Six4/5 Family Transcription Factor UNC-39 Controls the Development of RID Neuron in *Caenorhabditis elegans*

by

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A thesis submitted in conformity with the requirements for the degree of Master's of Science
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Abstract

Members of the Six4/5 family of homeobox transcription factors have been implicated in multiple human disorders, including type I myotonic dystrophy, branchio-oto-renal syndrome, and holoprosencephaly, suggesting a role for these factors in the nervous system development.

Using a forward genetics approach, we identified *unc-39*, a *C. elegans* homologue of the human *SIX5* gene, as a novel regulator of the development of a specific neuron, called RID. Our data support the role of *unc-39* early in *C. elegans* development and suggest a possibility of complete absence of RID neuron in *unc-39* mutants. *unc-39* mutant has a similar locomotion phenotype to the RID-ablated animals, which provides further support to the hypothesis that the absence of RID contributes to the locomotion phenotype observed in the mutant. We show that *unc-39* functions at multiple points in the lineage that gives rise to the RID neuron, and that its function is context-dependent.
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Specific contributions

Figure 1: Multiple sequence alignment was performed using CLUSTALW by Valeriya Laskova. The figure was adapted from Kawakami et al. (2000).

Figure 2: The figure was prepared by Valeriya Laskova.

Figure 3: The strain was made by Jyothsna Chitturi. The picture was taken by Jyothsna Chitturi. The figure was prepared by Valeriya Laskova.

Figure 4: Laser ablations of the RID neuron were performed by Jyothsna Chitturi. Videos, on-plate movement analysis and the figure were done by Valeriya Laskova.

Figure 5: The forward genetic screen was performed by Wesley Hung, Jyothsna Chitturi and Valeriya Laskova. The pictures were taken by Jyothsna Chitturi and Valeriya Laskova. The figure was prepared by Valeriya Laskova.

Figure 6: The figure was prepared by Valeriya Laskova. Multiple sequence alignment was performed using CLUSTALW by Valeriya Laskova.

Figure 7: The strains, pictures and the figure were generated by Valeriya Laskova.

Figure 8: The figure was adapted by Valeriya Laskova from Sulston et al. (1983).

Figure 9: The strains were generated by Valeriya Laskova and Jinbo Wang. All pictures, quantifications and the figure were prepared by Valeriya Laskova.

Figure 10: The strains were generated by Valeriya Laskova and Jyothsna Chitturi. All pictures, quantification and figures were prepared by Valeriya Laskova.
Figure 11: The strain, pictures and the figure were generated by Valeriya Laskova.

Figure 12: High-pressure freezing of the strains were performed by Mei Zhen, Valeriya Laskova and Jyothsna Chitturi. Preparation of the serial sections was performed by Douglas Holmyard. All images, analysis and 3D reconstruction were done by Valeriya Laskova.

Figure 13: Laser ablations of the RID neuron were performed by Jyothsna Chitturi. Videos, on-plate movement analysis and the figure were prepared by Valeriya Laskova.

Figure 14: The strains were generated by Valeriya Laskova and Jinbo Wang. All pictures, quantifications and the figure were prepared by Valeriya Laskova.

Figure 15: All strains, quantifications, pictures and the figure were prepared by Valeriya Laskova.

Figure 16: The figure was adapted by Valeriya Laskova from Doitsidou et al., 2010
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1 Introduction

1.1 Cell fate program and terminal differentiation

1.1.1 Transcription factors and their roles in cell fate determination

The diversity of neuronal types that constitute a functional nervous system is astounding. Beginning with the work of Ramond y Cajal, who first noted the morphological differences between different neuronal classes (1911), the field of developmental neurobiology has been making continuous advancement in deciphering the molecular basis of this diversity. We now know that a collection of factors, which are encoded by terminal differentiation genes, determine a specific identity of a given neuron. The identity of a neuron includes its morphology, the pattern of its synaptic connections, its neurotransmitter and receptor expression profiles, and its intrinsic excitability. These properties, in turn, dictate the function of a neuron in the context of the nervous system. One of the fundamental questions in developmental neurobiology, therefore, concerns the regulatory mechanisms that govern the differential expression of these factors.

