)l; dpy-19(n1347)III</em></td>
<td>53 (105/2)</td>
<td>15 (75/5)</td>
<td>38 (75/2)</td>
</tr>
<tr>
<td>- Sema II sub-library sub-totals</td>
<td></td>
<td>30</td>
<td>21</td>
<td>57</td>
</tr>
<tr>
<td>6 CeMe-2 sub-library</td>
<td><em>mut-2(r459)l; dpy-19(n1347)III; cdh-x(ev576::Tc1)V</em></td>
<td>20 (180/9)</td>
<td>10 (501/49)</td>
<td>56 (501/9)</td>
</tr>
<tr>
<td>- Grand Totals</td>
<td></td>
<td>27 Samples/Allele</td>
<td>9 Worms/Isolate</td>
<td>56 Worms/Allele</td>
</tr>
</tbody>
</table>

<sup>a</sup>The number shown is the average number of samples screened to obtain the allele of interest in that particular sub-library. In brackets is the number of sub-library samples divided by the number of isolated alleles.

<sup>b</sup>For comparative purposes, the number of small samples in the original library is given as the total number of small samples divided by 2.5, since each small sample contains an estimated 2.5 less genetic lines than the large samples.

<sup>c</sup>The number shown is the average number of worms screened to isolate the individuals carrying the allele of interest. In brackets are the number of worms assayed divided by the number of worms containing the desired allele.

<sup>d</sup>The number shown is the average number of worms screened per candidate sample to isolate the allele of interest. In brackets are number of thawed worms assayed divided by the number of isolated alleles.

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There were 570 alpha-pools in the original library, 390 of which were small samples and 180 of which were large samples. For comparative purposes, the total number of large samples in the original library includes the small samples divided by 2.5 plus the large samples.
3.3 Discussion

Although inherently useful for deciphering evolutionary relationships, sequence information is otherwise without use if not accompanied by functional analysis. Our expanding knowledge base of DNA sequences from a variety of organisms requires efficient functional analysis of targeted sequences, which has created the field of study known as functional genomics. With gigabases of sequence data now in hand, functional genomics seeks new ways to investigate the functional relevance of each gene systematically and catch up to the speed of sequencing projects. Presently, however, the methodology described herein may be one of the most efficient ways to generate loss-of-function chromosomal mutations in genes of interest in any animal model. Although RNAi is now in wide use, it is difficult to make absolute comparisons to other genetic data employing this technique. For this reason, it is imperative that chromosomal mutations in the loci of interest be generated so that firm genetic arguments can be made and that a reagent (the mutant) is produced for others to study.

Since the interest of reverse genetics has gained momentum in the past five years, one technique of targeted gene disruption in *C. elegans* has been widely successful. Searching for deletions of less than 3 kb in samples of genomic DNAs that represent frozen samples of mutated worms has resulted in the characterization of numerous genes that were not characterized or cloned using forward genetics (for example, Greenstein *et al.*, 1994; Vassilatis *et al.*, 1997; Pettitt *et al.*, 1996; Maryon *et al.*, 1996). The first mutagen used in this strategy was the Tc1 transposable element, which requires a laborious two step procedure. Some workers, however, have recently reported success in using a more direct, one step method of mutant isolation though the use of chemical mutagenesis that has the obvious advantage of an increased screening efficiency (Jansen *et al.*, 1997). One disadvantage in using a chemical mutagen in the construction of a library is the number of samples required to achieve an adequate success rate: Jansen *et al.* (1997) reported a success rate of 50% (n=10) when over 7000 samples of 150 lines
each were screened. Although the use of a chemical mutagen slightly exceeds our success rate of isolating Tc1 derived deletions\(^3\), the total number of samples screened to obtain alleles from our library is much less. Regardless, the techniques that improve screening efficiencies presented here can be used in any PCR based screen for DNA polymorphisms.

Several improvements on existing protocols for library construction and screening techniques were made, including novel pooling strategies and primer multiplexing strategies. These improvements enable the library to be screened for as much as eight different alleles in a single day. For example, to screen 960 samples from our library for a Tc1 insertion (in either orientation) into four genes, would require 200 PCRs\(^4\), while Zwaal et al.'s (1993) method would require 1920 reactions\(^5\) to screen for the same candidate alleles. These results amount to an increase in screening efficiency of nearly 10 times!

The techniques presented herein have enabled us to isolate 33 alleles for over 20 different laboratories, some of whom have gone on to derive deletions from their respective Tc1 alleles. For example, a deletion has been obtained from \(\text{lig-1(ev584::Tc1)}\) (X. Wang, P. Roy, J. Culotti, and A. Pawson, unpublished results). Interest in \(\text{lig-1}\) arose because of the apparent homology of \(\text{lig-1}\) to the Ephrins, which are ligands for the Eph receptors. As anticipated, the loss-of-function phenotype is related to the mutant phenotype of \(\text{vab-1}\), a putative Ephrin receptor. Curiously, we have also isolated several Tc1-deletion derivatives of \(\text{cdh-x, ecr-b, lig-3, and mab-20}\) that have no phenotype. The deletion within \(\text{cdh-x}\) removes the first four predicted exons, including the putative signal sequence. Several explanations could explain why \(\text{cdh-x}\) has no phenotype: First,

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\(^3\) The success rate of isolating deletions is calculated by multiplying the success rate of Tc1 isolation (.61) by the success rate of finding deletions within the sub-libraries that I have made or have made in close collaboration with others, namely for \(\text{els-1, mab-20, cdh-x, ecr-b, lig-1, ets-1, .(67)}\) resulting in an overall success rate of 41%.

\(^4\) Each of the twelve beta-pools are screened in triplicate for four primer combinations of four genes. One candidate address is chosen for each and the 15 individual samples plus the alpha-pool are screened in duplicate, resulting in one candidate individual sample per gene. (12 pools x 3 reactions each x 2 Tc1 orientations) + (15 samples x 2 reactions x 4 candidate addresses) + (1 alpha-pool x 2 reactions x 4 candidate addresses) = 200 reactions.

\(^5\) The 960 samples of Zwaal et al.'s (1993) library are organised into 30 pools, which are screened in quadruplicate for each primer combination. 30 pools x 4 reactions x 4 genes x 4 primer combinations = 1920 reactions.
the gene could be a recent duplication and is therefore functionally redundant, although no sequence evidence supports this supposition. Second, it is plausible that the cadherin repeats are themselves redundant and since the deletion removes only two out of eight cadherin repeats, it is plausible that enough protein is present to function adequately. Finally, it is possible that the phenotype of the cdh-x loss-of-function is too subtle to observe using common laboratory tools. The lack of observed phenotypes in ecr-b and lig-3 could also be explained using similar aforementioned explanations. Two of the three Tc1 derived deletion alleles of mab-20, however, did not show any phenotypes. Both mab-20(ev575) and mab-20(ev610) contain deletions that are larger than mab-20(ev574), which is a putative null allele of mab-20 (chapter 4). Each of the three mab-20 alleles removes exon 1, but differ in the extent of deleted untranslated and untranscribed DNA. mab-20(ev575) does not exhibit the mab-20 mutant phenotypes probably because it carries a linked duplication of the mab-20 locus (data not shown). The lack of phenotype in strains homozygous for mab-20(ev610) does not yet have a satisfactory explanation. It is important to consider, however, that the molecular screens that detect these alleles use a PCR assay that can amplify one to ten templates of one allele in a mixture of one million templates of another allele. Numerous forward genetic screens have reported bizarre rearrangements of mutated chromosomes making it plausible that these alleles that are detected via the very sensitive PCRs are nothing more than bizarre rearrangements that we call “weirdomorphs”. These results exclaim the need to isolate multiple alleles before making firm conclusions regarding the wild type function of any gene.

Finally, it is interesting to note that the frequency of germline Tc1 insertions per gene was predicted by Zwaal et al. (1993) to occur in about one in a thousand library cultures based upon forward genetic screens using Tc1 as the mutagen (Moerman and Waterston, 1990; Collins et al., 1987). This calculation is also based on the prediction that the PCR assay can only detect insertion events that result in at least 1% of animals within the sample carrying the allele of
interest. Out of the 44 requests for Tc1 alleles, we have a success rate of 61%; four of those requests resulted in two independent insertions each. These results approximate the calculated frequency of insertions that could be isolated per gene per generation (Zwaal et al., 1993).

3.4 Materials and Methods

3.4.1 Library Construction

General protocols for growing worm strains are as compiled by Wood, 1988. The library construction protocol has been modified from Andachi and Kohara (1995, personal communication), Rushforth et al. (1993), and Zwaal et al. (1993). MT 3126 [mut-2(r459)I; dpy-19(n1347)III] was chosen as the strain used to construct the library because of its intermediate rate of transposon transposition (Finney, 1987; Rushforth et al., 1993). Before culturing library samples, the first or second cousins of the worms used for library construction were tested for recent Tc1 transposition. A useful genetic marker for mutagen efficacy is unc-22; mutations in this gene will result in worms that twitch when bathed in 2% nicotine (Moerman et al., 1989). Compared to wild type, the MT 3126 strain had a many more twitchers when assayed in the nicotine solution.

A general outline of library construction is shown in figure 3.2. To construct the original library about 650 3.5 or 6 cm plates were seeded with two or five healthy MT 3126 worms, respectively, and stored at 20°C for 10 to 12 days. The collection of worms grown on 3.5 or 6 cm plates are respectively referred to as “small samples” or “large samples”. Small and large samples were generated to compare the efficiency of the recovery of putative positives. After the incubation period, the samples were screened for clearing of the bacterial lawn and starvation of many L1s. Severely contaminated samples were discarded. Those that grew well were assigned a sample number and were estimated to have gone through two to three generations that represent at least 170 genetic lines. M9 buffer (see appendix A) was used to collect the worms.
into correspondingly numbered 1.8 ml siliconized microcentrifuge tubes, after which the volume was reduced to 1 ml. Each sample was evenly aliquoted to four tubes, three of which were frozen at -70°C for long term survival (in worm freezing solution, appendix A) in separate freezers. The liquid from the fourth aliquot was removed and the worms were placed on ice for 5 min to stop the worms from wiggling. 15 μl of a sample lysis solution (appendix A) was added to the samples and were incubated at 60°C for 20 to 60 minutes. 50 μl of TE (pH 8.0) was added to the worm solution, mixed. “Sample DNA” was created by adding 7.5 μl of the lysate to each of two appropriately numbered tubes containing 500 μl of TE, and freezing at -70°C. The remaining sample lysate was pooled with 14 other samples, creating a total of about 40 “alpha-pools”. Each alpha-pool was treated in succession with phenol, phenol/chloroform, chloroform, and then precipitated with ethanol and NaAcetate, pH 5.2. 1200 μl of TE was added to the precipitate and the DNA was resuspended overnight at 55°C. The alpha-pool DNA was aliquoted to two tubes and stored in separate -70°C freezers. Alpha-pools derived from small or large samples are respectively referred to as small or medium alpha-pools. Alpha-pools derived from a mixture of the two sample sizes are referred to as medium alpha-pools.

In order to decrease false-positive signals presumably resulting from somatic insertions, and thereby increase screening efficiency, 64 accumulated alpha-pools were further strategically pooled into 12 “beta-pools”, representing three co-ordinates (figure 3.3, and appendix B). By screening the 12 beta-pools, a single alpha-pool address containing a putative germ-line Tc1 insertion could be identified. Individual samples that constitute the identified alpha-pool were then assayed for the presence of the putative Tc1 insertion into the gene of interest.

Tc1 derived deletions were obtained in an identical manner to the isolation of Tc1 alleles, except that instead of using MT 3126 as the founding strain of a library, the strain homozygous
for Tc1 alleles of interest are used. I call libraries created in this fashion “sub-libraries”. All the samples constituting the sub-libraries are made up from large (6 cm) plates.

3.4.2 Thermocycling

Nested primers were used for all PCR-based screening strategies (figure 1) except single worm PCR (see below). This technique allows the visualization of insertion and deletion specific amplicons when using a complex template mixture, such as alpha-pools, beta-pools (see below) or even the sample DNA. After thermocycling with the distal gene-specific primer (GSP) and Tc1-specific primer (TSP), the resultant template is diluted 66X with TE, followed by a second round of PCR with the proximal primer set. A typical 10 µl reaction for alpha-pool, beta-pool, and individual sample screens contains 1.0 µl of 10x reaction buffer without Mg²⁺ (Perkin-Elmer); 0.8 µl of 10 mM dNTPs (Bohringer); 0.8 µl of 25 µM Mg²⁺ (Perkin-Elmer); 0.4 µl of 10 µM primer 1; 0.4 µl of 10 µM primer 2; 1.0 µl of DNA Template; 0.325 µl of 5u/µl Amplitaq Gold (Perkin Elmer); and 5.3 µl of double-distilled water. When using multiple GSPs within the same reaction (multiplexing) twice the amount of TSPs are used.

The typical cycle conditions for all screens are 95°C for 9min, required to denature the DNA and activate the Amplitaq Gold taq enzyme; 32 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes; 72°C for 10 minutes to complete any partially completed templates. The reactions are then stored at 4°C. Four µl of the PCRs are added to 1 µl of 6X loading buffer/dye and 1 µl of water. The amplicons are visualized on 1% agarose gels (Sambrook et al., 1989).
3.4.3 Primers

PCR primers are designed with two purposes in mind; for screening the library for Tc1 insertions and for screening Tc1 derived deletions. I have used the following guidelines in choosing a set of 5' and 3' distal and proximal gene specific primers, (named GSP 1 & 2, and, GSP3 & 4, respectively in figure 1): The distal set of primers, GSP 1 and 4, should be between 2.5 and 3.5 kb apart. If the defined gene is less than 3 kb, then design primers to flank the gene within 400 bp on either side. GSP1 & 2 should be about 100 bp apart from each other as is for GSP3 & 4. Depending on the intended purpose of the desired alleles, the primers should flank regions predicted to encode essential domains. Alternatively, the exon encoding the initiator methionine could also be targeted for disruption. Each primer should be about 21 to 23 bases in length. To ensure strong anchorage, about three of six bases of the 3' end of each primer should be guanine or cytosine. The primers should have equivalent melting temperatures (Tm) of about 60°C, according to Tm= 17 + 1.46[2GC + AT]. Primers should be designed so that there are no palindromes greater than 3 bases, nor should there be any short direct repeats.

The sequence of the nested Tc1 primers (Plasterk, 1995) are as follows; L1, 5'-CGT GGG TAT TCC TTG TTC GAA GCC AGC TAC-3'; L2, 5'-TCA AGT CAA ATG GAT GCT TGA G-3'; R1, 5'-TCA CAA GCT GAT CGA CTC GAT GCC ACG TCG-3'; and R2, 5'-GAT TTT GTG AAC ACT GTG GTG AAG-3'.

3.4.4 Thawing Frozen Samples of C. elegans

A putative positive sample is thawed for 15 minutes at room temperature and then dumped onto a quadrant of a 10 cm OP50 bacterially seeded NGM plate. Samples were never pipetted onto the plate, as the worms are very fragile after being frozen. Samples numbered 1120 and above were frozen on their sides, so that the whole sample need not be thawed. Instead, a chunk of the frozen aliquot can be melted on a large plate.
Chapter Three

One to two days after thawing, about 100 to 300 larvae are cloned onto 3.5 cm OP50 NGM plates and stored at 20°C for three to four days. Those plates supporting fertile parents are assayed for the allele of interest by single worm PCR (SWPCR).

3.4.5 Single Worm PCR

SWPCR is used to molecularly assay an individual worm for the presence of an allele that contains a polymorphism visible on 1% agarose gel when compared to wild type. After thawing a sample, cloned fertile parents (Po) are assayed by SWPCR (Williams et al., 1992): A single Po is placed in 2 µl of single worm lysis buffer (Appendix A) in a 200 µl PCR tube. When multiple alleles are to be assayed, 2 µl of worm lysis buffer is used per allele. The tube and its contents are frozen on crushed dry ice, after which two drops of Sigma™ mineral oil is dispensed with a Pasture pipette onto the frozen pellet. To lyse the worm, the tube is incubated at 60°C for 60 minutes, followed by 15 minutes at 95°C to inactivate the enzyme. After being cooled to 4°C, a PCR master mix containing all reaction ingredients are added to the worm lysate while the tube is still in the thermocycler. The thermocycling program is then activated. To confirm the results of SWP, progeny of the assayed Po are assayed to insure germ line transmission.

The limit of template dilution of the single worm lysate was determined through serial dilution. After the single worm was lysed in 4 µl of SWL buffer, 3 µl of the lysate was two-fold serially diluted 7 times. 1 µl was then used in a standard 10 µl PCR reaction with one round of thermocycling.
3.4.6 Ultraviolet irradiation of the founding generation of some library samples

To test if ultraviolet (UV) irradiation enhanced the frequency of Tcl derived deletion alleles, L4 worms that founded several samples (see results) were treated with 34 mJ/cm² of UV energy in a Stratagene Stratalinker. Five to eight of the mutagenized L4s were used to seed 6 cm NGM plates as described in the Library construction section above.
Chapter 4

*mab-20* Encodes a *semaphorin II* Homologue and is Required to Prevent Inappropriate Epidermal Cell Contacts in *C. elegans*

All work reported herein was done by me, Peter J. Roy, except the following: Yuji Kohara isolated *yk80* and *yk100*. Hong Zheng isolated *evpZH11*, the *mab-20* gene, in a genomic library screen. Hong Zheng and I did the SSCP analysis on the two *mab-20* alleles. Others provided certain critical reagents such as antibodies and reporter constructs as indicated.
4.1 Introduction

During directed migrations, the leading edge of axons and cells must continually establish transient contacts to neighboring cells and/or the surrounding extracellular matrix (ECM). Cues expressed within the environment of the migrating soma or process may either promote or prevent the formation of actin-based extensions and further migration. The diversity in directed migrations during embryogenesis is dependent on the cell-autonomous expression of selected receptors for these guidance cues. For example, UNC-6 is a secreted ligand that is required for both ventrally and dorsally directed migrations of axons and other ectodermal and mesodermal cells in the nematode *C. elegans* (Hedgecock *et al.*, 1990; Ishii *et al.*, 1994). Whether or not a cell is attracted or repelled by UNC-6, which is expressed along the ventral midline (Wadsworth *et al.*, 1996), is dependent upon cell-autonomous co-expression of both the UNC-5 (Leung-Hagesteijn *et al.*, 1992; Hamelin *et al.*, 1993) and the UNC-40 surface receptors (Colavita and Culotti, 1998). Conversely, attraction to UNC-6 is thought to require UNC-40, but not UNC-5 (Chan *et al.*, 1996). Amazingly, this guidance paradigm is conserved from worms to mammals; for example, Keino-Masu *et al.* (1996) showed that the UNC-40-homologues Deleted in colorectal carcinomas (Dcc) and Neogenin bind the UNC-6 homologues Netrin-1 and 2, which are expressed in the ventral floor plate of the spinal cord (Serafini *et al.*, 1994, 1996). Clearly, the attraction or repulsion of a migrating cell or axon depends upon the cell-autonomous expression of cell surface receptors that recognize and initiate a response to guidance cues in the environment.

The Semaphorins are another family of ligands conserved from nematodes to humans. To date, they fall into seven classes depending on the modular nature of their domains, but all have a conserved ~500 amino acid “semaphorin” domain that defines the family (chapter 1; Adams *et al.*, 1996). Grasshopper Semaphorin I (G-Semal) was the first characterized Semaphorin to be reported (Kolodkin *et al.*, 1992) and was later shown to be required for PNS
axon outgrowth and axon extension (Wong et al., 1997). In contrast to G-SemaI, most other Semaphorin family members repel growth cone extensions (see chapter 1; Winberg et al., 1998; Koppel et al., 1997; Messersmith et al., 1995; Luo et al, 1993), and steer growth cones via contact mediated repulsion (Fan and Raper, 1995). Loss-of-function mutations in murine Sema-III/D support the findings that the Semaphorins repel migrating axons (Behar et al., 1996; Taniguchi et al., 1997). Mice deficient for Sema III/D also have pleiotropic cartilage abnormalities, including fused ribs and vertebrae, and cardiac defects. The nature of these non-neuronal defects, however, is poorly understood.

The response to the Semaphorins, like the response to UNC-6, is receptor dependent: The mammalian Neuropilin-1 receptor binds Sema III/D (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). A second receptor, Neuropilin-2, has a high affinity for Sema E and IV (Chen et al., 1997). In vitro studies have shown that Neuropilin-1 mediates the Sema III/D induced collapse of NGF, but not NT-3 dorsal root ganglia (DRG) responsive afferents in the dorsal spinal cord taken from Neuropilin-1 deficient mice (Kitsukawa et al., 1997). Mutant mice also have severe defects in PNS efferent fiber trajectories. Although no non-neuronal defects in the neuropilin-1 mutant mice were reported, constitutively expressed Neuropilin-1 results in nerve defasciculation and sprouting, along with cardiovascular defects, extra digits, and cardiac malformations (Kitsukawa et al., 1995). In principle, the vasculature defects may be explained by the promiscuity of the Neuropilin-1 receptor, as it also binds VEGF (Soker et al., 1998), but the underlying defects of the heart and limb phenotypes remain obscure.

In this chapter I show that we have cloned the C. elegans homologue of semaphorin II and have used transposon-mediated reverse genetics to obtain a putative null. Chromosomal linkage analysis and complementation tests revealed that the previously characterized mab-20 mutants (Baird et al., 1991; Chow and Emmons, 1994), for which the gene had not been previously cloned, encodes Semaphorin II. Hereafter, the gene and all mutant alleles will be
referred to as mab-20. The predicted protein product of mab-20 is called Ce-SemaII in order to conform to the nomenclature of the invertebrate Semaphorins. To find other genes within the Semaphorin or related signaling pathways, I have screened over half a million mutagenized F1 mab-20(bx61u) genomes for dominant enhancers of the mab-20 mutant phenotype, resulting in over 30 putative enhancers.

Besides having a role in guiding axon and cell migrations, a major function of Ce-SemaII is to regulate cell contacts within the developing epidermis, which is called the hypodermis in C. elegans. Mutant animals exhibit five primary phenotypes during hypodermal development: First, a large proportion of individuals die from internal contents being squeezed out during embryonic elongation. Second, escapers of the embryonic lethality hatch with severe body deformities. Third, a fraction of mutant embryos and young larvae have a forked tail whip. Fourth, at each larval stage the lateral hypodermal seam cells divide and in wildtype, the anterior daughters fuse to the surrounding hypodermal syncitium. In mutants however, the seam cell daughters occasionally do not detach from the lateral line. Finally, a drastic hypodermal reorganization occurs during the formation of the wildtype male tail. In semaphorin II mutants, subsets of the normally separate male tail sensory rays fuse. These mutant phenotypes correlate with the timing of expression of mab-20 transcriptional and translational reporters in these tissues. A detailed examination of the cellular basis of several of these phenotypes reveals that ectopic hypodermal cell contacts can explain each of the semaphorin II mutant phenotypes. I propose that Ce-SemaII is a ligand required to repel exploratory processes that would otherwise form ectopic cell contacts during hypodermal morphogenesis.

Finally, many other mutants such as mab-26 (Baird et al., 1991; Chow and Emmons, 1994), vab-6 (Brenner, 1974), vab-1 (George et al., 1998), lig-1 (X. Wang, P. Roy, J. Culotti, and T. Pawson, unpublished observations), unc-130 (B. Nash, P. Roy, and J. Culotti, unpublished observations), and mab-21 (Chow et al., 1995) have either embryonic and/or male tail
phenotypes that are at least superficially like those of mab-20 mutants. To further characterize the differences and similarities between the aforementioned mutants and mab-20 mutants, I investigate the cellular nature of many of the defects of these mutants in relation to one another.

4.2 Results

4.2.1 The Cloning of C. elegans semaphorin II

A C. elegans cDNA collection and sequencing project (Y. Kohara, personal communication) identified two clones with high homology to the semaphorin family of genes. The sequence information was used to design 3' primers to obtain the remaining 5' end of the mRNA by 5' RACE and SL1 PCR (see materials and methods). Splicing these cDNA fragments together resulted in the full-length semaphorin cDNA depicted in figure 4.1. The 2.45 kb clone was used to probe Northern blots of mRNA isolated from a mixed-stage population of C. elegans mRNA revealing a single band of a size predicted by the size of the cDNA (figure 4.1).

The full-length cDNA has 167 bp of 5' UTR, 285 bp of 3' UTR and a poly-A tail (figure 4.1). The cDNA is predicted to encode 657 amino acids comprising an amino-terminal signal sequence, followed by a 500 amino acid semaphorin domain, an immunoglobulin (Ig) domain, and a carboxy-terminal tail with no known homology. The semaphorin domain is defined by high conservation within the Semaphorin family of proteins. The only other Semaphorin that has the same domain configuration as this cDNA is Drosophila Semaphorin II (D-SemaII; Kolodkin et al., 1993). The predicted protein product of the C. elegans semaphorin II gene (figure 4.2) was therefore named C. elegans Semaphorin II (Ce-SemaII) because of the apparent homology to D-SemaII. Ce-SemaII encodes 13 of the 14 cysteines conserved within the semaphorin family. Seventeen of Ce-SemaII's 19 cysteines are conserved with 17 of D-SemaII's 26 cysteines. A blastp search for homologues within the GenBank sequence database using either the complete protein or the semaphorin domain of Ce-SemaII, reveals that D-SemaII (accession number
A. The construction of the full length *semaphorin II* cDNA. Two overlapping clones, *yk100* (not shown) and *yk80* (Y. Kohara, personal communication) were used to design primers to produce the 5′ RACE product and an SL1 PCR product. The Sma I site was used to splice the clones together to produce a full length clone, *evCe-SemalI*, which was sequenced (C). ATG, the predicted start codon; E, Eco RI; S, Sal I; B, Bam HI; Sm, Sma I; P, Pst I; H, Hind III; Stop, the predicted stop codon. Scale is indicated. B. A schematic of the predicted 658 amino acid protein product of *evCe-SemalI*. Yellow box, signal sequence; hatched box, semaphorin domain; 3′ circle, Ig domain. C. The sequence of the *evCe-SemalI* cDNA. The predicted initiator methionine and stop codons are underlined; the predicted poly-adenylation signal is double-underlined. The first intron is deleted in *mob-20*(*ev574*) and is indicated with a bold bracket. The missense mutation of *mob-20*(*bx179*) at position 563 that substitutes a thymine for a cytosine is indicated with a black circle. *mob-20*(*bx24*) has a tandem duplication resulting in bases 926 to 1971 (shown with brackets) inserted between bases 1971 and 1972. D. An autoradiograph of a northern blot of 5 μg of mixed-staged *C. elegans* poly(A)+ RNA probed with the full length *evCe-SemalI* cDNA labelled with P32. The resulting band is approximately 2.6 kb. The markers on the right of the blot are in RNA kilobase size units.

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**Figure 4.1** Isolation and Construction of a *C. elegans* *semaphorin II* full-length cDNA
Figure 4.2 A multiple sequence alignment showing conserved residues in Ce-SemaII

The alignment includes C. elegans Semaphorin II (Ce-SemaII, no accession number to date); Drosophila Semaphorin II (D-SemaII, Kolodkin et al., 1993, accession number L26083); mouse Semaphorin III (M-SemaIII, Puschel et al., 1995, accession number X85993); and Human Semaphorin V (H-SemaV, Sekido et al., 1996, accession number U28369). Conserved residues are boxed. The dots above the alignment indicate those residues that are conserved in immunoglobulin type-C2 domains. Residues not coded for in mab-20(ev574) are indicated in bold. The polymorphism of mab-20(bx61') that results in a change of proline to leucine is highlighted with a black circle. The sequence was aligned by eye using the Kolodkin et al. (1993) alignment as a guide and the Maligned program by Clark (1992).
L26083) has the highest identity scores of 1.4e-50, and 9.5e-47, respectively. Over the entire length of the predicted protein, Ce-SemaII is 32% identical and 41% similar to D-SemaII.

The full-length evCe-semaII cDNA was used to isolate a 15.4 kb genomic clone. Restriction enzyme analysis and sequencing of the coding region of this phage verified the cDNA sequence. The clone contains 7.7 kb 5' to the predicted initiator methionine and over 800 bases 3' to the polyadenylation site. A subclone of this phage, called pPRII.2 (figure 4.3), rescues the semaphorin II mutant ev574 (table 4.7 and below), lending further support to the idea that the genomic clone contains all elements required for proper functioning of the semaphorin II locus.

4.2.2 The Isolation of a C. elegans semaphorin II Mutant

Existing candidate mutants could not be tested for rescue with pPRII.2 since a physical map position for semaphorin II could not be ascertained. Conventional hybridization techniques to a genomic grid that contains a large portion of the C. elegans genome represented by overlapping genomic clones (Coulson et al., 1991) did not yield a signal when probed with the semall cDNA. This result suggested that the gene was located in a gap not covered by any clones on the genomic grid.

In order to obtain a semaphorin II mutant, a frozen library of worms containing an active and randomly distributed Tc1 transposon was made using the strain MT3126 (chapter 3; Collins et al., 1989; Zwaal et al., 1993). DNA samples from the frozen population were assayed for a Tc1 insertion into the semaphorin II locus using PCR, resulting in strain NW 1034 semaphorin II(ev573::Tc1) as illustrated in figure 4.3. The Tc1 transposon in ev573 is inserted into the first intron, 103 bp 3' to the adenosine base of the predicted initiator methionine. Animals homozygous for ev573::Tc1 exhibited no obvious phenotype. A frozen sub-library of NW 1034 was then built and screened for a deletion derivative of ev573::Tc1, resulting in the isolation of
Figure 4.3 mab-20 Alleles.
A. A schematic showing the mab-20 subclone (evpPRII.2), various mab-20 primers, and four alleles of mab-20. mab-20(ev573::Tc1) contains a Tc1 element inserted 103 bp after the initiator methionine. mab-20(ev574) contains a deletion that removes the first intron. mab-20(bx24) contains tandem duplication, and mab-20(bx61") encodes a leucine substitution for proline. Abbreviations not explained in figure 4.1 are as follows: X, Xba I; A, Asp 718; C, Cla I; N, Nco I, Pv, Pvu I; Sc, Sac I. Black boxes are predicted exons, red boxes and restriction sites are duplicated exons and sites, respectively. B. A Southern blot of genomic DNA from strains homozygous for indicated alleles cut with Xba I. The blot was probed with the Cla1'-sp718 fragment (A) labelled with 32P, revealing that mab-20(ev574) does not contain the 4.5 kb Xba I fragment (arrow) as predicted from single worm PCR using primers II.Y and II.10. C. A portion of the blot shown in (B), but striped and re-probed with the entire evCe-Semall cDNA as a loading control. e1490 is an allele of him-5 that is contained in all strains used, except N2. The number of integrated mab-20(+) transgenes relative to N2 in (B) and (C) was quantified using a phospho-imager (see table 4.7 and figure 4.29 for more details). D. A Southern blot of DNA from wildtype (N2) and mab-20(bx24) strains that is cut with various indicated enzymes and probed with the full length evCe-Semall cDNA. Note that mab-20(bx24) DNA samples cut with Cla I, Eco RI, Hind III, Pst I, Pvu II, Sac I, and Sal I, but not Asp 718 and Bam HI, all have polymorphisms consistent (asterisks) with RT-PCR results (not shown) and the model of gene structure presented in (A). E. RT-PCR from wildtype (N2), and two mab-20 alleles (bx24 and bx61") templates using various indicated primers. The primer combination of II.0 and IIR-O did not produce an amplicon of equal intensity with the mab-20(bx61") template (asterisk) compared to those of N2 and mab-20(bx24). Further investigation showed that the 3' most base recognized by primer II.0 was changed from cytosine to thymine in mab-20(bx61").
NW 1074 mab-20(ev574). PCR, southern analysis, and sequencing, show that ev574 has 1479 bp of DNA missing which includes part of the first intron, the first exon, and 641 bp 5’ to the predicted initiator methionine, thereby eliminating the predicted signal sequence (figure 4.3). Since no other methionine is present until the 120th amino acid, semaphorin II(ev574) is likely to be a null allele.

4.2.3 Mapping and Molecular Characterization of the semaphorin II Alleles

The deformed body phenotype (figure 4.13) of the semaphorin II (ev574) mutant was used to show linkage of ev574 to chromosome I (see materials and methods). Linkage analysis revealed that the ev574 DNA polymorphism and body shape irregularities are weakly linked to dpy-5(e61) in the gene cluster on linkage group I. Concurrent with the mapping experiments, phenotypic analysis revealed that the sensory rays of the ev574 homozygous male tail are fused, a phenotype referred to as male abnormal or mab (figure 4.18). The pattern of ev574 ray fusion is similar to those observed in mutants of mab-20 on linkage group I, and of mab-26(bx80) on linkage group IV (Baird et al., 1991). Both the mab-20 and mab-26 gene had not yet been cloned.

Complementation tests revealed that mab-20(bx24) and mab-20(bx61), but not mab-26(bx80), are in the same complementation group as ev574 (table 4.7). mab-20 therefore encodes Semaphorin II and maps to position –10.41 on chromosome I (Baird et al., 1991). I call the gene mab-20 and the predicted protein product Ce-SemaII.

SSCP analysis of genomic DNA from strains containing either bx24 or bx61 was performed. bx24 contains an internal tandem duplication as verified by PCR and Southern analysis (figure 4.3). The duplication is inserted precisely after the stop codon in the last exon and contains a 2.2 kb fragment of DNA normally found 5’ to the stop codon. The temperature sensitive allele bx61, however, has a missense mutation leading to the substitution of leucine for proline at amino acid 188. The effect of proline on the tertiary structure of proteins is consistent
with the temperature sensitivity of this mutation. The nature of the polymorphisms is consistent with the relative severity of the three \textit{mab-20} alleles (see the male tail section below and table 4.2).

The body shape and pattern of male sensory ray defects of \textit{mab-20} is very similar to both \textit{mab-26(bx80)}, and a second mutant named \textit{vab-6(e697)} (figure 4.8, figure 4.13, figure 4.18, table 4.7). It is plausible that these two mutants are in the Semaphorin II pathway since they may affect the same biological process that leads to the morphological defects observed in \textit{mab-20} mutants. \textit{mab-26(bx80)} and \textit{vab-6(e697)} were therefore included in the investigation of the cellular basis of the morphological abnormalities of \textit{mab-20} mutants.

\textbf{4.2.4 \textit{mab-20} Mutants have Defects in Nerve Cord Fasciculation, Cell Migration, and Pioneer Axon Guidance}

After isolating a \textit{mab-20} loss-of-function mutant, I investigated the role of \textit{C. elegans} Semaphorin II in axon guidance, nerve cord fasciculation and other cell migrations. Using the evls82b (\textit{unc-129''}:GFP (evpAC12); \textit{dpy-20}(+)) transgene that expresses GFP in the DA and the DB motor neurons (Colavita and Culotti, 1998), axon guidance and fasciculation was examined in a \textit{mab-20(ev574)} mutant background and compared to wildtype (figure 4.4). The cell bodies of the DA and DB neurons reside in the ventral cord and send axons longitudinally and then circumferentially to the dorsal cord. The axons are normally tightly fasciculated within the cords and rarely, if ever, have errant circumferential pioneer axon guidance defects (table 4.1). Seventeen per cent of \textit{mab-20(ev574)} mutants, however, have fasciculation defects, and 4\% of these animals show defective circumferential guidance defects in at least one of the DA or DB commissures (figure 4.4, table 4.1).

During the investigation of the role of Ce-SemaII in \textit{C. elegans} neural development, it was predicted that Ce-SemaII may have a similar role in regulating axon defascication from
Figure 4.4 Axon guidance defects of mab-20(ev574) mutants, mab-26(bx80) mutants, and animals that ectopically express Ce-SemaII. All larvae shown express the unc-129 neural-specific GFP reporter construct (unc-129":GFP (expAC12); dyp-20(+)). Red arrows point to defasciculated nerve cords. Red arrowheads point to misplaced nerve cord cell bodies. A. In a wildtype background, the DA and DB commissural axons (c) originate in the ventral cord (vc) and extend circumferentially to the dorsal cord (dc), which is out of the plane of focus. B. In many mab-20(ev574) mutant animals, the cell bodies of many DA and DB neurons do not tightly associate with the ventral cord (arrowheads). C. Some mab-20(ev574) animals have at least one DA or DB axon that does not reach the dorsal cord, and instead migrates in a ventral-sub-lateral position (red arrow). D. Some mab-20(ev574) mutants have fasciculation defects in either the ventral cord (shown), the dorsal cord or both. E. In mab-20(ev574); evls83[unc-129":mab-20 (expPRI.11); rol-6(su1006)] animals, axon guidance errors (arrow) and fasciculation defects (F) are significantly enhanced compared to evls83 or mab-20(ev574) homozygotes. mab-26(bx80) have axon guidance errors (G) and fasciculation defects (H) that are qualitatively and quantitatively similar to that of mab-20(ev574); evls83. All animals are shown from a ventral or a ventral-lateral view, except for (F) and (H) which are shown from a dorsal view. Scale bar ~20μm. I. The expPRI.11 construct was used drive the expression of Ce-SemaII from the unc-129" promoter by a fusion at the initiator methionine. Scale bar is 1 kb. Abbreviations are as in figure 4.3.
nerve cords at specific choice points like other invertebrate Semaphorins. It was hypothesized that over-expression of Ce-SemaII from the ventral cord might induce axon defasciculation within the nerve cord. Ce-SemaII was therefore expressed in the DA and DB motor neurons by using the \textit{unc-129 neural specific} (\textit{unc-129ns}) promoter to drive the ectopic expression of Ce-SemaII (\textit{evPRII.11}, figure 4.4) from the \textit{evls83 [unc-129ns:mab-20 (evPRII.11); rol-6(su1006)]} transgenes in a wildtype and \textit{mab-20(ev574)} mutant background. Three independent transgenic isolates of \textit{evls83} had mild guidance and defasciculation defects in a wildtype background, but greatly enhanced axon guidance and defasciculation defects in a \textit{mab-20(ev574)} mutant background relative to \textit{evls83} transgenes or \textit{mab-20(ev574)} mutants alone (table 4.1).

Interestingly, not only does \textit{mab-26(bx80)} have qualitatively similar neuronal defects as \textit{mab-20(ev574); evls83[unc-129ns:mab-20 (evPRII.11); rol-6(su1006)]}, the mutant also has similar frequencies of axon guidance and fasciculation defects (Table 4.1).

<p>| Table 4.1 Axon guidance defects of mab-20, ectopic mab-20, mab-26, and vab-6 |
|---------------------------------|-----------------|-----------------|---|</p>
<table>
<thead>
<tr>
<th></th>
<th>Genotype</th>
<th>% of Misguided</th>
<th>% of Defasciculate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>wildtype</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>mab-20(ev574)*</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>evls74[mab-20(+); rol-6(su1006)]</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>evls83a [unc-129:mab-20; rol-6(su1006)]</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>evls83b [unc-129:mab-20; rol-6(su1006)]</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>evls83c [unc-129:mab-20; rol-6(su1006)]</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>mab-20(ev574); evls83b [unc-129:mab-20; rol-6(su1006)]</td>
<td>28</td>
<td>82</td>
</tr>
<tr>
<td>8</td>
<td>mab-20(ev574); evls83c [unc-129:mab-20; rol-6(su1006)]</td>
<td>24</td>
<td>69</td>
</tr>
<tr>
<td>9</td>
<td>mab-20(ev574); evls83d [unc-129:mab-20; rol-6(su1006)]</td>
<td>22</td>
<td>66</td>
</tr>
<tr>
<td>10</td>
<td>mab-26(bx80)</td>
<td>28</td>
<td>49</td>
</tr>
<tr>
<td>11</td>
<td>vab-6(e697)</td>
<td>3</td>
<td>14</td>
</tr>
</tbody>
</table>

Axon guidance defects and cord defasciculation are reported as percentages of animals which contain them. To visualize the axons and nerve cords, all strains contained \textit{evls82b[evlspAC12 (unc-129 neural-specific promoter::GFP); dpy-20(+)]. In addition, all strains studied also contained him-5(e1490) except (*).}
Defects in the migration of a descendent of the left Q neuroblast, called PVM, in \textit{mab-20} mutants were also observed. QL is born well posterior to the vulva, which forms at the mid-body, and normally undergoes a short posterior then anterior movements which displace it slightly posterior to its birthplace (figure 4.5; Hedgecock \textit{et al.}, 1987; Sulston and Horvitz, 1977). The \textit{mec-4:gfp} reporter (M. Driscoll, Rutgers University) was used to visualize the six Q cell descendent mechanosensory neurons in wildtype and mutant animals. In 27\% of \textit{ev574} mutants, the PVM migrated near or past the vulva (table 4.2, figure 4.5). The extent of the anterior PVM migration in \textit{ev574} is similar to AVM, the right-sided analogue of PVM.

\begin{table}[h]
\centering
\caption{PVM migration defects} \label{table:pvm_migration}
\begin{tabular}{l|c|c|c|c|c}
\hline
Genotype & PVM Migration Equivalent to AVM & PVM Migration near the Mid-body & WT PVM Migration & N \\
\hline
wildtype & 0 & 0 & 100 & 200 \\
mab-20(ev574) & 14 & 13 & 73 & 184 \\
evls74a[mab-20(+); rol-6(su1006)] & 2 & 2 & 92 & 200 \\
evls74b[mab-20(+); rol-6(su1006)] & 0 & 0 & 100 & 208 \\
mab-26(bx80)* & 0 & 1 & 99 & 200 \\
vab-6(e697) & 4 & 0 & 96 & 200 \\
\hline
\end{tabular}
\end{table}

\textit{Table 4.2 PVM migration defects}

Migration defects are reported as percentages. All strains studied contained \textit{mec-4::GFP} (#137) except (*), which had and \textit{mec-4::GFP} (#154). WT, wildtype.

Finally, using differential interference contrast (DIC) optics it was observed that the final leg of the distal tip cell (DTC) migration was altered in \textit{mab-20(ev574)} mutants. The two DTCs are born at the mid-body on the ventral side near the presumptive vulval cells. Each DTC leads each somatic gonad arm through a series of directed migrations and turns (figure 4.6; Hedgecock \textit{et al.}, 1987). The DTCs first migrate in opposite directions away from mid-body on the ventral side, then undergo an orthogonal ventral to dorsal turn and migration, and then turn again and migrate proximally toward mid-body to meet each other dorsal to the developing vulva. Nine percent of \textit{ev574} DTCs underwent a third, distally oriented turn late in L4 (figure 4.6). The DTC defect in \textit{ev574} mutants was partially rescued by the \textit{mab-20} transgene \textit{evpPRII.2}, when present in the integrated array, \textit{evls74d[mab-20(+)(evpPRII.2); rol-6(su1006)]} (table 4.3).
Figure 4.5 The descendants of the QL neuroblast have migration defects in mab-20(ev574) mutants. A. A schematic of a ventral view of the migration paths taken by the descendants of the QL and QR neuroblasts in a wildtype background. The QL gives rise to PVM, SDQL, PQR and two cell deaths. The QR gives rise to the AVM, SDQR, AQR. The integrated transgene Is[mec-4:GFP: rol-6(su1006)], a gift from M. Driscoll, Rutgers University, can be used to follow the migration of the touch sensory neurons, including PVM and AVM of the Q neuroblast lineage (solid green circles). B. A schematic of a ventral view of the migration paths taken by the descendants of the QL and QR neuroblasts in a mab-20(ev574): Is[mec-4:GFP: rol-6(su1006)] background. In many of the mutant animals, the PVM takes on a similar migration path as the AVM, as shown in (C). It is unknown if SDQL and PQR are similarly affected in a mab-20(ev574) mutant background. C. The PVM of a mab-20(ev574): Is[mec-4:GFP: rol-6(su1006)] mutant animal has migrated past the vulva, approximating the path taken by the AVM. The photomicrograph is a composite of three photos of a ventral view. D. A second example of the PVM migrating past the vulva to nearly the equivalent position of the AVM in a mab-20(ev574): Is[mec-4:GFP: rol-6(su1006)] mutant background. All three panels (D1, D2, and D3) show the same portion of a single animal, but in different focal planes from a lateral perspective. In all of the photomicrographs, the vulva is out of frame to the right. Anterior is to the left. A, axon; VC, ventral cord. The scale bar represents approximately 20 μm.
**Figure 4.6 mab-20(ev574) mutants have distal tip cell (DTC) guidance errors.**

A. A schematic of the migration path (yellow arrows) of the anterior and posterior DTCs that lead the extension of the gonad. The DTCs first migrate distally, away from the presumptive vulva and along the ventral body wall muscle bands (not illustrated). The DTCs then make an orthogonal turn and migrate in a dorsal direction, then proximally along the dorsal muscle bands (not illustrated) towards the midbody. B. A DIC photomicrograph of a posterior gonad of a wildtype (N2) adult. Note the two orthogonal turns of the gonad (yellow arrows). The DTC is not in the plane of focus (asterisk). C. A schematic of the migration paths (yellow arrows) sometimes taken by DTCs in mab-20(ev574) mutants. D. A DIC micrograph of the gonad in a young mab-20(ev574) adult. Note that the DTC has led the migration of the gonad through two supernumerary turns (third and fourth yellow arrows). In (A) through (D) anterior is to the left and dorsal is up. The scale bar represents approximately 50 μm.
evls74d [mab-20(+); rol-6(su1006)] may have DTC defects on its own because of over-expression of Ce-SemaII from the many copies of mab-20(+) on the transgenic array (see below).

Table 4.3 Erroneous distal-tip-cell migrations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Supernumerary Turn</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>wildtype</td>
<td>1</td>
<td>101</td>
</tr>
<tr>
<td>mab-20(ev574)</td>
<td>9</td>
<td>131</td>
</tr>
<tr>
<td>mab-20(ev574); evls74d[mab-20(+); rol-6(su1006)]</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>evls74d[mab-20(+); rol-6(su1006)]</td>
<td>4</td>
<td>100</td>
</tr>
</tbody>
</table>

Each DTC was considered independently. All strains contain him-5(e1490).

4.2.5 mab-20 Mutants have Ventral Enclosure Defects

Initial observations of mab-20 mutants revealed that hermaphrodites have a low fecundity, a phenotype that is rescued both zygotically (data not shown) and with mab-20(+) transgenes (figure 4.7). A detailed examination reveals that in a given population, 84% of mab-20(ev574); him-5(e1490) embryos are obviously dead or have internal contents leaking from the ventral midline during the elongation stage of morphogenesis (figure 4.8). Presumably, the dead embryos are the result of leakage of cells from ventral midline. The embryonic defects of ev574 are partially rescued by a mab-20(+) transgene; only 28% of mab-20(ev574); evls74d[mab-20(+); rol-6(su1006)] doubles have embryonic defects (table 4.4). Incomplete rescue may be explained by the fact that 40% of evls74d[mab-20(+); rol-6(su1006)] animals exhibit a phenotype like that of mab-20 mutants; they die or burst at the midline. That mab-20(+) transgenes induce a mab-20(ev574)-like mutant phenotype in transgenic animals is a phenomenon I call phenocopy (see below for more details). Interestingly, when a mab-20(+) transgene is placed in the background of a mab-20 presumptive null, a significant reduction in embryonic lethality is observed even though each allele on its own results in a moderate or high penetrance of embryonic lethality (table 4.4).
A The Morphological Abnormalities of mab-20(ev574) are Rescued by the mab-20(+) Transgene evIs74d

![Bar chart showing penetrance of morphological abnormalities across different genotypes.]

B The Reduced Brood Size of mab-20(ev574) Parents is Partially Rescued by the mab-20(+) Transgene evls74b

![Bar chart showing the approximate average brood size per parent across different genotypes.]