In post-mitotic neurons, cascades of transcription factors, called terminal selectors, are proposed to bind to specific motifs in the cis regulatory sequences of the terminal differentiation genes, and induce the expression of these “terminal gene batteries” (Hobert, 2011). Some terminal selectors ascribe pan-neuronal qualities to the cell, meaning they induce expression of genes that are common to neurons in general, such as those coding for components of synaptic vesicles, their transport machinery, etc. Although these genes may also be expressed in other cell types, they are termed “pan-neuronal” due to their expression in all, or nearly all neurons. Other terminal selectors induce a subtype-specific role. For example, PROX1, a prospero-related homeobox transcription factor, is necessary for specification of the granule cell fate in the
dentate gyrus (DG) of mouse hippocampus. When Prox1 is knocked out in the post-mitotic DG granule cells, they are transformed towards the identity of the cornu ammonis (CA) pyramidal neurons (Iwano et al. 2012). Similarly, in C. elegans, two homeodomain transcription factors, TTX-3 and CEH-10, function together to induce cell fate of the AIY interneuron through cooperative binding to a specific cis regulatory element, called the AIY motif, found in a set of the AIY terminal differentiation genes. Upon the functional loss of TTX-3 and CEH-10, all known terminal differentiation characteristics of the AIY neuron are lost. These include loss of expression of a serotonin receptor SER-2, an orphan serpentine receptor SRA-11, KAL-1, which is a factor involved in axonal branching of AIY, as well as an acetylcholine transporter UNC-17. These factors are termed sub-type specific, since they are expressed in a restricted number of cells. However, in ttx-3 and ceh-10 mutants, AIY does not lose its general neuronal identity. This was assessed by monitoring expression of several pan-neuronal markers, such as UNC-119 and UNC-33, both of which are involved in axonal development. These markers were unaffected by ttx-3 and ceh-10 mutations (Altun-Gultekin et al. 2001).

The presence of motifs, specific for a given terminal selector, in the regulatory sequences of the “terminal gene battery” could suggest a “one selector – one neuronal sub-type” model. In this model, a single terminal selector would be necessary and sufficient to turn on all sub-type specific characteristics of a given neuron. However, it is becoming increasingly evident that this is not the case. Although some terminal selectors are restricted to a specific neuronal sub-type, many are expressed more broadly. This model cannot explain how a given terminal selector could induce different cell fates in several neuronal sub-types. A more plausible model considers a combinatorial effect. A given terminal selector can perform different functions, depending on the cell context it is in. For example, CEH-10, which is a PRB homeodomain
protein, plays a crucial role in the cell fate of the AIY neuron in *C. elegans*, by controlling the expression of all known AIY terminal differentiation genes, namely, SER-2, UNC-17, SRA-11 and KAL-1. However, CEH-10 does not have a profound effect in another neuron that also expresses it, called RID (Tsalik et al., 2003). The authors tested the expression of two RID terminal differentiation markers – a LIM family homeodomain protein LIM-4 and a serotonin receptor SER-2a, in *ceh-10* mutant background. Both of these terminal differentiation markers are known to be expressed in RID and are unaffected by *ceh-10* mutations (Tsalik et al., 2003). This context-dependent function of CEH-10 in the development of AIY and RID is likely achieved through binding with other transcription factors that are differentially expressed in these neurons. For example, in AIY neuron, CEH-10 physically interacts with TTX-3, and this complex activates transcription of the terminal gene battery (Wenick et al. 2004). However, in RID, the function of CEH-10 must be independent of TTX-3, since TTX-3 is not expressed in this neuron and therefore cannot serve as a binding partner to CEH-10. Therefore, it is rather a unique combination of terminal selectors in a given context that ultimately determines the fate of the neuron.

1.1.2 Homeodomain transcription factors and their role in development and cell fate determination

Homeobox genes code for transcription factors that contain a homeodomain. The homeodomain consists of conserved 60 amino acids, typically located closer to the C-terminus of a protein (Gehring and Hiromi, 1986). The homeodomain is comprised of a helix-turn-helix module, which binds the DNA in the major groove, and an N-terminal arm, which contacts the minor groove (Kissinger et al., 1990). One alpha-helix strand mediates DNA binding, while the second helix stabilizes the protein-DNA interaction (Berger et al. 2008). Homeodomain proteins
typically bind to the promoter regions of their target genes, and turn on cascades of other transcription factors. However, a single homedomain is often insufficient to achieve the desired sequence specificity. In addition to binding DNA, the homeodomains can also bind proteins, forming complexes with other transcription factors. The recognition sequences that are bound by homeodomain proteins vary. Moreover, the target recognition sequence of a homeodomain monomer may also differ from that of the complex. This way the complexes confer higher target selectivity. Homeodomain proteins typically recognize 7 or 8 bases in the target DNA sequence. Within this sequence, there are four base pairs – the TAAT motif, which is a canonical sequence recognized by the majority of homeodomain proteins (Desplan et al., 1998). The base pairs in the recognition sequence outside of the TAAT motif vary.