Figure 4.7. A double-blind study of the fecundity and the morphological defects of mab-20. Ten L4s from each of the following genotypes were cloned, wildtype (N2), a negative control evIs73[mab-20:GFP (evPRL1.67); rol-6(su1006)], mab-20(ev574), another negative control mab-20(ev574); evIs73, evIs74b[+][mab-20(+) (evPRL1.2); rol-6(su1006)], evls74d[mab-20(+) (evPRL1.2); rol-6(su1006)], unc-33(e204), mab-20(ev574); unc-33(e204) isolate number 2, and mab-20(ev574); unc-33(e204) isolate number 4. Also, thirty mab-20(ev574); evls74b[+] L4s and twenty mab-20(ev574); evls74d L4s were cloned. (*1) denotes the evls74b genotype before it had reverted to one that phenocopied mab-20(ev574) less (see table 4.7, figure 4.29 and text for details). Since it was difficult to objectively assess the penetrance of morphological abnormalities of various strains, a double blind experiment was done (see materials and methods for details). Although both the appearance (A) and the number (B) of all embryos, larvae and adults generated by each clone was recorded at days 1, 3.5, and 6, only that for day 3.5 larvae (black bars) and day 6 adults (grey bars) are reported here for clarity. A. Of note, the genotype that does not phenocopy mab-20(ev574) to a great extent (evls74d) can rescue the mab-20(ev574) morphological abnormalities while the evls74b transgene that does phenocopy the abnormalities, does not completely rescue the respective mab-20(ev574) phenotype. Curiously, the converse is true with respect to the average brood size (B). Note that the penetrance of the morphological abnormalities observed in day 3.5 larvae is consistently greater that that observed in day 6 adults. Two different mab-20(ev574); unc-33(e204) mutant isolates show a significantly different penetrance of morphological defects. Two isolates of the mab-20(ev574); unc-33(e204) double show that the low fecundity of mab-20(ev574) was not enhanced by unc-33(e204). The number of progeny scored per genotype far exceeds 200.
George et al. (1998) have shown that the leakage of internal contents from the ventral midline during elongation of vab-1 embryos is indicative of hypodermal ventral enclosure defects. As a result of the concentric forces during the elongation phase of embryogenesis (Figure 4.14; Priess and Hirsh, 1986), contents of the vab-1 mutant embryo are squeezed out via the incompletely sealed hypodermis at the ventral midline, resulting in embryonic death. Most animals that escape the embryonic lethality have morphological defects. When compared side by side, the embryonic defects of the mab-20 mutants examined are qualitatively indistinguishable from those alleles of vab-1 (table 4.4; figure 4.8).

From 210 to 230 minutes after first cleavage, the last cells to form the hypodermis are born (Schnabel et al., 1997; Sulston et al., 1983). By 250 minutes, two dorsal, two lateral and two ventral rows of hypodermal cells are arranged longitudinally (Podbilewicz and White, 1994) and sit on the embryo in a dorsal posterior locale, leaving the ventral neuroblasts exposed (figure 4.9). Ventral enclosure is required to cover the exposed ventral neuroblasts and the rest of the embryo with hypodermis. An excellent description of enclosure is given by Williams-Masson et al. (1997) and will be summarized here for clarity (figure 4.10). As the two dorsal rows of

<table>
<thead>
<tr>
<th>Table 4.4 Ventral enclosure defects</th>
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<tr>
<td><strong>Genotype</strong></td>
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<tr>
<td>----------</td>
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<td>1</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<td>4</td>
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<td>8</td>
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<td>9</td>
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<td>10</td>
</tr>
</tbody>
</table>

All strains contain him-5(e1490) except (*). The percentage of local bursting refers to the percentage of animals that had cells leaking out of either the head, mid-body, or posterior body regions at the ventral surface. The percentage of pleiotropic bursting refers to the leaking of cells throughout the length of the embryo, or severely deformed embryos. The percent dead refers to those embryonic shells that were filled with cellular debris and contained no defined cells or shaped-embryo. The percentage of wildtype (WT) refers to those animals that were older than the 2 fold stage and had at least partially elongated. Embryos prior to the 1.5 to 2-fold stage were not observed to have bursting defects, and although these animals were counted, the counts are not presented in the table. All strains were raised at 20°C.
Figure 4.8 A survey of embryos with ventral enclosure defects. Unless otherwise noted, all embryos are approximately at the threefold stage of development. If visible, the head of each embryo is indicated with a yellow arrow, while the tail is indicated with a yellow arrowhead. The internal contents that have spewed from the ventral midline are indicated with red arrows.

A. A wildtype embryo. The yellow arrow points to the head, which is below the plane of focus.
B. mab-20(ev574).
C. evls74a [mab-20(+) (evpPRII.2); rol-6(su1006)] which phenocopies mab-20 (ev574).
D. evls74b [mab-20(+) (evpPRII.2); rol-6(su1006)] which also phenocopies mab-20 (ev574).
E. evls83a [unc-129"::mab-20 (evpPRIII.11); rol-6(su1006)] which also phenocopies mab-20 (ev574) at a low frequency. Note that the embryo is at the 1.5 fold stage and that the ventral side of the head has burst.
F. evls102a [mab-20::GFP(evpPRIII.14); rol-6(su1006)] also phenocopies mab-20(ev574). Note that the animal is severely contorted.
G. mab-20(bx24).
H. mab-20(bx61")
I. mab-26(bx80).
J. vab-6(e697).
K. vab-1(e2027).
L. lig-1(ev658). All embryos have [him-5(e1490)]. Anterior is to the left. The scale bar is approximately 10 µm.
Figure 4.9. A summary of hypodermal morphogenesis in *C. elegans*. A. A schematic of an embryo at approximately 220 minutes after first cleavage. Six longitudinal rows of hypodermal cells sit on the embryo in a dorsal-posterior locale. Dorsal hypodermal cells are black, lateral hypodermal (seam) cells are green, ventral hypodermal cells are yellow. Miscellaneous blast cells are red. B. Soon after the longitudinal rows are established, the dorsal hypodermal cells intercalate and the hypodermis spreads ventrally to cover the ventral side (epiboly) from 250 to 390 minutes after first cleavage. C. After the embryo is covered with hypodermis, circumferential microfilaments (red ring) in hypodermal cells constrict the embryo (D), resulting in the vermiform shape of hatchlings at 800 minutes after first cleavage (E). Muscle cells are violet. H0-H2, V1-V6, and T are lateral hypodermal seam cells. P1/2- P11/12 inclusive, are P neuroectoblast cells. Drawings are not to scale and are interpretations from Podbielewicz and White (1994); Williams-Masson *et al.* (1997); and Priess and Hirsh (1986).
Figure 4.10 A review of ventral enclosure. Ventral enclosure has been described by Williams-Masson et al. (1997). A. Ventral enclosure is actively lead by the migration of the two pairs of leading ventral hypodermal cells, called "leader cells". The anterior pair of leader cells extend processes towards the ventral midline, while the posterior leader cells extend processes towards the anterior leader cells. The posterior ventral hypodermal cells, called "pocket cells" passively follow the leader cells. B. Upon contact with their respective contralateral homologues, the leader cells form tight junctions. A ventral cleft called the "ventral pocket", however, is left by the pocket cells. An actin based microfilament purse-string mechanism within the pocket cells closes the ventral pocket, allowing tight junctions to form between all ventral hypodermal cells (C). Green cells, lateral hypodermal "seam" cells; yellow cells, ventral hypodermal cells; red cells, miscellaneous blast cells. Anterior is to the left, and the ventral side is showing.
hypodermal cells interdigitate, the ventral rows of hypodermal cells on both sides of the embryo move towards the ventral midline to enclose the embryo within hypodermis. The ventrally directed epiboly is led by two anterior pairs of ventral hypodermal cells on either side of the embryo called leader cells. Only the leader cells extend substantial filopodia (Raich and Hardin, 1998); the anterior pair of leader cells extends processes ventrally towards the midline, while the posterior pair of leader cells extends filopodia anteriorly. After the contralateral leader cells meet and form junctions at the ventral midline, a ventral pocket is left by the trailing posterior ventral hypodermal cells, called pocket cells. A microfilament-based purse-string mechanism that operates within the pocket cells is thought to close the ventral pocket. Twelve of the seventeen pocket cells are P neuroectoblasts (P cells) and are arranged in two longitudinal rows (Podbilewicz and White, 1994). After ventral enclosure, all ventral hypodermal cells fuse into either the hyp-6 or hyp-7 syncitium except for the six pairs of P cells.

Since mab-20 mutants have phenotypes attributable to errors in ventral enclosure, the P cell-subset of pocket cells that play an essential role in ventral enclosure were examined in animals in the late stages of embryogenesis or just after hatching. After ventral enclosure is complete, the twelve ventral hypodermal P cells are arranged in two longitudinal rows (Podbilewicz and White, 1994). Each of the twelve rhomboid cells contact two seam cells on their dorsal side, one P cell on each ipsilateral side (terminal P cells contact only one ipsilateral P cell), and only one contralateral P. The arrangement of the P cells can clearly be seen through indirect immunofluorescence using the monoclonal antibody MH27, which recognizes JAM-1, a component of the apical zonulae adherins of all C. elegans epithelial cells (J.D. Hardin, personal communication; Waterston, 1988). MH27 was used to visualize the relative positions of the ventral hypodermal P cells in wildtype, and mab-20 and vab-1 mutants (figure 4.11). Seventy-eight out of one hundred mab-20(ev574) mutant hatchlings that had escaped the lethal ventral enclosure defects had P cells that contacted more than one contralateral P cell. These abnormal
P cell contacts may result in the observed starburst pattern of ventral hypodermal cells (figure 4.11). This arrangement of P cells is reminiscent of their earlier arrangement at the very completion of ventral enclosure, 400 minutes previously (Podbilewicz and White, 1994). In only one out of one hundred vab-1(e2027) animals did a P cell contact two contralateral neighbors. No wildtype (n=100), mab-26(bx80) or vab-6(e697) animals have any P cell defects. These results suggest that the P cells in mab-20 mutant animals may be able to form ectopic contacts with other P cells during ventral enclosure.

As described above, the Semaphorins repel growth cone extensions during axon migrations. Similarly, I hypothesized that without Ce-SemaII, some or all of the ventral hypodermal cells of the mutant embryos may extend ectopic processes that may establish ectopic contacts with other ventral hypodermal cells. Ectopic contacts between the ventral hypodermal cells could prevent the closure of the ventral pocket leading to the spewing of internal contents observed in the mab-20 mutants. Scanning electron microscopy (SEM) was used to examine the contacts between the ventral hypodermal cells of mab-20 mutants during ventral enclosure. SEM results show that some pocket cells of mab-20(ev574) form ectopic contacts to non-neighboring pocket cells before or during ventral enclosure (figure 4.12).

4.2.6 mab-20(ev574) Animals have Severe Morphological Abnormalities

The most obvious phenotype of ev574 homozygotes is the severe deformities in body size and shape (figure 4.13). Newly hatched animals are shorter than wildtype and have bumps and constrictions. More often than not, these morphological abnormalities are localized to the posterior mid-body region and are first observed during elongation of the embryo into a vermiform shape. To test if mab-20(+) transgenes can rescue the morphological abnormalities of mab-20(ev574), various strains were subjectively scored in a double blind test. Approximately 88% of larvae, and 55% of adults exhibit obvious morphological defects when
Figure 4.12 Scanning electron microscopy (SEM) reveals ectopic contacts between ipsilateral pocket cells during ventral enclosure. Except for (K) and (L), each SEM micrograph on the right is an enlargement of the yellow-boxed area of the SEM micrograph on the left. A & B. A lateral view of a control embryo [him-5(e1490)] at an early stage of ventral enclosure. Note that the ventral leader hypodermal cells (yellow arrows) and the trailing posterior-ventral pocket cells (blue arrows) are at a mid-lateral position. The green arrows in (B) illustrate that no prominent filopodia are observed in control embryos (or in mutants: see (E) and (I)) like that seen in the SEM micrograph of Williams-Masson et al. (1997) or the in vivo analysis by Raich and Hardin (1998). C & D. A lateral view of a control him-5(e1490) embryo at a later stage than (A), illustrating that each ventral hypodermal cell contacts at most two ipsilateral neighbours. E & F. A ventral-lateral view of a mab-20(ev574):him-5(e1490) mutant embryo at about the same stage of development as that in (C). Note that the posterior-ventral hypodermal cells make ectopic contacts (red arrows) before ventral enclosure is complete, which is never observed in control [him-5(e1490)] embryos. G & H. A ventral-lateral view of a him-5(e1490) embryo at a later stage than (C), whereby the leader cells, but not the pocket cells, have made contact and fused with their contralateral homologues. The processes emanating from the ventral blast cells (orange arrows) are unusual and may be artifactual. I & J. A lateral view of a mab-20(ev574):him-5(e1490) mutant embryo at about the same stage of development as that in (G). The embryo is shorter than that in (G) possibly because of the ectopic contacts (red arrows) between the anterior pocket cells. Some of the posterior pocket cells that are indicated with a blue arrow are tentative assignments. K. A ventral view of a control [him-5(e1490)] embryo prior to contralateral-leader cell contact at the ventral midline (approximated with a red line). Note that the pocket cells are just visible on the left side. L. A ventral view of a mab-20(ev574):him-5(e1490) after the leader cells have met and fused with their contralateral homologues. Note that the pocket cells are in a lateral position like those in (K). At this stage, however, the ventral pocket should be much smaller and shorter (compare with (G) and (K)), a defect that suggests that the hypodermis did not completely enclose the embryo and is now retracting towards the dorsal surface (Raich and Hardin, 1998). Anterior is to the left in all images. Scale bar is approximately 10 μm.
Figure 4.13 A survey of *mab-20*-like morphological defects. All animals shown are either L1s or young L2s, as determined by the developmental stage of the somatic gonad. A. Wildtype. B. A negative rolling control evls72[*mab-20::GFP(evpPR11.67); rol-6(su1006)]. C. *mab-20(ev574)*. D. evls74a(*mab-20(+) (evpPR11.2); rol-6(su1006)). E. evls74b(*mab-20(+) (evpPR11.2); rol-6(su1006)). F. *mab-20(ev574); evls74c(*mab-20(+) (evpPR11.2); rol-6(su1006)). G. *mab-20(ev574); evls74d(*mab-20(+) (evpPR11.2); rol-6(su1006)). H. evls83a[*unc-129"::mab-20 (evpPR11.11); rol-6(su1006)]. I. *mab-20(ev574); evls83b[*unc-129"::mab-20 (evpPR11.11); rol-6(su1006)]. J. *mab-20(ev574); evls102a(*mab-20::GFP(pPR11.14); rol-6(su1006)). K. *mab-20(bx24). L. *mab-20(bx61"). M. *mab-26(bx80). N. vab-6(e697). O. vab-1(e2027). All strains contain *him-5(e1490)*. Scale bar is approximately 25 μm.
progeny from ten ev574 clones are scored (figure 4.7). In contrast, the morphological defects of ev574 homozygotes are nearly completely rescued by evls74d[mab-20(+)(evpPRII.2); rol-6(su1006)]. Of course, control mab-20(ev574) mutant animals are not rescued by a non-functional mab-20:GFP reporter transgene called evls73[mab-20:GFP (evpPRII.75); rol-6(su1006)] (figure 4.7). These results suggest that a mutation within the mab-20(ev574) locus is responsible for the morphological abnormalities and that the DNA contained within the mab-20(+) (evpPRII.2) sub-clone is sufficient to rescue the morphological defects. It is interesting to note that like the phenocopy conferred by mab-20(+) transgenes on ventral enclosure (see above), the evls74b[mab-20(+) (evpPRII.2); rol-6(su1006)]) transgene also confers morphological defects that are qualitatively similar, if not identical to mab-20(ev574) (figure 4.7). Unlike evls74d however, which shows little mab-20(ev574) phenocopy and rescues mab-20(ev574), evls74b fails to completely rescue mab-20(ev574). As noted repeatedly throughout the results section, mab-20(+) transgenes can phenocopy most of the phenotypes conferred by mab-20 loss-of-function mutations, a phenomenon that is reportedly uncommon for other genes and will be addressed in later sections.

Since the morphological abnormalities of mab-20(ev574) are first manifested after embryonic elongation begins, I hypothesized a causal relationship between elongation in ev574 homozygotes and the morphological defects. To address this hypothesis, elongation was investigated in the mab-20 mutant background and compared to wildtype. Priess and Hirsh (1986) first demonstrated that the hypodermal cytoskeleton plays an essential role in the elongation and morphogenesis of C. elegans (figure 4.14). At about halfway through embryogenesis, animals are essentially transformed from a ball of cells to a vermiform shape without an increase in cell number or volume. As ventral enclosure nears completion, hypodermal microfilaments within the five longitudinal rows of hypodermal cells align
Chapter Four

Figure 4.14 *mab-20* mutants may have morphological defects as a result of an uneven distribution of force during elongation. Elongation has been described by Priess and Hirsh (1986). A. In wild type embryos, circumferential microfilaments within the hypodermis are aligned with respect to the left-right circumferential axis after the completion of ventral enclosure and are thought to generate a force (purple rings) that constricts the embryo into a vermiform shape. B. Microfilament disruption (using cytochalasin D) prevents embryonic elongation. C. Microtubule disruption (using colcemid, nocodazole, or griseofulvin) or the disruption of the embryonic sheath (using trypsin) results in irregularly shaped animals. Priess and Hirsh (1986) suggested that the microtubules and the sheath are required to uniformly distribute the force generated by the microfilaments. D. Hypothetically, the conformation of the hypodermal cells in *mab-20* mutants could restrict the conformation of the microfilaments, leading to an uneven distribution of the force generated during elongation. Green cells, seam cells; yellow cells, P cells. Anterior is to the left and dorsal is up. Drawings are not to scale.
circumferentially, parallel with the left-right (L/R) axis. These microfilaments contract to constrict the spheroid threefold. The force generated by the microfilaments during elongation is evenly distributed across the embryonic surface by the embryonic sheath and the hypodermal microtubules, without which animals develop bulges and constrictions. Since the conformation of the ventral hypodermal P cells is altered in mab-20 mutants, I further hypothesized that the disordered position of the ventral hypodermal cells may result in a concomitant alteration in the conformation of circumferential microfilaments during elongation. An oblique conformation of circumferential microfilaments may result in an uneven distribution of force like that suggested by Priess and Hirsh (1986) upon the application of microtubule inhibitors (e.g., colcemid) during or before elongation.

To investigate the conformation of the circumferential microfilaments, fixed embryos were briefly exposed to rhodamine-conjugated phalloidin to lessen the staining of internal structures that could obscure the visualization of the hypodermal F-actin (see materials and methods). Although the circumferential microfilaments in the ventral cells of mab-20 mutants sometimes conform to the starburst pattern of the ventral P cells observed with MH27 (figure 4.15), this conformation of microfilaments is not necessarily coincident with the deformities of the mutant animals.

Surprisingly, the most striking hypodermal microfilament defect in mab-20 mutants is in the lateral hypodermal seam cells (figure 4.15). The lateral hypodermal seam cell-microfilaments that neighbor the bumps of mutant embryos are skewed with respect to the L/R circumferential axis. The seam cell-microfilament defect is precisely coincident with the morphological abnormalities (compare panels A, C, and E in figure 4.15 and data not shown). Bumps along the mutant animals are only observed in areas where the microfilaments within two or more neighboring seam cells are aligned with respect to each other, but not with the L/R
circumferential axis. Microfilaments misaligned within a single seam cell, however, are not coincident with severe body deformities (compare panels A, E and F in figure 4.15), suggesting that two or more seam cells that contain skewed microfilaments are required for bump formation.

To address why the seam cell microfilaments were skewed with respect to the L/R circumferential axis, it was important to know if the defect was limited to the microfilaments, or if the skewed microfilaments reflected the conformation of the seam cells in which they were contained. To examine the spatial arrangement of the seam cells during embryonic development, the monoclonal antibody MH27 was used. In support of the latter hypothesis, MH27-stained \textit{mab-20(ev574)} homozygotes exhibited clustered seam cells (figure 4.11). Since the intestinal fluorescence of MH27-stained embryos was strong, the second side of the embryo could not be clearly scored for clustered seam cells. However, more than half of the sides examined in the mutant embryos contained seam cells that contacted more than two neighboring seam cells (table 4.5). The most frequently clustered cells involved H0, H1 and H2, and, V4, V5 and V6. Similar to \textit{mab-20} mutants, \textit{vab-6} mutants, but not \textit{mab-26} mutants, also exhibited significant seam cell clustering that more often involved the posterior seam cells rather than the anterior H seam cells (figure 4.11).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% of Sides Containing H Cell Defects</th>
<th>% of Sides Containing V and T Cell Defects</th>
<th>% of WT Sides</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{wildtype}</td>
<td>0</td>
<td>2</td>
<td>98</td>
<td>200</td>
</tr>
<tr>
<td>\textit{mab-20(ev574)}</td>
<td>25</td>
<td>31</td>
<td>44</td>
<td>208</td>
</tr>
<tr>
<td>\textit{mab-26(bx80)}</td>
<td>2</td>
<td>1</td>
<td>97</td>
<td>214</td>
</tr>
<tr>
<td>\textit{vab-6(e697)*}</td>
<td>12</td>
<td>20</td>
<td>68</td>
<td>101</td>
</tr>
</tbody>
</table>

One side of each embryo greater than 1.25 fold was scored for ectopic seam cell contacts. A contact was scored as ectopic if a seam cell contacted more than two neighboring seams. All strains contain \textit{him-5(e1490)} except (*).

During the course of examining circumferential microfilaments (see materials and methods) the body wall muscle arrangement could sometimes be seen, and in some \textit{mab-20(ev574)} mutant animals was perturbed (table 4.6). The 95 body wall muscles of \textit{C. elegans} are arranged as longitudinal rows that run along the entire length of the animal and are divided into
four quadrants. Two longitudinal rows of rhomboid shaped muscle cells make up each quadrant (Sulston and Horvitz, 1977). Although newly formed muscle cells appear normal, \textit{mab-20}(ev574) embryos stained for sarcomeric microfilaments with phalloidin exhibited a variety of defects in muscle arrangement including muscle cells crossing quadrant boundaries and a general disorganization of the four quadrants (figure 4.15). \textit{mab-20}(+) was able to rescue these defects since only 14% of the \textit{mab-20}(ev574); \textit{evl}s74d[\textit{mab-20}(+); \textit{evpPR}1.2]; \textit{rol-6}(su1006) animals had muscles that crossed boundaries (table 4.6). \textit{mab-26}(bx80) and to a lesser extent \textit{vab-6}(e697), had defects in the body wall muscle arrangement that qualitatively and quantitatively resembled those of \textit{mab-20}(ev574). Although perturbed muscle cell arrangement can cause body malformations (Chen \textit{et al}., 1994), the circumferential microfilament disorganization in the seam cells are more likely to be the root cause of the bulges since the latter phenotype is precisely coincident with the locale of the deformities in \textit{mab-20}(ev574) embryos. The body wall muscle defects may be only secondary to hypodermal disorganization. Indeed, in \textit{mab-20}(ev574) embryos where both the circumferential microfilaments and the body wall muscles could be observed, muscle cells only crossed quadrant boundaries when positioned beneath seam cells that contained skewed microfilaments (figure 4.15).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Quadrant Crossing Defects</th>
<th>Posterior Defects</th>
<th>Ventral Defects</th>
<th>Dorsal Defects</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{wildtype}</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>\textit{mab-20}(ev574)</td>
<td>73</td>
<td>1</td>
<td>42</td>
<td>58</td>
<td>61</td>
</tr>
<tr>
<td>\textit{evl}s74d[\textit{mab-20}(+); \textit{rol-6}(su1006)]</td>
<td>21</td>
<td>1</td>
<td>1</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>\textit{vab-6}(e697)*</td>
<td>23</td>
<td>5</td>
<td>12</td>
<td>18</td>
<td>19</td>
</tr>
</tbody>
</table>

The scores are reported as percentages of animals that exhibited the indicated defect. Anterior and posterior defects refer to an irregular arrangement of muscle cells within the quadrants situated anterior or posterior to the embryonic midbody, respectively. Ventral and dorsal defects refer to an irregular arrangement of muscle cells within the quadrants situated in either the two ventral or two dorsal body wall muscle quadrants, respectively. Quadrant crossing refers to body wall muscles that cross quadrant boundaries. All strains contain \textit{him-5}(e1490), except (*).

The expression patterns of \textit{HLH-1} and \textit{mab-20} before and during embryonic muscle development are intriguingly complementary when comparisons are made between figures 4.28
and 4.29 with Krause et al. (1990). Furthermore, the body deformities of \( hlh-1(cc450) \) mutants are strikingly similar to those of \( mab-20 \) mutants (Chen et al., 1994; Krause et al., 1995). \( hlh-1 \) encodes a transcription factor that belongs to the Myo D family, of which Drosophila \textit{nautilus} is also a member. Baker et al. (1995) found that when the epidermis is separated from mesoderm, \textit{Nautilus} fails to be expressed by the mesoderm. Similarly, I hypothesized that the morphological abnormalities of \( mab-20 \) mutants may result from in the inability of hypodermis to induce wildtype HLH-1 expression in mesoblasts, resulting in the observed muscle defects. It is also possible that HLH-1 prevents ectopic expression of Ce-SemaII, thereby resulting in the \( mab-20 \)-like deformities of the \( hlh-1(cc450) \) mutants. HLH-1 antibody (a gift from A. Fire) revealed that the expression pattern of HLH-1 was unchanged in the \( mab-20(ev574) \) mutant (data not shown). Furthermore, the \( mab-20 \) GFP reporter expression pattern was not changed in the \( hlh-1(cc450) \) mutants (data not shown). In light of these results, the morphological phenotypes of \( hlh-1 \) mutants are probably only superficially coincidental with \( mab-20 \) mutants and not causal.

By the time embryos hatch at 800 minutes after first cleavage (afc), many of the hypodermal cells are in multinucleated syncitia. Nuclei of the hypodermis are constrained to the ventral and lateral hypodermal ridges. The fourth hypodermal ridge, positioned dorsally, is devoid of hypodermal nuclei. The four hypodermal ridges exist between the four muscle quadrants and are produced by the constriction of hypodermal plasma between each quadrant of muscle cells and the tightly adjacent cuticle. The remaining hypodermal plasma is essentially shunted to the space between muscle quadrants, creating each ridge (Hedgecock et al., 1987). If the circumferential arrangement of microfilaments is locally disarrayed, as in the \( mab-20 \) mutants, an uneven distribution of force may result in bumps and constrictions like that observed when microtubules or the embryonic sheath is disrupted (Priess and Hirsh, 1986). I hypothesized that an uneven distribution of force within the hypodermis would restrict the hypodermal nuclei
to the bumpy regions and occlude them from the areas of constriction. Anti-LIN-26 antibodies were used to stain the nuclei of all hypodermal cells (Labouesse et al., 1996). In mab-20(ev574) hatchlings, the lateral hypodermal nuclei are indeed unevenly distributed along the lateral lines, in sharp contrast to the regular placement of wildtype hypodermal nuclei. The mutant nuclei accumulate in areas coincident with the bumps along the animal (figure 4.16).

A third defect in hypodermal ontogeny was observed in MH27-stained ev574 larvae and adults. The anterior seam cell daughters sometimes fail to break contacts with the seam and in turn are not able to fuse with hyp-7 (figure 4.11). A related phenotype of mab-20 mutants in the cuticle is clearly visible using DIC optics. In wildtype animals, the seam cells secrete a specialized grooved cuticle called the alae, which runs the length of the animal and whose lateral and lineal position reflects that of the seam cells (White, 1988). In mab-20 mutants, however, the path of the alae is often irregular and probably reflects the abnormal conformations of the underlying seam cells (data not shown).

4.2.7 mab-20 is Required for the Morphogenesis of Male Sensory Organs
The adult male tail is a specialized structure used for copulation. It is bilaterally symmetrical; each side has 9 sensory rays that project laterally and are embedded within a cuticular spade-shaped fan. The development of the male tail has been described in detail by Sulston et al. (1980) and will be summarized here for one side (figure 4.17). Differences in the male and hermaphrodite lineage are manifested early in the third larval stage of development (Sulston et al., 1977); the progeny of the posterior seam cells V5.ppp, V6.pap, V6.ppp undergo an extra round of division, and T.apap and T.appa continue to divide only in the male. Three more rounds of division give rise to 9 iterative groups of 5 cells and one cell death. Two of the five cells become hypodermal, two become neurons, and one becomes a structural cell that ensheaths the two neurons of the group, forming a hypodermal papillae. During the anterior-directed retraction of the entire tail at the fourth larval stage, the nine papillae remain attached to the
Figure 4.16 LIN-26 antibodies reveal hypodermal nuclei aggregation in mab-20 mutants. A. Wildtype [him-5(e1490)] animals stained with LIN-26 antibodies that recognize an antigen within all hypodermal nuclei. Note that the hypodermal nuclei are evenly distributed along the body length (arrow). B. A mab-20(ev574) mutant stained with the LIN-26 antibody. The hypodermal nuclei aggregate in regions coincident with bumps (arrows) and are occluded from the constricted body regions (arrowheads). Anterior is to the left. The scale bar is approximately 20 μm.
Figure 4.17 The development of the male tail. Shown here is a depiction of the morphogenesis of the left half of the bilaterally symmetric male tail. Gender-specific morphogenesis begins early in the third larval stage or L3 (A) and continues up to adulthood (L). A. Early in the third larval stage, the descendants of the posterior embryonic seam cells V5, V6, and T undergo the first of four divisions, generating nine ray precursor cells. V5 gives rise to ray one (R1), V6 to rays two (R2) through six (R6), and T to rays seven (R7) through nine (R9). B-K. Three more divisions give rise to one cell death, two sensory neurons, one structural cell that ensheaths the two neurons, and a fifth cell that either contributes to the male tail seam (SET) in the case of R1 through R5, or hyp 7 in the case of the remaining rays. The two neurons and structural cell of each ray precursor lineage are thought to have a greater adhesion for siblings and cousins than for second cousins, thereby forming ray clusters (F&G). The finger-like projections that are the mature sensory rays are generated by an anterior movement of the entire posterior relative to the ray clusters. The anterior movement of the hypodermis (into the page of H-K) covers the two axons which are ensheathed by the structural cell of each cluster. The anterior movement of the hypodermis also leaves behind the posterior cuticle, to which the sensory rays are attached, forming the spade-shaped fan (L). Ray precursor clusters 1, 5, and 7 are born in a dorsal position and respectively generate rays that have a dorsal sensory opening in the fan. Ray precursor clusters 3, 6, and 9 are born in a lateral position and respectively generate rays that have lateral sensory openings in the fan (except for R6, which does not have an opening). Ray precursor clusters 2, 4, and 8 are born in a ventral position and respectively generate rays that have a ventral sensory opening in the fan. Stylized drawings on the right are interpretations of the MH27 immunostained him-5(e1490) animals on the left. The numbers represent the ray precursor clusters. Phso is the phasmid socket. Anterior is to the left and dorsal is up. The scale bar is approximately 10 μm. The development of the male tail has been previously characterized in detail by Sulston et al. (1980) and Baird et al. (1991).

Cuticle and become surrounded by hypodermis, forming the finger-like rays of the adult.

Simultaneously, the cell bodies of the three ray cells retract sub-dermally. The ABp-derived V5 and T lineages give rise to rays one, and seven through nine, respectively. The ABa-derived V6 lineage gives rise to rays two through six.

*mab-20* mutant males are male abnormal (mab; figure 4.18). Table 4.7 illustrates the penetrance of fusions of rays 1 through 4, fusions of ray 6 to 4, and fusions of ray 7 to 9 for various mutants, including that of *mab-20(ev574)*. Ray 5 and 8 rarely fused to any neighbor.

Table 4.7 also shows that an integrated *mab-20(+) transgene nearly completely rescues the ray fusions of *mab-20(ev574)*. In addition, the table shows that *mab-20(ev574)* does not complement *mab-20(bx24) and *mab-20(bx61)*; transheterozygote combinations reveal a
penetrance of ray fusions that is between that normally conferred by the two alleles when in a homozygous state.

Baird et al. (1991) first used MH27 to characterize ray precursor cell positions during the formation of the male tail and showed the ray precursor clusters remained separated in wildtype but not in the mab-20(bx24), and mab-20(bx61) mutants. The position of the ray precursor clusters along the dorsal-ventral axis correlates with the position of the openings of the sensory rays: The clusters of rays 1, 5, and 7 are situated dorsally and the corresponding adult sensory endings open on the dorsal side of the fan. The clusters of rays 3 and 9 are positioned laterally and open at the margin of the fan. The clusters of 2, 4, and 8 are positioned ventrally and the corresponding adult rays open ventrally. These experiments were repeated for wildtype, mab-20(ev574), mab-20(bx24), mab-20(bx61), and evls74a[mab-20(+)(evpPR1.2); rol-6(su1006)] transgenic animals that phenocopy the mab-20 loss-of-function phenotype (figures 4.17, 4.19, 4.20, 4.21, and 4.22, respectively).

Also included in the analysis of the development of the male tail are mab-26(bx80), unc-130(ev505), unc-130(ev582), mab-21(bx41), vab-1(e2027) and lig-1(ev658) for comparison to mab-20 and mab-26 mutant tails (figures 4.23, 4.24, 4.25, and 4.26, respectively). Similar to the observations of the fused rays made with DIC optics, the mab-20 and mab-26(bx80) mutants reveal aggregations of ray clusters 1 through 4 and 6, and 7 to 9 when stained with MH27. These results differ from the interpretation of the mab-20 and mab-26 mutant phenotypes of Baird et al. (1991) and Chow and Emmons (1994), who suggest that ray 8 and 9 fuse, not rays 7 and 9. I also observe that cluster 5 is spatially isolated from cluster 4 and 6 by the R4.p-R6.p connection during early wildtype male tail development. Also, cluster 8 is isolated from cluster 7 and 9 by the R7.p-R8.p hypodermal cell connection. These observations suggest that those clusters that are spatially restricted from neighbors during early ontogeny are not able to fuse to other clusters in mab-20 and mab-26 mutants.
**Figure 4.18** A survey of male tail sensory ray fusions of various mutants. The male tails of various strains both discussed in this chapter and not are shown here for reference and for comparison to each other and *mab-20* mutant male tails. Except for (B) and (D), all tails were photographed with DIC optics. The different background colors are due to two different filters used on different occasions. Except for (A) through (D), only the fused rays are labeled. The rays within fusions were assigned labels based on several observations. First, the dorsal-ventral position of the sensory openings within fusions are like that of the rays that make up the fusion (see text for greater details; Baird et al., 1991). Second, the size of the fusion, together with those rays that are not fused, is indicative of the number and type of rays within the fusion. Finally, the patterns of ray precursor clustering as observed with MH27 immunostaining of many of the mutant male tails reveal a stereotypical pattern that is reflected in the fusions of the adult male tail (Baird et al., 1991). A. The tail of a wildtype male transgenic for evl579[unc-129::GFP(evpAC12); dpy-20(+)] that is highly expressed in a neuron of the dorsal rays 1, 5, and 7 as shown in the corresponding epifluorescent micrograph (B). C. A tail of a *mab-20(ev574)* mutant male. D. The corresponding epifluorescent micrograph of the tail of (C) showing that the neuronal expression pattern of evl582a does not change in a *mab-20* mutant tail compared to (B). E-H. The tails of males transgenic for wildtype *mab-20* array strains *evls74a[mab-20(+)] (evpPRII.2); rol-6(sa1006)], *evls74b[mab-20(+) evpPRII.2]; rol-6(sa1006)], *evls74c[mab-20(+) (evpPRII.2); rol-6(sa1006)], and *evls74d[mab-20(+)] (evpPRII.2); rol-6(sa1006)], respectively. I. A *mab-20(ev574); evls74c* phenotypically rescued male tail. J. A *mab-20(ev574); evls74d* phenotypically rescued male tail. K. *mab-20(bx24)*. L. A male tail from a *mab-20(bx51ts)* mutant raised at 20°C. M. A male tail of a *mab-20(ev574)/mab-20(bx24)* transheterozygote. N. A male tail from a *mab-20(ev574)/mab-20(bx51ts)* transheterozygote raised at 20°C. O. The male tail of an animal transgenic for evEx[mab-20::GFP (evpPRII.14); rol-6(sa1006)]. P. The male tail of an animal transgenic for evEx[unc-129ns::mab-20 (evpPRII.11); rol-6(sa1006)]. Q. *mab-26(bx80)*. R. *vab-6(e697)*. S. Tails mutant for vab-1(e2027) have swollen bursas that sometimes include rays 8 and/or 9. T. *lig-1(ev658)* mutants only have fusions of ray 8 and 9. U. *unc-130(ev505)* mutants generally have displaced rays and a couple of ectopic (e) rays. W. *mab-18(bx23)* mutants only have fusions of ray 4 to 6. X. *mab-19(bx38)* mutants have rays of the T lineage missing, i.e. rays 7, 8, and 9. Anterior is to the left and dorsal is up. Only the left half of the tail is shown in all micrographs except (E), where both halves are shown from a ventral perspective, and (F) and (H) where the right side is shown. Except for (A), (U), and (V), only the fused sensory rays are labeled. The scale bar is approximately 10μm. All strains shown contain *him-5(e1490)*.

In contrast to *mab-20* and *mab-26*, *unc-130* mutants often generate ectopic ray precursor clusters that sometimes result in an ectopic ray (figures 4.18 and 4.24). For example, figure 4.24 shows that midway through the fourth larval stage of an *unc-130(ev505)* mutant male, there are four ectopic clusters tentatively assigned to the R7, R8 and R9 lineage. In addition, there is fusion of the ray precursor clusters of R4, R5, and R6, and fusion of the ray precursor clusters of R7 and two ectopic ray clusters. These and other defects (see figure legends) are strikingly similar to those of *mab-21* mutants (figure 4.18 and 4.25, Chow et al., 1995), which are defective
Figure 4.19 The development of the *mab-20(ev574)* mutant male tail. (A) through (J) chronicle the development of *mab-20(ev574); him-5(e1490)* male tails from late in L3 (A and B) to late in L4 (I and J). Note that neighbouring ray precursor clusters adhere together early in morphogenesis (C and D) compared to figure 4.17(D) and 4.17(E). E & F. The ray precursor clusters of 1-4, and 6 are in direct contact with one another, as well as 7 and 9. G & H. Ray precursor cluster 1 and 2 are in direct contact, as well as 3, 4 and 6, and in another group, 7 and 9. I & J. Ray cluster 1-3 are in direct contact, as well as 4 and 6, and 7 and 9. Note that ray precursor cluster 5 and 8 are isolated from other clusters throughout morphogenesis. The stylized drawings on the right are interpretations of the MH27 immuno-stained animals on the left. SET, male tail seam; Phso, phasmid socket; the numbers represent ray precursor cells. Anterior is left and dorsal is up. The scale bar is approximately 10 μm.
Figure 4.20 The development of the *mab-20(bx24)* mutant male tail. (A) through (J) chronicle the development of *mab-20(bx24); him-5(e1490)* male tails from late in L3 (A and B) to late in L4 (I and J). Note that neighbouring ray precursor clusters adhere together early in morphogenesis (C and D) compared to figure 4.17(D) and 4.17(E). E & F. The ray precursor clusters of 1-4 are in direct contact with one another. G & H. Ray precursor cluster 1 and 2 are in direct contact, as well as 3 and 4, and in another group, 7 and 9. I & J. Ray cluster 1 and 2 are in direct contact, as well as 3 and 4. Note that ray precursor cluster 5 and 8 are isolated from other clusters throughout morphogenesis. The stylized drawings on the right are interpretations of the MH27 immuno-stained animals on the left. SET, male tail seam; Phso, phasmid socket; the numbers represent ray precursor cells. Anterior is left and dorsal is up. The scale bar is approximately 10 μm.
Figure 4.21 The development of the mab-20(bx61) mutant male tail. (A) through (J) chronicle the development of mab-20(bx61); him-5(e1490) male tails from late in L3 (A and B) to late in L4 (I and J). Note that neighbouring ray precursor clusters adhere together early in morphogenesis (C and D) compared to figure 4.17(D) and 4.17(E). E & F. The ray precursor clusters of 1-4 are in direct contact with one another, as well as 7 and 9. G & H. Ray precursor clusters 2, 3 and 4 are in direct contact. I & J. Ray cluster 3 and 4 are in direct contact, and possibly 8 and 9. The stylized drawings on the right are interpretations of the MH27 immunostained animals on the left. SET, male tail seam; Phso, phasmid socket; the numbers represent ray precursor cells. Anterior is left and dorsal is up. The scale bar is approximately 10 μm.
Figure 4.22 The development of the evls74a [mab-20(+)] (evpPRII.2); rol-6(su1006)] transgenic male tail. (A) through (H) chronicle the development of evls74a; him-5(e1490) male tails from late in L3 (A and B) to late in L4 (G and H). Note that neighbouring ray precursor clusters adhere together early in morphogenesis (C and D) compared to figure 4.17(D) and 4.17(E). E & F. The ray precursor clusters of 1, 2 and 3 are in direct contact with one another, as well as 4 and 6. Ray precursor cluster 9 cannot be seen. G & H. Ray precursor cluster 1 and 2 are in direct contact, as well as 3, 4 and 6, and in another group, 7 and 9. Note that ray precursor cluster 5 and 8 are isolated from other clusters throughout morphogenesis. The stylized drawings on the right are interpretations of the MH27 immuno-stained animals on the left. SET, male tail seam; Phso, phasmid socket; the numbers represent ray precursor cells. Anterior is left and dorsal is up. The scale bar is approximately 10 μm.
Figure 4.23 The development of the \textit{mab-26(bx80)} mutant male tail. (A) through (H) chronicle the development of \textit{mab-26(bx80); him-5(e1490)} male tails during the fourth larval stage. Note that the pattern of the neighbouring ray precursor clusters that adhere together in the \textit{mab-26} mutant male tails is strikingly similar to that of the \textit{mab-20} alleles. A & B. The ray precursor clusters of 1-4 and 6 are in direct contact with one another, as well as 7 and 9. C & D. Ray precursor cluster 1-4 and 6 are in direct contact, as well as 7, 8, and 9 in another group. E & F. Ray cluster 1-4 and 6 are in direct contact with each other. G & H. Ray precursor clusters 2, 3, and 4 are in contact with each other, and possibly 7 and 9. The stylized drawings on the right are interpretations of the MH27 immuno-stained animals on the left. SET, male tail seam; Phso, phasmid socket; the numbers represent ray precursor cells. Anterior is left and dorsal is up. The scale bar is approximately 10 \textmu m.
Figure 4.24 The development of *unc-130* mutant male tails. (A) through (E), and (F) through (J), respectively, chronicle the development of *unc-130(ev505); him-5(e1490) and uncs-130(ev582); him-5(e1490) throughout the fourth larval stage. In the case of the most severe allele, *ev505*, the ray precursor clusters are sometimes a group of four cells (A), compared to a group of three in both wildtype (figure 4.17D and 4.17E) and *mab-20(ev574)* (figure 4.19C and figure 4.19D). These ectopic cells may be those that normally undergo programmed cell death. The second phenotype observed during *unc-130* mutant male tail morphogenesis is the generation of entire ectopic cluster(s), which are denoted as eX. These ectopic clusters probably result in the ectopic rays observed in mature males (figure 4.20). The third phenotype observed, like that of *mab-20* and *mab-26* mutant male tails, is the adherence of neighbouring clusters. However, unlike *mab-20* and *mab-26* mutants, groups of clusters 4 and 6 sometimes include cluster 5 (C) and ectopic clusters (B). Also, like *vab-l(e2027)* and *lig-1(ev658)*, clusters 8 and 9 often fuse in *unc-130* (C, G, H, I, and J). Finally, in contrast to wildtype, and *mab-20*, and *mab-26* mutant development, where the R6.p cell fuses to hyp-7, the R6.p cells of *unc-130* mutants fuse with the SET (E, I, and J), like that of *mab-21* mutants (figure 4.25). Animals are stained with the MH27 antibody. SET, male tail seam; Phso, phasmid socket; the numbers represent ray precursor cells. Anterior is left and dorsal is up. The scale bar is approximately 10 μm.

in a gene that encodes a product that is thought to be translocated from the cytoplasm to the nucleus upon cell signaling (Chow et al., 1996).

Besides fused rays and ectopic rays, a third type of morphological defect that can be observed in male tails with DIC microscopy is a swollen bursa, as seen in *vab-l(e2027)* mutant male tails (figures 4.18 and 4.26). The swollen bursa may result from an irregular arrangement of tail hypodermal cells, including hyp-8, hyp-9, hyp-10, and hyp-11, before male tail morphogenesis begins (unpublished observations). Finally, a fourth type of defect in male tail morphogenesis occurs in *mab-19* (figure 4.18), *egl-5*, and *mab-5* mutants where selected rays are absent because of fate alterations within the V5, V6, or T lineage. The four different types of hypodermal defects in male tail morphogenesis reported here for various mutants, namely fusions, ectopic ray formations, swollen bursae and rays in absentia, illustrate the variation in mutant sensory ray development. Hence, the male tail defects observed in *mab-20* mutants are probably not a general default mechanism. Indeed, Ce-SemaII may have the same function in the development of the ray precursors as in the ventral hypodermal and seam cells: To prevent formation or stabilization of ectopic contacts between cells.
The development of the \textit{mab-21(bx41)} mutant male tail. (A) through (E) chronicle the development of \textit{mab-21(bx41); him-5(e1490)} male tails from late in L3 to late in L4 using the MH27 antibody that recognizes zonulae adherins. The first observable phenotype during the development of the \textit{mab-21(bx41)} mutant male tail is the presence of an ectopic ray precursor cluster (e) in (B). A second phenotype of the developing \textit{mab-21(bx41)} male tail is the adherence of ray precursor clusters 4, 6, e, and 8. Finally, by comparing (D) and (E), the R6.p (D, arrow) fuses to the male tail seam (SET), instead of the surrounding hyp-7 as in wildtype development. Numbers represent ray precursor cells. Anterior is left and dorsal is up. The scale bar is approximately 10 μm.
### Table 4.7. Male tail sensory ray fusions

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The body of the table gives the percentage of ray fusion to a neighbor within one side of a male tail. The column heading “>2R” refers to the percentage of male tail sides that had more than two rays within a single fusion. The evls74 is series of four integrated transgenes (a, b, c, and d) of the genotype [mab-20(+)(evPpRII.2); rol-6(su1006)] that are derived from the integration of the same extra-chromosomal array. evls74b was initially highly penetrant for the morphological and sensory ray phenotypes (*1), but sub-culture yielded an isolate that had severely reduced phenotypes (*2). All animals contained him-5(e1490).

#### 4.2.8 mab-20 Loss-of-Function Does Not Change the Fate of Rays that Fuse

It is thought that each sensory ray of the male tail may express a particular identity and that in some mab mutants, the identity of the rays that fuse are equivocated (Baird et al., 1991; Chow and Emmons, 1994). To investigate if the fused rays in mab-20(ev574) mutants is the result of a change in ray identity, the expression of a ray-specific marker was examined in a him-5(e1490).
background and compared to the expression in a \textit{mab-20} (ev574); \textit{him-5} (e1490) mutant background. In a wildtype and a \textit{him-5} (e1490) background, the \textit{evls82b} [unc-129\textsuperscript{*}:GFP; \textit{dpy20}(+)] transgene expresses relatively high levels of GFP in a neuron of rays 1, 5, and 7. If the identity or fate of the ray precursor clusters changes in a \textit{mab-20} (ev574); \textit{him-5} (e1490) genetic background, then fusions that involve rays 1 and 7, but not ray 5, since it rarely fuses with other rays, are predicted to have a corresponding change in the expression pattern generated from \textit{evls82b}. A strain carrying \textit{mab-20} (ev574); \textit{evls82b}; \textit{him-5} (e1490) revealed that the neuronal expression pattern in the sensory rays remains unchanged when compared to \textit{evls82b}; \textit{him-5} (e1490) (panels A through D, figure 4.18). In fusions involving ray 1 or 7, only the anterior-most neuron in the fusion expressed the reporter at high levels, as expected if the identity of the ray precursor clusters and the rays is not altered in the mutant background.

4.2.9 \textit{mab-20} is Required in the Morphogenesis of the Tail Whip

Besides having defects in ventral enclosure, morphogenesis, larval seam fusions, and male tail fusions, \textit{mab-20} mutants also have defects in the generation of the whip of the tail. In \textit{mab-20} (ev574) embryos, first-stage (L1) and second-stage (L2) larvae, about a third of the animals have a forked tail (figure 4.13). Using the demonstration version of the SIMI Bio Cell CD ROM\textsuperscript{TM} freely available from R. Schnabel (Schnabel et al., 1997), I have traced the path of divisions and migrations that lead to the generation of the two precursor cells that generate the left and right homologues that produce the tail whip (figure 4.27). The cells that generate the tail whip are descendants of cells that are situated in the anterior of the embryo. Somehow, the left and right tail progenitors must end up juxtaposed at the extreme posterior end of the embryo. Although the orientation of the plane of cell division may contribute to the anterior to posterior path taken by the descendents of ABpr and ABpl, several descendents also undergo short-range migrations to situate the precursors of the whip cells in a posterior locale. Once the two
progenitors are juxtaposed, they divide, each producing a hyp-10 cell that generates the hypodermis of the tail, and a spike cell that extends a bundle of filaments that provide a scaffold for the extension of the whip (Sulston, 1988). The two wildtype hyp-10 cells fuse to produce the binucleated hyp-10 tail syncitium. DAPI staining reveals no difference in the locales of the two hyp-10 nuclei between \textit{mab-20(ev574)} and wildtype. Interestingly, a fraction of \textit{evls74a}, \textit{evls74b}, \textit{mab-26(bx80)}, and \textit{vab-6(e697)} embryos and young larvae also have forked tails.

\textbf{4.2.10 \textit{mab-20} Reporters are Expressed During Hypodermal Ontogeny and Male Tail Morphogenesis}

With all of the caveats knowingly associated with reporter constructs (outlined in chapter 5), two types of \textit{mab-20} reporter constructs were nevertheless made. Both \textit{evpPRII.67} and \textit{evpPRII.75} (not shown) are transcriptional fusions between 2.5 kb of genomic DNA 5' to the predicted initiator methionine of \textit{mab-20(+)} and a \textit{gfp} cassette in the pPD95.67 and pPD95.75 vectors (gifts from A. Fire), respectively (figure 4.28). The \textit{evpPRII.14} construct is a translational fusion between Ce-SemaII and GFP, which is inserted into the second predicted exon of \textit{mab-20(+)} and contains the same upstream regulatory sequence as \textit{evpPRII.2} (figure 4.29). Animals transgenic for \textit{evpPRII.14}, but not \textit{evpPRII.67} and \textit{evpPRII.75}, phenocopy \textit{mab-20} mutants (figures 4.13 and 4.18).

Animals carrying either the \textit{evls72[mab-20:GFP (evpPRII.67); rol-6(su1006)]} transgene or the \textit{evls73[mab-20:GFP (evpPRII.75); rol-6(su1006)]} transgene show early GFP expression at the 40 to 50 cell stage in every cell except Ear, Eal, Epr, and Epl at about 100 minutes after fertilization (figure 4.28). A little later, expression is lacking in the Msppa, Msppp, Mspap,Mspaa, Dp, Caa, Cpa, Cap, and Cpp cells. All cells that do not express GFP at this time have either gastrulated or are in the process of gastrulating. At about 240 to 350 minutes, the cells of the dorsal posterior hypodermis, which is made up of progeny from C, have a low level or no GFP, as does the gut,
Figure 4.27 A schematic of the lineage and migrations that result in the proper positioning of the two tail spike cells. Each yellow or green circle represents successive posterior daughters or movement of daughters that originate from either ABpl, or ABpr, respectively. The drawing shows a dorsal view of the path of the cells that generates either the left (●, ABplpppppp) or right (●, ABprpppppp) progenitor that each give rise to a hyp-10 and tail spike cell. ABp divides at 30 minutes after the first cleavage (afc). The tail spike progenitors are born around 245 minutes afe. Anterior is to the left and right is up. Each cell center is traced from a 345 degree tilt of embryo number 1 of the demonstration version of SIMI Bio Cell CD ROM™ (Hutter and Schnabel, 1997).
Figure 4.28 mab-20 promoter activity. All animals shown are transgenic for evls72 [mab-20:GFP (evpPRII.67); rol-6(su1006)]. A. The construct evpPRII.67 has 2.4 kb of DNA 5' to the predicted initiator methionine of mab-20 driving GFP expression from A. Fire's pPD95.67. Although evpPRII.67 has a nuclear localization signal, GFP is also observed in the cytoplasm.

B & C. A dorsal view of a 40 to 50 cell stage embryo. GFP expression is seen in all cells except Ear, Eal, Epr, Epl, and P4. D & E. A left-lateral view of an embryo at about 110 minutes after first cleavage (afc). Expression is seen in all cells except Caa, Cap, Cpp, and Cpa.

F & G. A right-lateral view of an embryo at the nearly equivalent stage as in (D) exhibiting GFP expression in MSppp, MSppa, MSpaa, MSPap, Da and Dp. H & I. A dorsal-lateral view of an embryo at about 240 minutes afc. Descendants of C that become the dorsal hypodermis show down-regulated expression of GFP (arrows), as do the descendants of E which produce the gut (not shown). J & K. A lateral view of an L4 male exhibiting GFP in seam cells (arrows).

L & M. A ventral view of an adult male. GFP is highly expressed in the posterior third.

N & O. A dorsal lateral view of an adult male tail. GFP is observed in the hypodermis of the male sensory rays (one ray is indicated with an arrow).

P. A dorsal-lateral view of GFP expression in some of the neurons that make up the dorsal cord (dc). The circumferential neurons are indicated with yellow arrowheads.

Q. A ventral-lateral view of a hermaphrodite. GFP is expressed in the specific neuron (HSN), the vulva muscles (red arrowheads), and many neurons of the ventral cord (vc), and the circumferential neurons (yellow arrowheads) that enter the dorsal cord. In micrographs B-O, each DIC photo is followed by the corresponding epifluorescent micrograph. Anterior is to the left in all micrographs. Scale bar is approximately 20 μm.
A

mab-20(+) (evpPRII.2)

mab-20::GFP (evpPRII.14)

1 kb
Figure 4.29 A GFP expression pattern generated from a transgene encoding a tagged version of Ce-SemaII. All animals shown are of the following genotype, evls102a [mab-20(+):GFP (evpPRII.14); rol-6(su1006)]; him-5(e1490). The evls102 series of transgenes encode a translational fusion between Ce-SemaII and GFP. A. A schematic of the GFP-tagged Ce-SemaII construct evpPRII.14. The GFP-encoded fragment inserted into exon 2 of the mab-20(+) subclone evpPRII.2, was derived from pPD95.75 (a gift from A. Fire). Green boxes represent GFP exons; abbreviations are as indicated in figure 4.3. The wildtype mab-20(+) subclone evpPRII.2 is included for reference. In (B) through (M) each epifluorescent micrograph is preceded by the respective animal observed with DIC microscopy, except for (F) and (G). B & C. A dorsal view of an embryo. GFP is first observed before 250 minutes after first cleavage (afc). D & E. A dorsal view of a second embryo. Subtle dorsal hypodermal stripes (arrows) of differential reporter expression is observed at about 340 minutes afc. High expression is observed in the seam cells (F, arrow) and is otherwise nearly ubiquitously expressed (F & G). In (H) through (M), dorsal is up. The GFP reporter is observed in the 9 Rn.ps of the male tail at 30 hrs (H & I, arrows) and 38 hrs (J & K) of development. The same animal shown in (J) & (K), but in a different focal plane, exhibits reporter expression in the descendants of the 9 Rn.ps at 38 hrs of development. The arrow in micrographs (J) through (M) indicates an Rn.p (J & K) and it’s descendants (L & M). Anterior is to the left. Scale bar is approximately 20 µm.

which is descended from E. Most other cells in the embryo express GFP. Reporter expression is observed in the seam cells throughout development, and is highly expressed in the posterior third of L4 and adult males. Neuronal expression of GFP from the mab-20 promoter includes ALA, ALN, PLN, CAN, CEP, GLR, HSN, PVP, RID, RES, RME, the post-deirid ganglia, and other neurons within the nerve ring and the ventral and sub-lateral cords. Other cells that exhibit GFP expression in evls72 or evls73 transgenic animals include the body wall muscles, the enteric muscles, and the sex muscles (of both the hermaphrodite and male), hyp-10, the primary and secondary vulval precursor cells, and probably hyp-7 at low levels.

Animals transgenic for the translational fusion reporter, evls102a[mab-20::GFP (evpPRII.14); rol-6(su1006)], first exhibit GFP at approximately 240 minutes of development and is ubiquitous within the embryo (figure 4.29). No significant difference in expression levels is observed between any of the cells at this stage, except for the gut blast cells, which exhibits relatively lower GFP levels, and the seam cells which exhibit relatively higher GFP expression levels. Nearly ubiquitous expression is seen at all embryonic stages thereafter.
Ce-SemaII::GFP expression generated from the *evls102* transgene is highly regulated within the seam cells of both hermaphrodites and males at each larval stage. It appears as though expression is turned on before each seam cell divides and in each daughter thereafter, but is shut off during or after the anterior daughter fuses with the surrounding hyp-7 hypodermal syncitium. Other hypodermal expression during larval development is not evident in hermaphrodites. No seam cell expression in young and old adult hermaphrodites or males was observed (data not shown).

About 35 hrs after hatching, mid-L3 males express Ce-SemaII::GFP in the 9 descendents of V5, V6 and T that give rise to the ray clusters, called Rn cells (figure 4.29), along with some light background expression from the preanal, lumbar, dorsal rectal and cloacal ganglia. At 38-40 hrs, when ray papillae are just barely visible, the descendents of the 9 Rn’s exhibit GFP fluorescence. No surrounding hyp-7 cells exhibit GFP expression. The interior neural ganglia however, are about as bright as the ray precursors at this stage. This pattern of expression continues until adulthood.

Other cells that are observed to express Ce-SemaII::GFP in the hermaphrodite include the hermaphrodite specific neurons (HSNs) only at the L4 stage, vulva cells A to F throughout development, the migrating distal tip cells only at the L4 stage, and several neurons within the nerve ring and ventral cord. Expression in males also includes cells in the aforementioned posterior ganglia, the migrating male linker cell beginning at the L3 to L4 transition, and several neurons within the nerve ring and ventral cord.

### 4.2.10 Ce-SemaII is Required in a Dose-Dependent Manner

To test if *mab-20(+) *coding sequences could rescue any or all of the *mab-20(ev574)* mutant phenotypes, the *evls74[mab-20(+)](evpRII.2); rol-6(su1006)]* transgenic arrays were created in a wildtype background with the intention of later passing the array into the mutant background.
Surprisingly, animals carrying both extra-chromosomal and integrated versions of the array in a wildtype background were nearly indistinguishable from mab-20(ev574) homozygotes, a phenomenon previously described as phenocopy. Two of the four integrated lines, namely evls74a and evls74b, were highly penetrant for both the body deformities (figure 4.13) and the male tail fusions (figure 4.18, table 4.7). The two lines that did not have a highly penetrant phenotype in a wildtype background, evls74c and evls74d, were able to completely rescue the mab-20(ev574) recessive mutant phenotypes. The phenocopy is copy number dependent since the penetrance was reduced when the integrated arrays were made heterozygous (table 4.7).

Southern blot and phospho-imager analysis of the genomic DNA isolated from wildtype and strains containing the evls74 transgenes revealed a correlation between the mab-20(+) gene dose and the penetrance of the phenocopy (table 4.7, figure 4.30). Interestingly, the transgene evls74b initially induced a high penetrance of mab-20(ev574) phenocopy (designated as evls74b1) but after prolonged sub-culture, evls74b induced much less morphological abnormalities (designated as evls74b2) (table 4.7). The loss of the penetrance of mab-20(ev574) phenocopy is presumably due to a loss of mab-20 copy number within the array, as evls74b2 has a low mab-20(+) copy number (figure 4.30). Finally, animals transgenic for arrays encoding a GFP-tagged version of Ce-SemaII (Ce-SemaII::GFP), namely evls102[mab-20::GFP (evpPRII.14); rol-6(su1006)] (figure 4.29), also showed mab-20(ev574) phenocopy. Three integrated lines that were highly penetrant for the defects, namely evls102a, evls102b, and evls102c exhibited a relatively high level of GFP epifluorescence. In contrast, GFP is not observed in the evls102d line, which shows a low penetrance of phenocopy (data not shown). These results further support the idea that Ce-SemaII over-expression may somehow disrupt the function of the Semaphorin II pathway.
A. An autoradiograph of a Southern blot of genomic DNA isolated from wildtype (N2), and strains homozygous for evls74{mab-20(+) (evpPRII.2); rol6(su1006)}; him-5(e1490). Various other controls, including that of him-5(e1490) alone was also done (not shown). The genomic DNA was digested with the Eco RI restriction enzyme and probed with the full length evCe-Semall cDNA and an els-1 genomic fragment to control for the DNA quantity loaded on the gel (2N standard). A phosphoimager screen was exposed to the blot overnight and the intensity of the experimental band was measured to determine the mab-20 copy number in each strain.

B. A comparison of the mab-20 copy number to the extent of mab-20(ev574) male tail phenocopy induced by the various transgenic strains. Approximate numbers are given in table 4.7.

Figure 4.30 mab-20(ev574) phenocopy induced by mab-20(+) transgenic arrays is copy number dependent.
4.2.11 Ce-SemaII Expression from the \textit{unc-129 restores specific} Promoter Results in a \textit{mab-20} Mutant Phenocopy

As reported above (table 4.1; figure 4.4), Ce-SemaII expression in ventral cord motor neurons driven by the \textit{unc-129} promoter from transgenic arrays in the background of \textit{mab-20(ev574)} results in a significant enhancement of the fasciculation and axon guidance errors compared to \textit{mab-20(ev574)} alone. It was surprising to observe, however, that the strains transgenic for \textit{evIs83[unc-129::mab-20(+) (evPRII.11); rol-6(su1006)]} in a wildtype background partially phenocopies both the gross morphological abnormalities and sensory ray defects of \textit{mab-20} mutants (figure 4.13 and 4.18). The spectrum of ray fusions in strains transgenic for \textit{evIs83[unc-129::mab-20(+) (evPRII.11); rol-6(su1006)]} was similar to \textit{mab-20(ev574)}, but much less penetrant (table 4.8). In contrast to \textit{evIs74c} and \textit{evIs74d}, which could rescue \textit{mab-20(ev574)} and phenocopy the \textit{mab-20} mutants to a small extent in a wildtype background, \textit{evIs83} does not apparently rescue any defects of \textit{mab-20(ev574)} (table 4.8).

Given that \textit{mab-20(+) transgenic animals phenocopy mab-20 mutants} and that it is likely that the phenocopy is dependent on \textit{mab-20(+)} over-expression, it is curious that \textit{evIs83[unc-129::mab-20(+) rol-6]} strains that express Ce-SemaII from a heterologous promoter phenocopy \textit{mab-20} mutants. To investigate the possibility that \textit{evIs83} induces \textit{mab-20} mutant phenocopy because Ce-SemaII is over-expressed from the heterologous \textit{unc-129} promoter in similar tissues as the \textit{mab-20(+) transgenes}, the hypodermal expression pattern generated by the \textit{unc-129 promoter} from the \textit{evIs82b[unc-129::GFP; dpy20(+)]} (Colavita and Culotti, 1998) transgene was studied in detail (figure 4.31). Expression of GFP was first seen at about 120 minutes after first cleavage, and just after gastrulation begins. The patch of cells that express GFP are probably from the ABplla and the ABarp lineage, but not the E, C, or MS lineage. Later, at about 300 minutes after first cleavage, expression in the 8 E cells is evident, along with light expression in the dorsal and lateral hypodermal cells. Some anterior neuroblasts also express the
Table 4.8. Male tail sensory ray fusions in strains that ectopically express Ce-SemII from the unc-129" promoter

<table>
<thead>
<tr>
<th>Genotype</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
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<th>R6</th>
<th>R7</th>
<th>R8</th>
<th>R9</th>
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<td>4</td>
<td>4</td>
<td>0</td>
<td></td>
<td>317</td>
</tr>
<tr>
<td>mab-20(ev574)</td>
<td>92</td>
<td>99</td>
<td>97</td>
<td>96</td>
<td>3</td>
<td>63</td>
<td>92</td>
<td>12</td>
<td>87</td>
<td></td>
<td>200</td>
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<td>3</td>
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<td>0</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td></td>
<td>117</td>
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<td>evls83b[unc-129&quot;:mab-20(+); rol-6(su1006)]</td>
<td>19</td>
<td>32</td>
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<td>0</td>
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<td>2</td>
<td>4</td>
<td></td>
<td>115</td>
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<tr>
<td>evls83d[unc-129&quot;:mab-20(+); rol-6(su1006)]</td>
<td>9</td>
<td>15</td>
<td>14</td>
<td>0</td>
<td>5</td>
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<td>92</td>
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<td>0</td>
<td>87</td>
<td>87</td>
<td>17</td>
<td>87</td>
<td>99</td>
<td>104</td>
</tr>
<tr>
<td>mab-20(ev574); evls83c</td>
<td>77</td>
<td>91</td>
<td>97</td>
<td>92</td>
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<td>22</td>
<td>90</td>
<td>110</td>
<td>111</td>
</tr>
<tr>
<td>mab-20(ev574); evls83d</td>
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<td>99</td>
<td>100</td>
<td>97</td>
<td>0</td>
<td>77</td>
<td>85</td>
<td>24</td>
<td>83</td>
<td>108</td>
<td>100</td>
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</table>

The body of the table gives the percentage of ray fusion to a neighbor within one side of a male tail. The column heading ">2R" refers to the percentage of male tail sides that had more than two rays within a single fusion. The animals were raised and the counts were done at room temperature. All strains contained him-5(e1490).

reporter at this time. At the onset of male tail morphogenesis at about 30 to 32 hrs post-hatching, light expression from evls82b in R1.p and R3.p can be seen, along with high expression in R5.p and R7.p. Other Rn.ps may express the transgene, but are obscured by the bright GFP in the enteric muscles. Later, at about 34-39 hrs after hatching, GFP is obviously expressed in the SET and R5.p and R7.p. During the retraction of the ray precursor cluster cell bodies at 40 to 42 hrs post-hatching, expression from the evls82b reporter is limited to one neuron from ray 5 and 7. A third neuron, possibly belonging to ray 8 or 9, can also be traced directly to the papillae. In the adult male, GFP expression from evls82b is high in a neuron of ray 1, 5, and 7. In some animals, light GFP expression is observed in the other ray neurons.

Given that the hypodermal expression pattern driven by the unc-129" promoter is similar to many of tissues that express mab-20 reporters and that Ce-SemII over-expression from mab-20(+) transgenes results in mab-20 mutant phenocopy, it is not surprising that evls83[unc-129":mab-20(+); rol-6] transgenes also confer morphological abnormalities and male tail defects like that of mab-20 mutants.
4.2.12 Other genes of the Semaphorin pathway

Although many of the phenotypes of mab-26(bx80) have a lower penetrance than those of mab-20(ev574), the obvious phenotypic similarities between the two mutants suggest that they may act in the same biological process, genetic pathway, or may even directly interact with each other. To address the former hypothesis, the mab-20(ev574); mab-26(bx80) double mutant was constructed. If the two genes act in independent parallel pathways that control male sensory ray morphogenesis, the penetrance and expressivity of ray fusions in the double mutant would be expected to increase relative to each single mutant, if at least one mutation is null as suspected for mab-20(ev574). If, however, at least one of the mutants is a null and the two genes act in the same pathway, then the penetrance and expressivity would not be expected to increase, since the function of the pathway is eliminated by the null mutation. Comparing rows 1, 2, 4, and 6 of table 4.9 reveals that all ray fusion counts of the double mutant are the same or lower than those of mab-20(ev574) except for the percentage of ray 6 fusions with ray 4, which is increased by 18%. The increase of ray 6 to ray 4 fusion translates into an increase of the percentage of pleiotropic ray fusions (those involving more than two rays), since ray 4 is almost always fused to ray 3 in mab-20(ev574). The lack of synergy or additivity in the effects of these mutations is consistent with the idea that at least mab-20(ev574) or mab-26(bx80) is a null and that these two genes act in the same biological process or perhaps even the same genetic pathway.

Chow and Emmons (1994) reported that a loss-of-function in mab-5, the C. elegans Antennapedia HOM-C/HOX gene cluster homologue, dominantly enhances the ray fusion phenotypes of both mab-20(bx61) and mab-26(bx80) heterozygous mutants. Similarly, they showed that a loss-of-function in egl-5, the C. elegans Abdominal B homologue, dominantly suppresses the ray fusion defect of mab-26(bx80) heterozygous mutants. To test if mab-5, egl-5, and lin-39 (another HOM-C/hox gene cluster homologue) loss-of-function mutations affect the expression pattern of mab-20, doubles of the hox mutants and the mab-20 GFP reporters were
examined for altered mab-20 expression. All of the mab-5(e1239); evls72, mab-5(e1239); evls102a, egl-5(n486); evls72, egl-5(n486); evls102a, lin-39(n709); evls72, and lin-39(n709); evls102a doubles showed no gross difference in the expression patterns of the mab-20 reporters when compared to evls72 and evls102a alone during male tail development and/or embryogenesis. The effects of mab-20 and mab-26 mutations on the expression patterns of mab-5, egl-5, and lin-39 were not examined.

A vertebrate homologue of the C. elegans protein UNC-33 (Li et al., 1992) called CRMP-62 was shown to be required for the transduction of a Semaphorin III signal in a Xenopus oocyte assay (Goshima et al., 1995). Unlike the mab-20 mutant phenotype, C. elegans unc-33 mutants are primarily defective in axon guidance. Although unc-33 mutants have no obvious morphological defects like those of mab-20 mutants, the results of Goshima et al. (1995) prompted the investigation of possible genetic interactions between unc-33 and mab-20. Four separate lines of the mab-20(ev574); unc-33(e204) double were created. Observation with the dissection microscope clearly showed that two of the four lines grew slower were generally less healthy. The progeny of ten representative parents of each phenotypic class were subjectively scored for morphological defects and growth rates in a double blind experiment. Figure 4.7 shows that mab-20(ev574); unc-33(e204) line number four had more defects than mab-20(ev574), but line number two had fewer defects. These results suggest the possible existence of a genetic modifier present in one or both of the parent strains used to produce the double mutant. After the discovery of the male tail defects of mab-20(ev574) mutants, the sensory ray fusions of unc-33; mab-20 double mutants were unambiguously and objectively scored. If unc-33 mutations can enhance the frequency of sensory ray fusions in the male tail in animals heterozygous for a mab-20(ev574) mutation, then unc-33 may be in the Semaphorin II pathway of C. elegans. Table 4.9 shows that such animals do not have an increase in the frequency of sensory ray fusions.
Chapter Four

Table 4.9. Possible genetic modifiers of *mab-20*(ev574)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>R1</th>
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<th>R8</th>
<th>R9</th>
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<td>0</td>
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<td>4</td>
<td>4</td>
<td>0</td>
<td>317</td>
</tr>
<tr>
<td>2 <em>mab-20</em>(ev574) <em>mab-20</em>(ev574)/+</td>
<td>92</td>
<td>99</td>
<td>97</td>
<td>96</td>
<td>3</td>
<td>63</td>
<td>92</td>
<td>12</td>
<td>93</td>
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<td>700</td>
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<tr>
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<td>30</td>
<td>73</td>
<td>96</td>
<td>93</td>
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<td>100</td>
<td>93</td>
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<td>9</td>
<td>91</td>
<td>86</td>
<td>111</td>
</tr>
<tr>
<td>6 <em>mab-26</em>(bx80): <em>mab-20</em>(ev574)</td>
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<td>99</td>
<td>98</td>
<td>93</td>
<td>0</td>
<td>81</td>
<td>85</td>
<td>11</td>
<td>85</td>
<td>100</td>
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<tr>
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<td>8 <em>mab-26</em>(bx80)/+ <em>mab-26</em>(bx80)/+</td>
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<td>0</td>
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<td>13</td>
<td>0</td>
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<tr>
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<td>1</td>
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<td>1</td>
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<td>0</td>
<td>5</td>
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<td>94</td>
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</tbody>
</table>

The body of the table gives the percentage of ray fusion to a neighbor within one side of a male tail. Unless otherwise noted, the counts were done either at RT or at 20°C. The column heading ">2R" refers to the percentage of male tail sides that had more than two rays within a single fusion. All strains contained *him-5*(e1490). *mab-20*(ev574); *vab-6*(e697) and *mab-20*(ev574); *vab-1*(e2027) double mutants could not be constructed during two or more attempts. Although these are negative results, the outcome of repeated attempts suggests that each double mutant may be nearly 100% embryonic lethal. If either *ev574* or *e697* and *e2027* are null alleles, then the increase in embryonic lethality (table 4.4) is indicative that *vab-6* and *vab-1* function in pathways that are independent of *mab-20*. (*1) and (*2) indicate that each heterozygote was constructed and scored on two independent occasions.

Finally, a genetic screen was done to find other genes within the *mab-20* pathway. If worms that contain an already weakened *mab-20* pathway are mutagenized, then genes that can further weaken the *mab-20* pathway may be more easily revealed through mutation than if mutated in a wildtype background. EM 253 [*mab-20*(bx61); *him-5*(e1490)] parents, that are nearly indistinguishable from wildtype at the permissive temperature, were mutagenized with 0.5% to 1% EMS at day 0. Approximately 60 large 10 cm OP50-seeded plates were seeded with 50 mutagenized parents. The mutant F1 progeny were screened for putative dominant enhancers of the *mab-20* phenotype at days 3, 4, 5 and 6. A total of 600 000 mutagenized genomes were visually screened for individuals that had severe morphological deformities similar to those caused by the *mab-20*(ev574) deletion. Table 4.10 summarizes the results of the screen for dominant enhancers of the *mab-20* morphological defects. The non-outcrossed putative enhancers fall into four phenotypic classes in the background of the *mab-20*(bx61) homozygous
mutation, including those that had a body shape like mab-20 mutants, those that had dumpy body shapes like dpy-5 mutants, those that had a more twisted body shape like vab-6 mutants, and those that had dumpy body shapes and abnormal male tails. A subset of these mutants are being characterized by other members of our laboratory.

Table 4.10 Candidate enhancers of the morphological defects of mab-20(bx61)

<table>
<thead>
<tr>
<th>mab-20(ex574)-like</th>
<th>dpy-5-like and Mab</th>
<th>vab-6-like</th>
<th>dpy-5-like</th>
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<td>6.7X5</td>
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<tr>
<td>20.5.21</td>
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</tr>
<tr>
<td>6.7.32</td>
<td></td>
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</tbody>
</table>

The body of the table gives the names of the mutants that fall into four phenotypic classes when observed as non-outcrossed doubles with mab-20(bx61).

4.3 Discussion

The development of the C. elegans embryo requires numerous cell-position rearrangements, and short and long range migrations. In doing so, cells must continually make and break contacts with surrounding cells. The results presented above suggests that Ce-SemaII either prevents ectopic contact formation or breaks transient contacts normally made between cells of the hypodermis. Several observations show that the putative ligand is used throughout the development of the worm to prevent or break contacts formed during migrations, cell intercalation, ventral enclosure, cell divisions, and contacts formed due to birth position. I propose a non-autonomous model for Ce-SemaII function whereby the specificity of destabilizing transient contacts is dependent on the activation of receptors on the surface of the cells capable of making contacts.
4.3.1 *mab-20* Ventral Enclosure and Morphological Defects may be Due to Ectopic Hypodermal Cell Contacts

The most detrimental phenotype of *mab-20* mutants are ventral enclosure defects that lead to embryonic lethality. Both SEM and MH27 antibody *in situ* analysis suggests that ventral hypodermal pocket cells, including the P neuroectoblasts, make ectopic contacts with one another, which probably results in the inability of the hypodermis to completely enclose the embryo at the ventral midline in *mab-20(ev574)* mutants. The incompletely sealed hypodermis most probably leads to the lethal ejection of internal contents from the ventral midline. In embryos that escape this incompletely penetrant defect, the ectopic contacts between the ventral hypodermal cells probably results in the observed starburst pattern of P cells in hatchlings.

The ectopic pocket cell contacts could be the result of three or more processes going wrong: First, at about 250 minutes after the first cleavage, the hypodermal progenitors are lined up in six longitudinal rows. At this time, each of the 6 ventral P cells on either side of the embryo contact two lateral V seam cells, except for P11/P12, which only contacts V6 (Podbilewicz and White, 1994). Since *mab-20(ev574)* mutants have a disrupted seam cells arrangement, the arrangement of P cells, might also be disrupted. P cells might then be misaligned with their respective contralateral partners at the midline near the completion of ventral enclosure and be forced to make ectopic contacts. This possibility is unlikely however, since the arrangement of the vast majority of the individual V seam cells are normal in the hatchlings that have the starburst arrangement of P cells.

A second explanation for the ectopic contacts between the ventral hypodermal cells involves the short-range migrations of the P cell-progenitors that results in the linear arrangement of P cells (figure 4.32). For example, the progenitor of the P9/P10 and P11/P12 cells must migrate past the progenitor of P3/P4, P5/P6, and P7/P8. P9/P10 must then intercalate between P7/P8 and P11/P12, which are in direct contact (P. Roy, observations from the
Figure 4.32 Numerous short range migrations set up the linear arrangement of P cells. Panels (A) through (J) chronicle the development of the left ventral neuroectodermal P cells from 123 minutes after first cleavage (afc) to 350 minutes of development. A. 123 minutes afc. ( ), ABplapaa; ( ), ABplappa; ( ), ABplapap. B. 143 minutes afc. ( ), ABplapaap; ( ), ABplappaa; ( ), ABplapap; ( ), ABplapapa; ( ), ABplapapp. D. 185 minutes afc. E. 200 minutes afc. ( ), P7/P8, ABplappapp; ( ), P9/P10, ABplapappap; ( ), P11/P12, ABplappappa. F. By 215 minutes afc, all of the P cells are born. ( ), P1/P2, ABplapaapp; ( ), P3/P4, ABplappaa; ( ), P5/P6, ABplappaap. G. 245 minutes afc. H. 275 minutes afc. I. 320 minutes afc. J. By 350 minutes of development, the P cells are linearly arranged parallel to the longitudinal axis of the embryo. Anterior is to the left and dorsal is up. Each cell center was traced from a demonstration version of SIMI Bio Cell CD ROM™ (Hutter and Schnabel, 1997).
demonstration version of SIMI Bio Cell CD ROM\textsuperscript{TM} (Schnabel et al., 1997)). If the P7/P8 cell contact to P11/P12 cannot be broken, and/or if the contacts made by the P9/P10-P11/P12 progenitor to neighboring cells during migration are not broken, then a disruption of the linear pattern of the 6 P cells on either side of the embryo may occur. Like the first possibility, the disrupted linear pattern of P cells could result in ectopic P cell contact formation at the ventral midline and is equally unlikely for the aforementioned reasons.

Finally, it is plausible that Ce-SemaII repels or inhibits the extension of processes from the passively trailing pocket cells during ventral enclosure (figure 4.33). SEM has demonstrated that without \textit{mab-20}, the pocket cells may extend ectopic processes like those of the anterior leader cells. These findings are sufficient to explain both a disarrayed ventral pocket and the numerous ectopic P cell contacts observed in \textit{mab-20} mutant hatchlings. Detailed observations by Raich and Hardin (1998 and personal communication) suggests that the pocket cells do indeed extend filopodia. These extensions, however, are shorter and more transient than their leader cell counterparts. It is tempting to speculate that the cue that limits the extension of filopodia from the pocket cells, but not the leader cells is Ce-SemaII.

Regardless of exactly how the ectopic contacts form between the pocket cells, the observation that embryos burst at the ventral midline during enclosure is sufficient to explain the high frequency of embryonic lethality in \textit{mab-20(ev574)} mutants. It is therefore likely that the ventral pocket does not seal properly, in a manner that is superficially similar to \textit{vab-1} mutants (George et al., 1998). Although staining mutants with MH27 revealed numerous erroneous contacts between ipsilateral and contralateral P cells in \textit{mab-20(ev574)} hatchlings, only one of one hundred \textit{vab-1(e2027)} hatchlings had an ectopic P cell contact. This result suggests that although the \textit{mab-20(ev574)} and \textit{vab-1(e2027)} mutants similarly burst at the midline during elongation, they do so for different reasons. George \textit{et al.} (1998) suggest that \textit{vab-1} functions
Figure 4.33 A model of the function of Ce-Semall during ventral enclosure. A. In contrast to wildtype ventral enclosure (figure 4.10), ubiquitously expressed Ce-Semall may be required to prevent the extension of ectopic processes from the pocket cells that trail behind the anterior ventral hypodermal leader cells. Without mab-20, ectopic contacts are made by the pocket cells, in turn leading to the formation of an irregular ventral pocket (B). The microfilament based purse-string mechanism (B, black line that surrounds the hole) cannot close the irregular ventral pocket, leading to the expulsion of internal cells during elongation (C). Green cells, seam cells; yellow cells, ventral hypodermal cells; red cells, miscellaneous blast cells. Anterior is to the left, right is up.
to regulate the closure of the ventral pocket. Four dimensional analysis of ventral enclosure of *vab-1* mutants revealed that in some embryos the leading cells fail to migrate or migrate slowly towards the ventral midline. In other *vab-1* embryos the pocket does not close, and in others still, the pocket closes but then later breaks open. In contrast, I hypothesize that *mab-20* does not function to regulate the closure of the pocket, but instead regulates the contacts that form between the cells of the ventral pocket. The terminal phenotypes of *mab-20* and *vab-1* mutant embryos may nevertheless be the same; the pressure of elongation forces internal contents through the open or disarrayed ventral pocket, in turn killing the embryos.

The ectopic P cell contacts of *mab-20(ev574)* probably cannot account for the severe morphological defects seen in embryos that have survived elongation. As mentioned above, the force that powers elongation derives from contraction of parallel circumferentially oriented microfilaments within the hypodermis (Priess and Hirsh, 1986). It is plausible that the starburst organization of *mab-20* mutant P cells could constrain the conformation of the microfilaments within the P cells. This abnormal arrangement of microfilaments could then lead to an uneven distribution of constriction force that in turn leads to the observed morphological defective and shortened bodies. Indeed, if the circumferential force becomes unevenly distributed by either the disruption of the microtubule network or the embryonic sheath, the embryos are shorter and have a variety of shape abnormalities, including constrictions and bulges (Priess and Hirsh, 1986). However, several lines of evidence suggest that the aberrant P cell conformation is not likely to be the primary cause of the morphological deformities. First, mutant animals are observed to have severe bulges without disarrayed microfilaments within the P cells. Second, *vab-6(e697)*, and to a lesser extent *mab-26(bx80)*, have *mab-20(ev574)*-like body morphologies without ectopic P cell contacts. Finally, disarrayed microfilaments within clustered seam cells are precisely coincident with the morphological defects of *mab-20(ev574)* and *vab-6(e697)* and are sufficient to explain the abnormal body morphologies. There is no discounting that the aberrant
P cell conformation may be a contributing factor to the morphological defects of mab-20(ev574), but is not by itself causal.

The most obvious larval phenotype of the mab-20(ev574) mutant is the severe morphological defects in body shape. As mentioned above, the circumferential microfilaments within clustered seam cells are skewed relative to the left-right circumferential axis during elongation. In addition, the clustered seam cells are precisely coincident with the locale of the morphological deformity or bump. Investigating hypodermal ontogeny with MH27 revealed that the seam cells are clustered along the length of the embryo in a manner that is reminiscent of earlier stages of seam cell ontogeny. The positions of the seam precursors leading up to the genesis of the 10 seam cells were determined using the demonstration version of the SIMI BioCell CD ROM™ freely available from R. Schnabel (Hutter and Schnabel, 1997) (figure 4.34). By 275 minutes of development, the two lateral rows of hypodermal seam cells have been generated by a rearrangement of the relative positions of numerous cells, including a few short-range migrations. For example, on the left side of the embryo, the lateral hypodermal precursor of V5 (ABplapapaa) migrates posteriorly, past the precursors of V1 and V2 (ABarppapa), V3 (ABplappap), and V4(ABarppappa) to the target destination between V4 and V6 (ABarppappp). The V5 precursor must then intercalate between V4 and V6, which are in direct contact with each other, to form a contiguous lateral line called the seam. In contrast to the dynamics required to order the body seam cells such as V4, V5, and V6, the head seam cells, H0, H1, and H2 maintain their initial contacts. The H0/H1 precursor (ABplaaapp) directly contacts the H2 precursor (ABarppaaa), and divides along the anterior/posterior axis, resulting in H0 and H1, the latter of which maintains the original contact to the H2 precursor. The end result of cell division and rearrangement is that each lateral hypodermal cell contacts only two other neighboring seam cells. These events generate a lateral line of 10 hypodermal cells on both sides of the embryo; H0, H1, H2, V1 through V6, followed by the T seam cell.
Figure 4.34 The generation of a linear arrangement of seam cells. The figure chronicles the generation of the right ventral hypodermal seam from a lateral view (A-F) and a dorsal view (G-L). A & G. 160 minutes after first cleavage (afc). (O,H0/H1), ABarpapp; (●, V5/QR), ABpapp; (●, H2), ABarpapp; (●, V1/V2), ABarpapp; (●, V3), ABpapp; (●, V4/V6), ABarpapp; (●, T), ABpapp. B & H. 185 minutes afc. C & I. 200 minutes afc. (O, H0), ABarpapp; (●, H1), ABarpapp. D & J. 215 minutes afc. (●, V1), ABarpapp; (●, V2), ABarpapp; (●, V4), ABarpapp; (●, V6), ABarpapp. E & K. 245 minutes afc. F & L. 275 minutes afc. Anterior is to the left. Each cell center was traced from a demonstration version of SIMI Bio Cell CD ROM™ (Hutter and Schnabel, 1997).
In short, many of the conformations of the mab-20(ev574) seam clusters look as if the V5 seam cell migration was arrested early, or that it failed to intercalate between V4 and V6. It is possible that cell contacts between V5 and the cells that it normally migrates past do not break, and/or the V4-V6 contact can not be broken. A similar argument can be made for clusters involving other seam cells, including H0, H1 and H2: At about 185 minutes after first cleavage, the progenitor of H0/H1 directly contacts the progenitor of H2 and two neighboring cells (ABplapaa and ABarapaapa) which also contact the progenitor of H2. If the transient contacts between these four cells were made stable, the positions of the daughters of the H1/H2 or H2 progenitors may be constrained along a dorsal-ventral axis,instead of the normal anterior-posterior axis, resulting in the H0, H1, and H2 clustered pattern that is often observed. I postulate that Ce-SemaII is required to prevent ectopic contacts between the seam cell progenitors during their ontogeny and surrounding cells.

A favored model to explain why hypodermal microfilaments are skewed in mab-20 mutants is that microfilament polarity is determined by seam cell polarity. I hypothesize that the clustering of seam cells and/or the formation of ectopic contacts during their ontogeny in a mab-20 mutant background results in skewed seam cell polarity. Hence, those skewed seam cells contain skewed microfilaments during elongation. An alternative model is that microfilament polarity is dependent upon extracellular cues that are independent of seam cell polarity. By this model, microfilament polarity could be abnormal irrespective of the seam cell axes.

The first model that explains skewed microfilaments in mab-20 mutants is favored since skewed microfilaments are only observed in seam cells that are clustered and often shaped as if their axes are turned by 90 degrees. However, these results merely indicate a coincidental relationship between microfilament polarity and seam polarity. Until markers for the polarity of seam cells become available, a causal relationship cannot be addressed.
Independent of how microfilaments become skewed, I hypothesize that during the elongation of \(mab-20\) mutants the contraction of the misaligned microfilaments, relative to microfilaments that are aligned with the L/R circumferential axis, results in an uneven distribution of force along the surface of the embryo (figure 4.35). This uneven distribution of force in turn results in the bulging of hypodermis surrounding the clustered seam cells. Consistent with this model is the finding that the hypodermal nuclei within the lateral hypodermal ridges of \(mab-20\) mutant hatchlings are absent from constricted body regions and are restricted to non-constricted regions.

### 4.3.2 Contacts between the Ray Sensory Precursors Persist in \(mab-20(ev574)\) Male Tails

During the development of the male tail sensory rays, ray precursor clusters 1, 2, 3, 4, 6, and 7, and 9 are born very close to neighboring clusters. The nine clusters on each side of the tail then become distinct. In \(mab-20\) mutants, the aforementioned clusters fail to separate. The phenotypes of \(mab-20(bx24)\) and \(mab-20(bx61)\) were first characterized by Baird et al. (1991), and later by Chow and Emmons (1994). These reports interpret the posterior-most ray fusion in \(mab-20\) and \(mab-26\) mutant males as containing rays 8 and 9. My results suggest that the posterior ray fusion in these mutants are between rays 7 and 9. Three lines of evidence support this conclusion: First, DIC microscopy reveals that of the three posterior rays, the non-fused ray has a ventral sensory opening, like that of ray 8. Second, MH27 in situ of mid-L4 males suggests that the clusters of ray progenitors 7 and 9 fuse. Third, the \(unc-129\) neural specific reporter results in high expression of GFP in the neurons of rays 1, 5, and 7. The posterior fusion of \(mab-20\) male sensory rays show high GFP expression within the anterior half of the fusion, suggesting that ray 7 has fused to another ray.

A model for why sensory rays fuse in some \(mab\) mutants is that the ray identities equivocate (Baird et al., 1991; Chow and Emmons, 1994). While changing a cell's expression
Figure 4.35
pattern might arguably be called an identity change, the fact that the \textit{unc-129} reporter retains high expression in only one of the anterior-most neurons within fusions involving ray 1 and those involving ray 7, would suggest that ray identity has not changed in \textit{mab-20(ev574)} mutants. I propose that the response of the ray precursors to the absence of Ce-SemaII is the same as that of the seam cells and the P neuroectoblasts. That is, Ce-SemaII expression in the vicinity of ray precursors repels contacts between neighboring ray precursor clusters. In the absence of Ce-SemaII, the clusters establish and maintain strong contacts. I also propose that contacts between the longitudinally oriented 9 Rn.p cells are refractile to infiltration of the exploratory processes of the clusters, thereby preventing the fusion of 5 and 8 to other clusters (compare figures 4.17 with 4.19, 4.20 and 4.21).

A phenotypic analysis of \textit{vab-1(e2027), lig-1(ev658), mab-21(bx41) and unc-130} mutant male tail development suggests that there may be several genetic programs controlling different aspects of sensory ray development. For example, \textit{unc-130} and \textit{mab-21} may control the fate of the sister of Rn.a that undergoes programmed cell death, and the fate of the R6.p cell which normally fuses to hyp-7, and perhaps the surface properties of ray precursor clusters 3 through 9. \textit{vab-1} and \textit{lig-1} may control the fate and/or the surface properties of ray clusters 8 and 9. Finally, \textit{mab-20}, \textit{mab-26}, and to a lesser extent, \textit{vab-6}, probably control the surface properties of all ray precursor clusters, determining whether or not to extend exploratory processes at any one particular time.

4.3.3 A Neomorphic Phenotype may Reveal an Underlying \textit{semaphorin II} Pathway in the Commissural Axons

The errors in cell migration, nerve cord fasciculation and circumferential pioneer axon guidance in \textit{mab-20} could be secondary defects due to the errant positioning of cues from a disorganized hypodermis. Alternatively, these cells may require Ce-SemaII as a redundant ligand for
fasciculation and circumferential guidance. In support of the later supposition, ectopic expression of Ce-SemaII from the DAs and DBs during their migration results in a significant increase in the percentage of errors in guidance and fasciculation in a \textit{mab-20(ev574)} mutant background. Since over expression of Ce-SemaII induces morphological and male tail phenotypes that are identical to \textit{mab-20(ev574)}, it is possible that over expression of Ce-SemaII in the DAs and DBs from transgenic \textit{mab-20(+) arrays} similarly disrupts the \textit{semaphorin II} pathway with respect to the migrating neurons. By ectopically expressing Ce-SemaII from and therefore in the vicinity of the migrating axons in a \textit{mab-20} mutant background, two effects are hypothesized: First, the \textit{semaphorin II} pathway in the DAs and DBs is disrupted. Second, the hypodermal source of the primary cues required for circumferential guidance and fasciculation (eg, Wadsworth et al., 1996) is disorganized by the \textit{mab-20} mutation. The resulting synthetic phenotype may thereby reveal a Semaphorin II-sensitive pathway within the migrating axons. Amazingly, the frequency of synthetic defects in the DAs and DBs is nearly equivalent to those found in \textit{mab-26(bx80)}, a mutant that exhibits most of the mutant phenotypes of \textit{mab-20}. In support of the activity of Ce-SemaII on migrating axons, the repulsive migratory response of \textit{Drosophila} motor axons to D-SemaII is dependent on the relative levels of D-SemaII that surround the axons (see short-range repulsion in chapter 1; Winberg et al., 1998).

4.3.4 A Model for Ce-SemaII Activity

Many cell rearrangements and cell and axon migrations require contacts to be actively made and broken with neighboring cells. Given a global distribution of a repulsive ligand, the autonomous regulation of a cell-surface receptor could enable temporal and spatial regulation of cell-cell contacts. Together with cues that promote lamellapodia and filopodia extension, tight regulation of the adhesion to neighboring cells may be achieved during migrations and cell rearrangements. In the case of cell rearrangements, a loss of the function of the repulsive molecule would result in
ectopic contacts and perhaps a permanent ectopic adhesion to neighboring cells. Long range cell and axon migrations may similarly be affected if the specification of the migratory path heavily depends on inhibitory forces that restrain migratory "wandering".

One model to explain the lethal and morphological phenotypes observed in \textit{mab-20} mutants is that Ce-SemaII is required to prevent or repel the extension of cellular exploratory processes such as lamellapodia and filopodia or is required to destabilize inappropriate cell-cell contacts during hypodermal morphogenesis. Since \textit{mab-20} is nearly ubiquitously expressed during seam ontogeny, ventral enclosure, tail whip formation, and the genesis of the male tail sensory rays, it is unlikely that the regulation of Ce-SemaII activity is dependent on the spatial and temporal regulation of Ce-SemaII expression. Instead, the model predicts that the temporal and spatial regulation of a co-factor, activator, receptor or any downstream signaling mechanism is required for the regulation of the activity of the \textit{semaphorin II} pathway. \textit{mab-26} is an excellent candidate for a highly regulated upstream or downstream element of the \textit{mab-20} pathway. Since the phenotypes of \textit{mab-26} mutants are so similar to \textit{mab-20} mutants, \textit{mab-26} may act very close to \textit{mab-20}, perhaps as a co-factor or a receptor of Ce-SemaII, akin to the relationship between UNC-40 and UNC-6 (Hedgecock \textit{et al.}, 1990; Ishii \textit{et al.}, 1992; Chan \textit{et al.}, 1996). For example, it is plausible that the expression of \textit{mab-26} during male tail development would be limited to the ray precursor cells during their ontogeny. Likewise, since \textit{vab-6} is required more in embryonic hypodermal morphogenesis than that of the male tail, VAB-6 expression may be tightly may be limited to the seam cells during their ontogeny.

\textbf{4.3.5 Concluding Remarks}

As previously mentioned, the Semaphorins have been repeatedly shown to repel migrating axons, but have not been implicated in the guidance of cell migrations to date. The results presented here suggest that not only may Ce-SemaII be involved in axon guidance in the
nematode, *C. elegans*, but it may also be a global cue that regulates short range migrations and rearrangements of hypodermal cells. Like the Semaphorins that have been characterized in other organisms such as Drosophila and Mouse, Ce-SemaII may also function to prevent or repel further extension of cell processes, perhaps by inducing localized filopodial and lamellapodial collapse. Not only have these investigations of *mab-20* revealed a novel function for the Semaphorin Superfamily, but they have also led to unique insights into the development of *C. elegans*. For example, it was previously unknown that the embryonic seam cells and P neuroectoblast cells could establish ectopic contacts with one another. In addition, the studies of *mab-20(+)*) presented here have led to the suggestion that body wall muscles may be restricted from wandering in a lateral orientation by the seam cells. Finally, it is interesting to note that over-expression of a protein product (Ce-SemaII) may have the same dire consequences as the loss-of-function of the respective gene. How this happens is unknown. The questions raised by these and other results presented here may help us to better understand metazoan development.

4.4 Materials and Methods

Standard molecular biology methods were used (Sambrook *et al.*, 1989) unless otherwise noted. General procedures used for the culture, maintenance, and storage of the nematodes are compiled by Wood (1988). All strains not isolated in our lab were obtained from the *C. elegans* Genetic Center, care of T. Stiernagle (The University of Minnesota).

4.4.1 Molecular Biology

To increase the efficiency of cloning PCR products, the pBluescript KS (+) vector (Stratagene) was digested with Eco RV, and then incubated at 72°C for 2 hours with 10 mM dTTP and 1 unit of Taq polymerase (Bohringer). All DNA fragments used to probe blots were radiolabelled with P\(^{32}\) using the random priming method of Feinberg & Vogelstein (1983). DNA sequence was
analyzed using the programs of Staden (1987).

4.4.2 cDNA Construction

The lambda ZAPII (Stategene) cDNA clones yk80 and yk100 were provided by Y. Kohara and excised in vivo. To obtain the 5' end of the cDNA, the RACE procedure was used (Frohman et al., 1988) on total RNA from a mixed stage population of wildtype nematodes (N2). First strand cDNA was synthesized using the mab-20 specific primer II.RO (5'-GGG ATC AAG GGA GCA GAG AG-3'), complementary to bases +1452 to +1473 with respect to the cDNA. A polymerase chain reaction using an oligo d(T) primer and the gene specific primer II.RI (5'-GAA TTC GGC AGG CAA AAG TGC-3'), complementary to bases +1361 to +1382, generated a 1.25 kb product from a first-strand cDNA template tailed with cATP, and was then cloned into pBluescript KS (+). A polymerase chain reaction using the splice leader sequence I (SL1) 5' primer (Krause and Hirsh, 1987) and the mab-20 specific primer II.RI generated a 1.55 kb product which was cloned into pBluescript KS (+). The full length cDNA was spliced together using the SL1 PCR product's 5' end to Bam HI, the 5' RACE product's Bam HI to Sma I fragment, and yk80 3' from the Sma I restriction site. The cDNA was sequenced (Sanger et al., 1977) in its entirety, and compared to other Semaphorin family members using the Maligned program (Clark, 1992).

4.4.3 RNA Blots

The entire semaphorin II cDNA was released from its vector using polylinker sites Xba I and Xho I, and used to probe a Northern blot of 5 μg of total RNA from a mixed-stage population of wildtype nematodes (N2), prepared as described in Spence et al. (1990).
4.4.4 Genomic Clones

1.3 x 10^6 plaques from an EMBL3 genomic phage library were screened using a 1.25 kb semaphorin II cDNA fragment released from its vector using the Eco RI cDNA restriction sites. Only 1 out of 10 positive isolates contained the predicted mab-20 gene in its entirety. evpZH13 was demonstrated by restriction analysis, subclone sequencing, and transgenic rescue of the mutant, to contain an entire functional mab-20 gene. To obtain recombination of separately injected DNAs to form transgenic arrays the DNAs must have sequence in common (Mello et al., 1991). To this end, all but 800 bp of the 5' end of pZH13 was subcloned into pBluescript, resulting in pPRII.2. The transcriptional fusion reporters pPRII.67 and pPRII.75 were constructed by inserting 2.5 kb of DNA 5' to the predicted initiator methionine of mab-20 inserted into the GFP vectors pPD95.67 and pPD95.75 (a gift from A. Fire), respectively.

4.4.5 Tc1 Library

The strategy for the construction of our Tc1 library is an extension and modification of previous work (Zwaal et al., 1993; Y. Andachi and Y. Kohara, personal communication) and is described in detail in chapter 3. Five young MT 3126 (Collins et al., 1989) adults were placed onto more than 500 OP50 seeded 6 cm NGM plates, each of which represents one sample. The siblings of the library’s founding generation were assayed for an active Tc1 mutagen by placing the worms in 1% nicotine and looking for a high frequency of “twitching” mutants (a phenotype of unc-22 loss-of-function) compared to N2 (Moerman et al., 1989). The plates were then stored for 10 to 12 days at 20°C, after which they were examined for clearing of the bacterial lawn and presence of many starved L1s. Using siliconized plasticware, the worms were collected, and each sample was divided into four aliquots of 250 µl each. To three of the four conical tubes, 250 µl of worm freezing solution was added before freezing at -70°C. Each aliquot was stored in a separate
The worms from the fourth tube were concentrated, 15 µl of ProK lysis solution (appendix A; Plasterk, 1995) was added, and the tube was placed at 60°C for 20 to 60 minutes. Fifty µl of TE (pH 8.0) was mixed in, and 7.5 µl of the worm lysate was dispensed to two tubes containing 500 µl of TE, creating the “Sample DNA” which is stored -70°C. “Alpha-pools” were created by pooling the remaining lysate from 15 samples, which were then cleaned by organic extraction and precipitated with ethanol and .3M NaAcetate, pH 5.2. The alpha-pool DNA was resuspend in 1200 µl of TE at 55°C over night, split into two aliquots, and stored in separate -70°C freezers.

4.4.6 Screening for Tc1 Alleles and Deletion Derivatives

Alpha-pool DNA was screened for an insertion allele in mab-20 using nested PCR with gene specific primers II.A (5’-TAC AAT TAC GAT GTC TTC TCC G-3’) at position -264 to -242 relative to the initiator codon of the gene, and II.B (5’-TAG GAC TAA AGA GGC ACA CAG C-3’) at position -229 to -209, and Tc1 specific primers R1 and R2 (Plasterk, 1995). AmpliTaq Gold (Perkin-Elmer) was used in all PCR reactions. The PCR screening conditions were 9 min at 95°C, 32 cycles of 30 sec at 95°C, 30 sec at 57°C, 2 min at 72°C, then 10 min at 72°C. The samples of candidate alpha-pool 90 were then screened for the corresponding amplicon. Eight of 25 isolates from sample 72 contained the desired allele, mab-20(ev573::Tc1), as assayed by single worm PCR (Plasterk, 1995). The transposon of mab-20(ev573::Tc1) was inserted between positions +103 and +104.

To obtain a deletion allele of mab-20, a sub-library was constructed and screened in the same fashion as the original, with the exception of using NW 1034 [mab-20(ev573::Tc1); mut-2(r459); dpy-19(n1347)] as the founding strain. To screen the alpha-pools of the sub-library,
PCR was carried out using \textit{mab-20}-specific primers \texttt{II.Y} (5'-ATG GAA CAT TGG GAC GCA
GCT CGG-3') at position-2.7 kb, \texttt{II.Z} (5'-CTA GTA TAG CTC AAG ATT CGG-3') at position
-2.6 kb , \texttt{II.9} (3'-GTC ATT AGA AGT TTC CGC GTG-5') at position +834 to +855 and \texttt{II.10}
(3'-CGT GAC GCC TAG ACC CTA AGT CCA-5') at position +991 to +1015. Sample 853 contained four out of 109 isolates that carried \textit{mab-20(ev574)}, a 1.3 kb deletion whose 3'
breakpoint is at position +835. \textit{mab-20(ev574)} was outcrossed with N2 more than 11 times for
phenotypic and genetic analysis.

\textbf{4.4.7 Mapping}

From \textit{mab-20(ev574)/+; dpy-5(e61)/+} heterozygotes, 115 animals that had no morphological
defects like that of \textit{mab-20(ev574)} homozygotes but were dumpy like that of \textit{dpy-5(e61)}

dehomozygotes were cloned. Based on Mendelian ratios, only 23 of the expected 76 clones threw
\textit{mab-20(ev574)} homozygous progeny, showing \textit{mab-20} linkage to chromosome I.

By inference, \textit{mab-20(bx24)} and \textit{mab-20(bx61)} were suspected not to complement \textit{mab-20(ev574)}.
\textit{mab-20(bx61ts); him-5(e1490)} males raised at the permissive temperature (16°C)
were crossed to \textit{mab-20(ev574)} hermaphrodites. Male cross progeny raised at 25°C were scored
for ray fusions. Since neither \textit{mab-20(bx24)} nor \textit{mab-20(ev574)} male homozygotes can

successfully mate, the complementation test between these two alleles was done by crossing
\textit{mab-20(bx24)/+; him-5(e1490)} males to \textit{mab-20(ev574); evfls73[mab-20:GFP (evpPRII.75);
rol-6(su1006)]} hermaphrodites. All male cross progeny were scored for ray fusions. Although
50% of the cross-progeny males would normally be expected to have sensory ray fusions based
on Mendelian ratios, the high frequency of lethality during the embryonic development of \textit{mab-20(ev574)}
homozygous mutants embryos (49% n=452) suggests that only approximately 33% of
the cross progeny males will have fusions if the two alleles are not complementary. As
predicted, 28% (n=203) of cross progeny males exhibited male tail fusions of an expected
expressivity that is between that of the ev574 and bx24 homozygotes.

4.4.8 Transgenics

Extra-chromosomal arrays of mab-20(+)(evPRII.2); rol-6(su1006) were generated by co-microinjection of the DNAs into N2 oogonia (Mello and Fire, 1995) at a concentration of 27.5 ng/μl and 100 ng/μl, respectively. F1 and F2 transformants were selected based on the dominant rolling phenotype induced by rol-6(su1006). The extra-chromosomal arrays were integrated into random double stranded breaks in the chromosomes generated by 3000 rads from a cesium source, resulting in chromosomally inserted arrays evls74a, evls74b, evls74c, and evls74d.

When constructing doubles with mab-20(ev574) and evls75, evls76, or evls77, or triples with him-5(e1490), the marker used to identify mab-20(ev574) was the 1.6 kb amplicon generated in single worm PCRs with mab-20 specific primers II.2 and II.9 (see above). If 16/16 siblings from a single clone had the mab-20(ev574) deletion amplicon, the allele was considered homozygous.

Before it was known that mab-20(ev574) homozygotes had sensory ray fusions, the ability of the evls74 transgenes to rescue mab-20(ev574) was assayed through subjectively scoring the morphological abnormalities conferred by the mab-20(ev574) homozygous mutation in different genetic backgrounds in a double blind experiment. Ten L4-staged larvae of the following genotypes were seeded onto OP50-seeded NGM plates (see appendix A) which were then numbered by a second person (Dr. J Pierce) at day 0: mab-20(ev574), the negative control evls73[mab-20:GFP (evPRII.75); rol-6(su1006)], evls74b+ (a transgene which confers an obviously high penetrance of mab-20(ev574) phenocopy), evls74d (a transgene which confers an obviously low penetrance of mab-20(ev574) phenocopy), and 20 L4s of mab-20(ev574); evls74b, and mab-20(ev574); evls74d. All progeny of the 10 parents were scored either (+) or (-) for mab-20(ev574)-like morphological defects on the first, third and sixth days of the
experiment. The number of progeny thrown by each parent was also noted, and later used as a rough measure of the fecundity of each strain counted. I looked at the crib sheet detailing the genotype of each numbered plate upon completion of the counts on day 6.

4.4.9 Light and Epifluorescence Microscopy and Immunostaining
Male tail ray fusions were scored by mounting 1mM levamisole treated animals on 2% agarose pads for observation by DIC optics at 1000X using a Leica microscope (Leitz DMR).

For immunostaining, animals were permeabilised as outlined in Finney and Ruvkun (1990), and incubated overnight at room temperature with a 1:1000 dilution of MH27 (Francis and Waterston, 1991), or a 1:1000 dilution of anti-LIN-26 (Labouesse et al., 1996), or a 1:1000 dilution of anti-HLH-1 (Chen et al., 1994). The next day, after several washes, the animals are incubated in a 1:128 dilution of a fluorescein or rhodamine-conjugated goat anti-rabbit secondary antibody (Sigma Immuno Chemicals).

Sarcomeric microfilaments were observed by fixation in 1% paraformaldehyde followed by an overnight incubation with rhodamine-conjugated phalloidin (Molecular probes Inc.). Circumferential microfilaments were observed by fixation in 4% paraformaldehyde and 0.5% gluteraldehyde, followed by a brief 5 minute exposure to rhodamine-conjugated phalloidin as outlined in Priess and Hirsh (1986). Although this procedure is intended for viewing the circumferential microfilaments during elongation, the sarcomeric microfilaments were sometimes observed, and cells outlines were always visible.

4.4.10 Scanning Electron Microscopy
Embryos were harvested from nearly confluent plates and isolated in 0.71 M NaOH in 29% Javex Bleach for 10 minutes, followed by at least 3 rinses in egg salts (see appendix A) or M9 Buffer (see appendix A). Embryos were then treated for about 8 minutes in a chitinase-
chymotrypsin solution to remove the egg shell (see appendix A; Edgar, 1995). The enzymatically-treated embryos were then passed numerous times through a drawn-out pasture pipette to mechanically remove the embryonic sheath (Priess and Hirsh, 1986). The embryos were then pipetted onto coverslips submerged in M9 buffer and coated with either 2% silane (Sigma-A3648) in acetone or with 0.1% poly-L-lysine (Sigma-P8920). (The poly-L-lysine is a far better adhesive substrate than the silane). The M9 was then removed and 2% formaldehyde in fixation buffer (0.15 M cacodylate buffer; 2 mM MgSO₄) was added to the embryos attached to the coverslips. Samples were then washed in fixation buffer, dehydrated through an acetone series and critical-point dried (Ris, 1985). Specimens were coated with 1-2 nm of gold and viewed with a JOEL 820 scanning electron microscope.
Chapter Five

A General Discussion and Future Directions
5.1 Introductory Remarks

The purpose of this chapter is to briefly review the results described in each of the three data chapters and discuss them with respect to each other and some recent findings. In addition, I outline experiments that could be done in the future to further our understanding of the subject matter and answer any outstanding questions.

5.2 The Molecular and Genetic Characterization of three semaphorin Genes in C. elegans

I set out to clone C. elegans homologues of the semaphorin family upon realizing that they are ancient secreted and transmembrane molecules present in grasshoppers, flies and humans (Kolodkin et al., 1993) that might be involved in pioneer axon guidance (Kolodkin et al., 1992). At the time, the C. elegans sequencing consortium was in its infancy and GenBank database searches for previously sequenced C. elegans semaphorins were fruitless. As described in chapter 2, degenerate PCR led to the cloning of C. elegans semaphorin I (els-1). This gene is now retrospectively called C. elegans semaphorin Ia (els-1) since another class I molecule has since been discovered in C. elegans (see below). The cosmid that contains the els-1 gene, called F14B11, resides just to the left of the cosmid that contains the unc-54 gene. Based on the genetic position of unc-54, els-1 resides on the right arm of linkage group (LG) I at +27.21 map units relative to dpy-5 at 0.00 map units.

As detailed in chapter 2, loss-of-function alleles of els-1 have not yet been identified or isolated. However, two Tc1 alleles of els-1 called ev586::Tc1 and ev587::cis-Tc1 have been generated. Although transposable elements can be efficiently spliced from transcripts during RNA processing (Rushforth and Anderson, 1996), it is plausible that the Tc1s of ev586::Tc1 and ev587::cis-Tc1 that are positioned in intron 1 may have subtle effects on Ce-SemaIa function. To address this issue, ev586::Tc1 and/or ev587::cis-Tc1 could be placed in trans to deficiencies that uncover the els-1 locus. If ev586::Tc1 or ev587::cis-Tc1 have any effects on Ce-SemaIa
function, strains hemizygous for either allele may exhibit hypomorphic phenotypes. In any event, future experiments should work towards obtaining a deletion derivative of either Tc1 allele of *els-1*.

An EST of a second *C. elegans semaphorin* was identified by a cDNA-sequencing project initiated by Yuji Kohara (personal communication). This cDNA fragment was used to construct a full-length cDNA and obtain a genomic fragment that presumably contains all of the essential elements of the gene since it can completely rescue the loss-of-function phenotypes induced by mutation. Sequence analysis revealed that the second *semaphorin* encoded a product with a similar domain structure as *Drosophila* Semaphorin II (D-SemaII). The predicted product was therefore named *C. elegans* Semaphorin II (Ce-SemaII). The isolation of a Tc1-derived deletion allele of *C. elegans semaphorin II* (ev574) led to the finding that *mab-20*, a previously characterized mutant for which the gene had not been cloned (Baird *et al*., 1991; Chow and Emmons, 1994), encodes Ce-SemaII. *mab-20* genetically maps to the left arm of LG I at –10.41 map units relative to *dpy-5*.

PCR and Southern analysis reveals that worms homozygous for *mab-20* (ev574) lack *mab-20* sequence that includes DNA 5' to the predicted initiator methionine, the first exon, and part of first intron. Since there is no other methionine until halfway through the semaphorin domain, it is likely that *mab-20* (ev574) is a molecular null. However, this supposition cannot be tested genetically by asking if ev574 hemizygotest have a similar or worse phenotype than ev574 homozygotes, since no chromosomal deficiencies uncover the *mab-20* locus. Until such a deficiency becomes available, it cannot be said that ev574 is a genetic null of *mab-20*. If antibodies against Ce-SemaII were generated, as suggested below, one could easily test if *mab-20* (ev574) is a protein null.

Finally, Y. Kohara’s EST project also identified a third *semaphorin* homologue (Kohara, personal communication). Screens of *C. elegans* genomic databases revealed that the *C. elegans*
Sequencing Consortium Group recently sequenced the third *semaphorin* and can be obtained from “ftp://ftp.sanger.ac.uk/pub/databases/C.elegans_sequences/EMBL_FORMAT/I/D1037.embl.”. The predicted initiator methionine is positioned at base number 25251 relative to base zero of cosmid D1037. The gene contains 11 putative exons (figure 5.1). Multiple sequence alignments (Clark, 1992; figure 5.2) and a Kyte-Doolittle hydrophobicity plot (data not shown) of the virtual mRNA predicts a signal sequence, a semaphorin domain, a transmembrane domain and a cytoplasmic tail of unknown homology. I call this gene *C. elegans* *semaphorin* *Ib* (*els-3*) and the predicted protein product Ce-SemaIb in recognition of the class I domain arrangement and the previously described *semaphorins* in *C. elegans*. Sequence analysis of the semaphorin domains of Ce-SemaIa, Ib, and II reveal that Ce-SemaIb is the least identical to the other two homologues, while Ce-SemaIa and II share the greatest identity (Table 2.1; see chapter 2). The approximate genetic map position of *els-3* is at -3.5 map units relative to *dpy-5* on the left arm of LG I. Intriguingly, all three Semaphorin homologues of *C. elegans* reside on LG I, the significance of which is unknown.

To date, little functional data concerning *els-3* has been produced or reported. However, PCR primers have been designed and generated in anticipation of screening our Tc1 library for *els-3::Tc1* alleles (figure 5.1). With an *els-3::Tc1* allele in hand, it would be easier to generate an *els-3* loss-of-function allele. Not only might an *els-3* mutant enable the functional characterization of Ce-SemaIb through genetic and phenotypic analysis, but in combination with mutant alleles of the other *semaphorins*, it could reveal functional redundancies. That is, the *C. elegans* Semaphorin family could redundantly function in developmental processes that may not be revealed by single mutations alone. In addition to the pursuit of *els-3* genetic lesions, *els-3* PCR primers have also been designed to amplify exon fragments for the use in RNA interference experiments (see below and figure 5.1).
**A. els-3**

ATG

1 2 3 4 5 6 7 8 9 10 11

**B. els-3 Primers**

ATG

IB.3+IB.4

IB.1+IB.2

IB.RNA1F IB.RNA3F

IB.RNA1R IB.RNA4R

IB.5+IB.6

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**Figure 5.1 The els-3 gene and PCR primers.** A. The cosmid D1037 carries the els-3 gene which is predicted to encode at least 11 exons (numbered). B. Six nested PCR primers were designed for use in screening a Tc1 or direct-deletion library and are numbered IB.1 through IB.6. IB.1 begins at 22 bp after the predicted initiator methionine, 5'-AGA TTG TGG TGG TGG GAT GC-3'; IB.2 begins at 146 bp, 5'-AGA GAC ACC TGA TTA GTG GC-3'; IB.3 begins at 942 bp, 5'-CAG ATT ACT CCA TTT GAC ACA G-3'; IB.4 begins at 1078 bp, 5'-CTC GGC TAC CTA GAG CTG TG-3'; IB.5 begins at 3874 bp, 5'-TTG ATG ACT TCA GTG GC-3'; IB.6 begins at 3988 bp, 5'-GAA CAC CGA CAG ACA CC-3'. In addition, four PCR primers were designed for the purpose of constructing templates for use in the production of dsRNA. IB-RNA1F begins at base number 2289 bp relative to the predicted initiator methionine codon, 5'-GGT ACT CTT CTT CTT CTC CG-3'; IB-RNA2R begins at 2805 bp, 5'-ACA TAC TTT GCT TTA TCA GTG G-3'; IB-RNA3F begins at 3123 bp, 5'-TCA GAA ATC TCT GCC AAC CC-3'; IB-RNA4R begins at 3674 bp, 5'-CTA CTA TTT TTC AGG GCC GC-3'. ATG, the predicted initiator methionine; *, the predicted stop codon. The scale bar represents 1kb.

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### 5.3 Semaphorin Expression Patterns in *C. elegans* and *Drosophila*

As a first approach to investigate the expression patterns of the Semaphorins in *C. elegans*, monoclonal antibodies that were raised against G-Semal (Kolodkin et al., 1992) and D-SemaII (Winberg et al., 1998) were used to immunostain fixed worms. Unfortunately, the G-Semal mAb 6F8 antibody did not recognize any antigens of *C. elegans* and the D-SemaII antibodies...
Figure 5.2 A multiple sequence alignment that compares the predicted els-3 gene product Ce-Semalb to other Semaphorins. For Semaphorin acronym names and accession numbers, see figure 2.3. The predicted signal sequence of Ce-Semalb is underlined and the predicted transmembrane domain is boxed. Ce-Semal is otherwise known as Ce-Semala. The sequences were aligned by eye using the alignment in Kolodkin et al. (1993) as a guide. The sequences were arranged using the Maligned program (Clark, 1992).
19C2, 5B10 and 11H1 either recognized no C. elegans antigen or non-specifically hybridized to fixed animals.

Instead, els-1 and mab-20 gene activity was assayed by using 5' regulatory sequences to drive the expression of lac-Z and GFP reporter genes from transgenic arrays. els-1:Lac-Z reporters generated an interesting pattern of expression that was probably hypodermal in origin and increased in intensity from anterior to the posterior. The graded expression pattern, however, was not corroborated by els-1:GFP reporters possibly because of the molecular nature of the different reporter genes. In contrast to the activity of the 5' regulatory sequences of mab-20, which drives GFP reporter expression in a large number of neurons, the els-1:GFP transgene is expressed in only one neuron. Other differences in the spatial expression patterns generated by the two sets of C. elegans semaphorin reporters include enteric muscle and sperm expression from the els-1:GFP transgene, but not from mab-20:GFP transgenes. In addition, the onset of gene activity of els-1 may be slightly earlier than mab-20 and is certainly more restricted; els-1:GFP is expressed at about the 50-cell stage in the E cell lineage. In contrast, nearly all cells except those of the E and P cell lineage express the mab-20:GFP transgene during equivalent stages. Another major difference in the expression patterns is that els-1:GFP is expressed in the distal tip cells from L2 to L4, while GFP-tagged Ce-SemaII is expressed only late in L3 to mid L4. Finally, reporters of both semaphorins are expressed in body wall muscles.

A direct comparison of the semaphorins in C. elegans with those in Drosophila reveals some analogy. Like els-1 and mab-20, both Drosophila Semaphorins are expressed in the presumptive epidermis. In addition, the relative extent of epidermal expression of D-SemaII and GFP-tagged Ce-SemaII compared to that of D-SemaI and the els-1:GFP reporter is quite similar. The expression patterns of C. elegans and Drosophila Semaphorin homologues also differ in many analogous tissues. For example, SemaI, but not SemaII, is extensively expressed throughout the nervous system of Drosophila. In C. elegans, the converse may be true. Also,


els-1:GFP is expressed in sperm, while germ line expression of the Drosophila Semaphorins is limited to SemaII. Without knowing the localization of the endogenous Semaphorins in C. elegans, however, it is difficult to make concrete assessments of the functional significance of the differences in expression with their respective homologues in Drosophila. Another layer of complexity is added upon considering that C. elegans has a second SemaI, while Drosophila has another SemaII (M.Tessier-Lavigne and C.S. Goodman, personal communication), the expression patterns of which have yet to be characterized. It is plausible that future investigations may reveal that the sum of the Semaphorin expression patterns in the worm will be analogous to the sum of the expression patterns of the fly.

It should be noted that expression data obtained from transgenes containing either transcriptional or translational fusions to a reporter gene should be interpreted with caution. For example, it is unlikely that the transgene contains all of the elements used to regulate the expression of the endogenous gene, even in the case of reporter-tagged proteins that completely rescue mutant phenotypes. By removing a DNA fragment from its native environment, cis and trans regulatory elements may not only be taken out of context, but some are probably missing, in turn leading to an expression pattern that is spatially and temporally inaccurate. Second, such expression patterns often do not take into account sub-cellular localization, nor can they usually report extracellular expression patterns. Third, the extra-chromosomal or integrated arrays that carry the transgenes contain copy numbers many times that of the endogenous gene. Not only is it impossible to compare absolute or relative levels of expression, but multiple copies of the transgene could have unexpected autoregulatory consequences on the expression pattern. Finally, given that transgenic reporters of the transcriptional fusion-type encode no gene product of interest, and that those of the translational fusion-type result in the over-expression of the protein of interest, the endogenous post-translational regulatory mechanisms may or may not impinge upon the resultant expression pattern. On-the-other-hand, there is no substitute for the
data that results from the four-dimensional reconstruction of an *in vivo* expression pattern that can only be conveniently achieved with a GFP-tagged version of the gene of interest and a multiphoton laser scanning microscope (MPSLM, Mohler *et al.*, 1998). Considering all of the caveats of transgenic reporters however, the expression patterns driven by 5' regulatory sequences can be extremely convenient and informative. Together with functional data, expression data generated from transgenic reporters can help in model building. Addressing the proposed functional models of Semaphorin Ia and II in future investigations would obviously be facilitated by the production of antibodies that specifically recognize each of the three *C. elegans* Semaphorins.

5.4 Targeted Disruptions in *C. elegans* Semaphorins

While expression patterns may indicate when and where a gene may function, only genetic and epigenetic analysis can seriously address the purpose of gene function. One of the more challenging technical aspects of *C. elegans* molecular biology has been the genetic characterization of known genes through targeted mutagenesis, otherwise known as reverse genetics. While homologous recombination does occur in worm cells, current techniques that select for gene replacement events cannot efficiently exploit the low frequency at which recombination occurs. Targeted disruption of gene function has instead relied on two very different techniques. The first is RNA interference (RNAi). Although it is not understood how RNAi mediates gene disruption, it is clear that it is an epigenetic effect (as review by Tabara *et al.*, 1998). That is, by injecting, feeding or soaking worms in double stranded RNA of coding sequence of interest, the function of that product is disrupted without changing genomic sequence. It should be noted however, that even if an antibody suggests the absence of a protein product, it is difficult to be certain that the RNAi-induced phenotype is due to the disruption of the targeted product and not other homologues. To be certain of specificity, a genetic mutation is
at least required. Nevertheless, like transgenic reporter constructs, RNAi can be a convenient first step in the analysis of gene function.

Five constructs containing subclones of each of the three *C. elegans* semaphorin genes have been made for use as templates in double stranded (ds) RNA production (data not shown). *els-1* and *els-3* dsRNA were injected separately into worms expressing *mab-20*:GFP, which enables the immediate visualization of any defects in circumferential and/or longitudinal axon guidance amongst other phenotypes. Although preliminary results suggest that RNAi cocktails targeted against two regions of *els-3* may result in precocious axon outgrowth and migration from the HSNs, further characterization of this phenotype is required. Unfortunately, no phenotype was observed upon RNAi targeting of a single *els-1* region. Yet to be injected is a single *mab-20*-RNAi cocktail, a second *els-1*-RNAi cocktail, and combinations of all three *semaphorin* dsRNAs. Since RNAi often approximates the null phenotype (Fire *et al.*, 1998), injecting *mab-20*(ev574) and wildtype worms with *mab-20* dsRNA could address whether ev574 is a genetic null allele of *mab-20* or not. Obviously, these experiments need to be pursued further in the future.

Before the widespread use of RNAi, constructs containing putative dominant-negative and antisense coding regions of *els-1* were made and injected into worms as described in chapter 2. Surprisingly, significant proportions of transgenic animals were constipated. The investigation of the cellular basis of this defect was deferred until a genetic mutation of *els-1* could substantiate the phenotype. In addition to the pursuit of Tc1-derived *els-1* deletions, an examination of the *els-1::*Tc1 alleles in trans to a large chromosomal deficiency as suggested above may also demonstrate a genetic role of *els-1* in defecation. In retrospect however, it may be worthwhile to at least examine the cells that directly control defecation, namely the enteric muscles and the two motor neurons that regulate their contraction, namely AVL and DVB in the background of the constipation-inducing transgenes. This could easily be achieved by passing
the putative dominant-negative els-l transgenes into various GFP reporter lines, including els-
l:GFP which is expressed in the enteric muscles and possibly in the AVL.

The second technique commonly used to obtain targeted gene disruptions is to screen a library of mutant worms for genomic polymorphisms in the gene of interest. As described in chapter 3, the library can be made up of worms mutagenized with a chemical or with a transposon. With the former, isolated polymorphisms may directly lead to a loss-of-function, while the latter mutagen requires an extra step to obtain a transposon derived deletion. For historical reasons, I constructed a Tc1 library and not a direct-deletion library to target several genes such as els-l and mab-20 for mutation. My Tc1-library design and the pooling and screening innovations has enabled our lab to efficiently isolate over 30 Tc1-alleles for various laboratories. In addition, I have generated or have helped to generate numerous Tc1-derived deletions, including those for mab-20. Unfortunately, no Tc1-derived deletion has been obtained for els-l. However, in trying to obtain an els-l::Tc1 allele, a cis-Tc1 allele (ev587) was isolated. The value of this allele, and another like it in mab-20, may be that a high rate of Tc1 excision could drastically enhance the frequency in which the DNA flanked by the two Tc1 elements is repaired using the homologous chromosome as a template. It is known that Tc1-induced chromosomal lesions can be repaired from an exogenous templates present on extra-
chromosomal arrays and that small base pair mutations can be introduced in this way (Plasterk and Groenen, 1992). A high frequency of deletion of the DNA flanked by the cis-Tc1s could hypothetically increase the repair frequency for the flanked DNA. It may therefore be possible to replace or introduce exogenous DNA sequence into the targeted region that is flanked by cis-
Tc1s with strategic selection markers and/or reporter genes etc. that culminate in mutation by gene replacement. Future experiments need to resolve the plausibility of this proposed technique.
Finally, a direct-deletion library consisting of a relatively small number of EMS-mutagenized wildtype genomes has been constructed (L.Z. and J.G.C., unpublished results). Although a positive control consisting of a sample that was “doped” with a known deletion validated the screening techniques, preliminary screens for novel deletions have been unsuccessful. While the sample size is most likely too small, the large number of primers that have been collected from the various Tc1 screens could be used to screen the small deletion library to assess the success rate of isolating alleles. Many other labs have also tried building direct-deletion libraries and have failed to isolate targeted deletions. It is plausible that the worm strains used to seed the libraries that have been successfully screened (Jansen et al., 1997) contain an undefined mutation(s) that somehow facilitates the creation of detectable deletion alleles that can be isolated. It would be prudent to obtain a stock of these strains from successful laboratories if further construction of direct-deletion libraries is to be pursued.

5.5 The Phenotypic Analysis of mab-20 mutants

At least five events are aberrantly affected in mab-20(ev574) mutants during hypodermal morphogenesis. First, some homozygous embryos burst from the ventral midline after ventral enclosure. Second, animals that escape the embryonic lethality develop severe morphological defects during elongation. Third, normally distinct sensory rays of the developing male tail fuse together. Fourth, anterior larval seam cell progeny often fail to dissociate from their siblings and cannot fuse with the surrounding hypodermis disrupting the alae cuticular pattern. Finally, some mutant embryos and larvae have split or forked tails. A detailed investigation into the cellular basis of the first three phenotypes suggests that Ce-SemaII is required to repel ectopic contact formation between hypodermal cells.

Both immunostaining and SEM analysis of mab-20(ev574) mutant embryos reveals that the ventral hypodermal pocket cells establish ectopic contacts with ipsilateral cells during ventral
enclosure. The ectopic contacts between the pocket cells apparently interfere with proper hypodermal enclosure of the embryo, resulting in the observed expulsion of internal contents from the incompletely sealed midline during elongation. These results suggest that Ce-SemaII is required to repel filopodial or lamellapodial extensions from the pocket cells, but not the leader cells, which in turn prevents neighboring cells from forming and/or stabilizing ectopic contacts. Recent evidence using a GFP-tagged HMP-1 marker and MPLSM suggests that pocket cells transiently extend small filopodia, compared to leader cells that extend longer filopodia to the ventral midline (Raich and Hardin, 1998). Interestingly, if the leader cell migration is genetically disrupted, the short transient filopodia of the anterior pocket cells become stable and extend to the midline. This suggests that although the pocket cells can extend substantial filopodia, they are normally prevented from doing so. I hypothesize that the filopodia of the pocket cells normally express receptors that mediate a repulsive response to Ce-SemaII, while leading anterior ventral hypodermal cells do not. Without Ce-SemaII, the filopodia of the pocket cells are no longer repelled and in turn become more stable and substantial. I further hypothesize that each cellular defect that results in the five aforementioned hypodermal phenotypes is due to ectopic contact formation mediated by filopodia that are normally repelled by Ce-SemaII. Two experiments are proposed to address this hypothesis. First, using the HMP-1::GFP marker and MPLSM, the filopodial extended from cells in mab-20 mutants could be compared to those in wildtype animals. It is predicted that the ventral hypodermal cells, the embryonic and larval seam cells and the ray precursor cells of the male tail will extend substantial filopodia towards neighboring cells in mab-20 mutants but not in wildtype. Second, a detailed examination of the pattern of the Ce-SemaII receptor is anticipated to reveal an expression pattern that is limited to the leader cells and not the pocket cells. Also, the expression of the Ce-SemaII receptor is predicted to be expressed in the embryonic seam cells during their ontogeny, the anterior larval seam cell daughters, but not the posterior daughters that establish contacts with each other, the
ray precursor cells of the developing male tail, and the hyp-10 precursor cells during their migration to the posterior tip of the embryonic tail to generate the whip.

What is the Ce-SemaII receptor(s)? Since mab-26 and vab-6 mutants have many phenotypes in common with mab-20 and as such are candidate genes in the semaphorin pathway, they were further investigated side by side with mab-20 for many of the experiments described in chapter 4. The fact that mab-26(bx80) has the same spectrum and expressivity of ray fusions as mab-20(ev574) and that it does not significantly enhance the ray fusion expressivity of mab-20(ev574) suggests that the two genes may be in the same pathway. As described in the introduction, Plexin is a receptor for viral Semaphorins (Comeau et al., 1998) and preliminary genetic evidence from Drosophila suggests that Plexin I is a D-SemaI receptor. Excitingly, the C. elegans genomic sequencing consortium has recently sequenced a Plexin II homologue that resides close to the mab-26(bx80) genetic locus on the left arm of LG IV at -29.20 map units relative to dpy-13 (G. Dalpe, personal communication). In collaboration with Scott Emmons and colleagues (Albert Einstein College of Medicine, Bronx, NY), we plan to investigate the possibility that mab-26 encodes Plexin II and that it is a receptor for Ce-SemaII. Through transgenic rescue experiments, SSCP analysis, expression studies, and ectopic expression experiments, the possibility of Plexin II mediating the ability of Ce-SemaII to repel filopodial extension will be investigated. In addition, if a Ce-SemaII or Plexin antibody were available or if tagged versions of each existed, a direct interaction between the two products could also be investigated.

Although mab-26 mutants have morphological defects like those of mab-20, the penetrance of mab-26 defects is relatively low. By reviewing the penetrance and/or expressivity of the male tail defects (table 4.9), the erroneous seam cell contacts (table 4.5), and the ventral enclosure defects (table 4.4) exhibited by mab-20, mab-26, and vab-6 mutants, it becomes obvious that unlike mab-26 and vab-6, Ce-SemaII is required in all three processes. Although it
is unknown if \textit{mab-26} and \textit{vab-6} are nulls or at least hypomorphic for all functions, the aforementioned data suggests that \textit{mab-26} may be principally required in male tail development, and \textit{vab-6} in seam cell ontogeny and neither in ventral enclosure. These results imply that even if \textit{mab-26} encodes a receptor, there must additional receptors that mediate Ce-SemaII activity in different spatial and temporal locales.

Since the principle defect of \textit{vab-6(e96)} mutants is restricted to abnormal seam cell conformations that possibly result in the observed morphological abnormalities, is VAB-6 a seam cell-receptor for Ce-SemaII? Two lines of evidence suggest that it is unlikely that VAB-6 is a receptor that mediates Ce-SemaII action. First, preliminary results indicate that \textit{vab-6} encodes a novel intracellular protein. Gratien Dalpe (personal communication) has demonstrated that multiple independent genetic lines that contain arrays that carry cosmid F10C2 can rescue the \textit{vab-6} mutant phenotype. F10C2 contains only 8 kb of genomic sequence and physically maps relatively near the genetic map position of \textit{vab-6} on the left arm of LG III at \(-27.17\) map units relative to \textit{unc-32}. GeneFinder suggests that F10C2 contains only one complete gene, which is predicted to encode an intracellular protein whose only known motifs are three TPR domains that may mediate protein-protein interactions. For example, eight TPR domains are required to mediate binding of CDC28 to type 2A phosphatase (Ollendorff and Donoghue, 1997). To date however, it is uncertain as to exactly how VAB-6 functions in seam cell development and embryogenesis. Further investigations into the expression pattern of VAB-6 may help resolve this issue.

The second line of evidence that suggests that VAB-6 is not a Ce-SemaII receptor is that genetic experiments reveal that \textit{mab-20} and \textit{vab-6} probably act in parallel genetic pathways. If two genes are thought to be in the same genetic pathway and if either allele used to make the double mutant is a null, then the resultant double mutant phenotype is predicted not to be more severe than either mutant alone. Although the double heterozygote could easily be constructed, a
mab-20(ev574); vab-6(e96)III double mutant line could not be isolated on several attempts. Although this is a negative result, it may indicate that the double mutant has a highly penetrant early embryonic lethal phenotype and suggests that the two genes function in different pathways. In addition, mab-20(bx61); vab-6(e96)III doubles contain morphological defects that are subjectively more severe than either mab-20(ev574) or vab-6(e96) mutant alone (G. Dalpe, personal communication). Together these results suggest that vab-6 and mab-20 probably act in parallel pathways that regulate the relative positions of seam cells.

As previously mentioned, the second major hypodermal phenotype observed in mab-20 mutants is severe morphological abnormalities. The proposed model suggests that Ce-Semaphorin II is required to repel or prevent ectopic seam cell contacts initiated during a period when the seam cell progenitors undergo numerous short-range migrations and rearrangements. The resulting irregular seam cell conformation is proposed to lead to seam cell microfilaments that are skewed with respect to those in other hypodermal cells aligned with the left-right circumferential axis. Since the circumferential microfilaments are required to transduce the force that drives elongation (Priess and Hirsh, 1986), the skewed microfilaments in mab-20 mutants are predicted to result in an uneven distribution of force during elongation. The non-uniform force is predicted to result in animals with numerous bumps and constrictions. This model predicts that each seam cell has a dorsal-ventral (D-V) axis that is used by the cytoskeletal network to align microfilaments during elongation. Until recently, there was no corroborating evidence that supported the existence of autonomous D-V or anterior-posterior (A-P) axes in the embryonic seam cells that was independent of the alignment of the circumferential microfilaments during elongation. It is possible that microfilament alignment in the seams is non-autonomously regulated. Conveniently, Zhu and Hedgecock (personal communication & 1998) recently revealed that a GFP-tagged version of MIG-15 is localized to the D-V borders and then to the anterior-posterior (A-P) borders of embryonic seam cells during their ontogeny and ventral
enclosure. Not only does this observation corroborate the notion that the hypodermal seam cells express markers indicative of a D-V and A-P axis, but it also provides a tool to address the model of *mab-20* morphological abnormalities. Future experiments can now examine the distribution of MIG-15::GFP in *mab-20* and wildtype mutant embryos to compare the axes of the seam cells relative to the circumferential microfilaments.

It is intriguing that *mab-20* mutants contain body wall muscle (BWM) cells that cross quadrant boundaries only beneath seam cells whose D-V axes are apparently skewed with respect to the D-V axis of worms. This observation suggests that the D-V borders of the lateral hypodermal seam cells secrete a short-range repulsive cue(s) that normally prohibits muscle cells from straying laterally. If the seam cells become skewed, the BWMs may have safe passage through the lateral boundary since they only encounter the A-P boarders of the seam. A genetic dissection of the ability of BWM cells to cross the quadrant boundary could reveal the molecular nature of how the BWMs are restricted to their respective quadrants.

Finally, to identify other genes that may be required for Ce-SemaII-mediated function, I initiated a screen for mutants that dominantly enhance the hypomorphic *mab-20* body deformities as described in chapter 4. Thirty putative enhancers were isolated, of which a subset are currently being characterized by Gratien Dalpe (personal communication). In addition, Gratien Dalpe and Richard Ikegami are screening for additional dominant and recessive enhancers of the *mab-20* hypomorphic body malformations (personal communication). Besides identifying the receptor(s) that may mediate the embryonic functions of Ce-SemaII, it is predicted that regulatory and downstream signaling components may also be identified. As anticipated during the onset of my investigation into the Semaphorins, the unique power of *C. elegans* genetics will be exploited to gain a better understanding of how the Semaphorins function throughout development. It is fortunate that some of the characterized phenotypes of
mab-20 are readily apparent at the level of the dissection microscope, which enables our lab to screen millions of mutagenized genomes for genes that may interact with the semaphorins.

5.6 General Summary

The Semaphorins are a large family of secreted and transmembrane molecules that are known to be multifunctional axon guidance cues. Although the Semaphorins can elicit filopodial collapse, axon outgrowth and perhaps even attraction, the characterization of the guidance functions of Semaphorins have been limited to axons. Interestingly, Semaphorin mutants in Drosophila, mouse, and humans reveal that they may have additional embryonic functions in organ patterning and tumor suppression that is not well understood. To elucidate mechanisms involved in semaphorin function in vivo, I have partially characterized semaphorin Ia and Ib, and have intensely investigated semaphorin II in C. elegans. Through reverse genetics, I have created a putative null mutation in semaphorin II. In doing so, I have further developed techniques of targeted mutagenesis that have enabled the isolation of over 40 alleles of interest to various laboratories.

I have demonstrated here that semaphorin II mutations result in errors in axon guidance, fasciculation, and certain cell migrations. The most penetrant phenotypes of mab-20 mutants however, are embryonic death due to ventral enclosure defects, pleiotropic body malformations in escapers, and fused sensory rays in male tails. I have shown that ectopic contacts between a limited number of hypodermal cells may explain all of the hypodermal phenotypes in the semaphorin II mutants. These results not only suggest that Ce-SemaII is required to inhibit exploratory processes from establishing permanent contacts, but that the Semaphorins may play a broad role throughout animal development. Finally, the characterization of the semaphorins in C. elegans has allowed numerous questions to be addressed through further experimentation.
Undoubtedly, these future experiments will lead to a better understanding of the Semaphorins and how guidance cues in general, function in multiple developmental processes.
References


Appendix A

Recipes
### 2X MRWB
- KCl: 160.0 mM
- NaCl: 40.0 mM
- Na₂EGTA: 20.0 mM
- spermidine HCl: 10.0 mM
- PIPES (pH 7.4): 30.0 mM
- Methanol: 50.0 %

### TTB
- Tris-HCl (pH 7.4): 100.0 mM
- triton X-100: 1.0 %
- EDTA: 1.0 mM

### 40X BO₃
- H₃BO₃: 1.0 M
- NaOH: 0.5 M

### Redox Buffer
- Potassium Ferricyanide: 50.0 mM
- Potassium Ferrocyanide: 50.0 mM

### β-galactosidase Staining Solution
- NaH₂PO₄ (pH 7.5): 0.8 mM, 250.0 μl
- MgCl₂: 1.0 M, 2.0 μl
- SDS: 1 %, 4.0 μl
- Redox Buffer: 100.0 μl
- X-GAL: 2 %, 20.0 μl

### Cosmid Solution I
- Glucose: 50 mM
- EDTA: 10 mM
- Tris-HCl (pH 7.8): 25 mM

### Cosmid Solution II
- NaOH: 0.2 M
- SDS: 1.0 %

### Cosmid Solution IV
- Tris-HCl: 50 mM
- NaCl: 150 mM
- β-mercaptoethanol: 7 mM
- Rnase: 20 μg/ml

### Egg Salts
- NaCl: 118 mM
- KCl: 48 mM
- CaCl₂: 2 mM
- MgCl₂: 2 mM
Hepes (pH 7.4) 0.025 mM

**Chitinase-Chymotripsin Solution**

Chitinase (Sigma C7809) 5 units
α-chymotrypsin (Sigma C4129) 10 mg
egg salts 1 ml
Penicillin-Streptomycin (Sigma P3539) 10 μl

Mix the solution on ice, let sit several hours or overnight, filter sterilize. Store refrigerated or freeze in 100 μl aliquotes.

**NGM Agar** (Wood, 1988)

NaCl 3.0 g
agar 17.0 g
peptone 2.5 g
ddH₂O 975.0 ml

Autoclave, then add the following using aseptic techniques and mix:

CaCl₂ 1M 1.0 ml
MgSO₄ 1M 1.0 ml
potassium phosphate buffer (pH 6) 1M 25.0 ml
cholesterol (5mg/ml in EtOH) 1.0 ml

**M9 Worm Buffer** (Wood, 1988)

KH₂PO₄ 3.0 g
Na₂HPO₄ 6.0 g
NaCl 5.0 g
MgSO₄ 1M 1.0 ml
ddH₂O to 1 liter

**2X Freezing Solution** (Wood, 1988)

NaCl 5.85 g
KH₂PO₄ 6.8 g
glycerol 300.0 g
NaOH 1M 5.6 ml
ddH₂O to 1 liter

After autoclaving, add
MgSO₄ 1M 300 μl

**Sample Lysis Solution** (Y. Andachi and Y. Kohara, personal communication)

NaCl 5M 100 mM 200 μl
Tris-HCl (pH 8.5) 2M 100 mM 500 μl
EDTA (pH 7.4) 500 mM 50 mM 1 ml
SDS 10% 1 % 1 ml
β-mercaptoethanol 1 % 100 μl
proteinase-K 20 mg/ml 100 μg/ml 50 μl
ddH₂O to 10 ml
**Single Worm Lysis Solution** (Plasterk, 1995)

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Appendix B

The Composition of the Tc1 Library
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The pools of the library were constructed for several different purposes. The original library (OL) was seeded with MT 3126 [mut-2(r459); dpy-19(n1347)III] and constructed in order to obtain many different Tcl alleles. It is noted that it is uncertain if mut-2(r459) is linked to linkage group I (Collins et al., 1989).

The first sub-library (SL1) was seeded with NW 1033 [mut-2(r459); els-1(ev586::Tcl); dpy-19(n1347)III] and was constructed in hopes of isolating an els-1 loss-of-function mutation.

Sub-library two (SL2) was seeded with NW 1034 [mab-20(ev573::Tcl); mut-2(r459); dpy-19(n1347)III] and was constructed in hopes of obtaining a mab-20 loss-of-function.

The third sub-library (SL3) was seeded with NW 1037 [mut-2(r459); dpy-19(n1347)III; cdh-x(ev576::Tcl)V] and constructed in hopes of obtaining a cdh-x loss-of-function mutation.

The fourth sub-library (SL4) was seeded with NW 1050 [mab-20(ev573::Tcl); mut-2(r459); els-1(ev586::Tcl); dpy-19(n1347)III] and was constructed in hopes of obtaining loss-of-function alleles in both els-1 and mab-20.

Sub-library five (SL5) was seeded with NW1058 [mut-2(r459); els-1(ev587::cis-Tcl); dpy-19(n1347)III] in hopes of isolating loss-of-function alleles in els-1 at a relatively high frequency.

The sixth sub-library (SL6) was seeded with NW 1087 [mab-20(ev573::Tcl); mut-2(r459); dpy-19(n1347)III; lig-1(ev584::Tcl)IV; cdh-x(ev576::Tcl)V; ets-1(ev583::Tcl)X] and was constructed with the purpose of isolating loss-function alleles in each of mab-20, lig-1, cdh-x, and ets-1.

Finally, the seventh sub-library (SL7) was seeded with NW 1080 [gly-2(ev581::Tcl); mut-2(r459); gly-1(ev571::Tcl)II; dpy-19(n1347)III] with the hopes of isolating loss-of-function mutations in gly-1 and gly-2.
Appendix C

The Organization of $\alpha$-Pools within $\beta$-Pools
The organization of the alpha-pools within beta-pools. Each alpha-pool belongs to three beta-pools. For example, alpha-pool 30 belongs to beta-pools X1, Y1 and Z1. Similarly, alpha-pool 1030 belongs to beta-pools X2, Y3, and Z4.