The homeobox was first discovered through *Drosophila melanogaster* mutants that exhibit homeosis, or, homeotic transformations, where a body part is abnormally developed to exhibit characteristics of another body part (Bateson, 1894). Two gene clusters, the bithorax complex (BX-C) and antennapedia complex (ANT-C), are expressed in anatomical units called parasegments (PS), which are comprised of the posterior end of one body segment and the anterior end of the next segment. There are fourteen parasegments in the *Drosophila* embryo. ANT-C cluster genes have a role in ascribing anterior identity to PS1-5, while genes in the BX-C cluster induce the posterior identity of PS5-13 (Lewis, 1978; Kaufman et al., 1979). The genes were later found to contain a conserved sequence, termed homeobox (McGinnis et al., 1984).

There are numerous homeobox genes, and their protein products are classified into families based on evolutionary sequence conservation. The following homeodomain protein classes have been recognized in humans: ANTP, PRD, POU, LIM, PROS, CUT, SINE, HNF, TALE, ZF, CERS (Holland et al., 2007). The role of homeobox genes in patterning cell types during
development is evolutionarily conserved. A well-studied group of homebox genes, the Hox genes, is involved in patterning of the body segments in diverse bilateral species, from insects to humans. The characteristic feature of these genes is their grouping in clusters, called Hox clusters, along a chromosome and their expression pattern along the head-tail axis in the order that corresponds to their position in the cluster, a phenomenon termed colinearity. In *Drosophila*, there are two homeotic clusters, while mice and humans have four (HOX-A through HOX-D). These genes determine the type of body parts that are going to be formed at that particular segment that expresses them (Caroll, 1995). Hox genes act high in the regulatory cascade, controlling transcription of each other, various transcription factors and morphogens. For example, in *Drosophila*, two Hox proteins, Ultrabiothorax (UBX) and Abdominal-A (ABD-A), induce expression of *Decapentaplegic (Dpp)* gene in the visceral mesoderm (Bienz, 1994). DPP is a secreted morphogen, which regulates normal gut morphology in this specific region. In addition to serving as transcriptional activators, Hox proteins can perform a repressive function. For example, the same UBX and ABD-A Hox proteins, repress the expression of *Distal-less (Dll)* gene in the epidermis of the abdomen in *Drosophila*. DLL is itself a homeodomain transcription factor, which induces formation of the appendages. By locally blocking the expression of *Dll*, UBX and ABD-A ensure that the appendages are not ectopically formed in the abdomen (Vachon et al., 1992). The execution of such complex functions, as patterning of body segments by Hox proteins, requires their involvement in various aspects of development, such as cell cycle, apoptosis, cell adhesion and migration (Pearson et al., 2005).

In addition to the homeobox, some homeodomain proteins have other signature domains that perform specific functions. Homedomain proteins are sorted into classes based on the identity of these secondary domains. For example, in addition to the homedomain, the LIM-homedomain
(LIM-HD) transcription factors have two conserved zinc finger motifs, termed the LIM domain, positioned N-terminally to the homeodomain. The LIM domain confers protein-protein interaction, which allows for LIM-HD transcription factors to function as a component of transcription factor complexes that dictate various aspects of development, including neuronal fate specification (Appel et al., 1995).

### 1.1.3 SIX family transcription factors

A rather unique family of homeobox genes coding for transcription factors that fall into the SINE class is the *Six* family. The first member of this family, *sine oculis* (*so*), was discovered to play a crucial role for normal *Drosophila* eye development (Cheyette, 1994). To date, members of the *Six* family have been identified in many species, including nematodes, rodents and humans (Seo et al., 1999). SIX transcription factors share the SIX domain (SD), which consists of 110-115 amino acids. The homeodomain of the SIX family is divergent from a classic homeodomain, lacking two conserved amino acids, arginine and glutamine in helix 1 at positions 5 and 12, respectively. In the classic homeodomain transcription factors, this arginine is necessary for binding to the TAAT motif in the recognition sequences of the target genes. Consistently, the DNA sequences that are known to be bound by some SIX family members do not contain the TAAT motif (Kawakami, 1996). AREC3/SIX4 homeodomain protein was found to bind the ARE sequence in the regulatory element of a Na+/K+ GTPase α1 subunit. Other members of the SIX family, SIX2 and SIX5, also bind to this element through their SD and HD in the gel retardation assays. However, SIX3 does not bind this sequence (Kawakami, 1996). The SD domain is typically positioned to the N-terminus of the HD, and the SIX family is further divided into three groups, based on the sequence similarity of the conserved domains (SIX1/2, SIX3/6 AND SIX4/5) (Figure 1). A unique characteristic of the SIX4/5 subfamily is a large region C-
terminal to the HD that contains a transcription activation domain, which is lacking in other SIX families (Kawakami et al., 1996; Ohto et al., 1999).

**Figure 1:** Structures of human Six proteins. Percentages indicate degree of homology of SD and HD between respective members of the Six family. In addition to the Six (SD) and homeodomain (HD), members of the Six4/5 family have a C-terminal activation domain (AD).

The members of the SIX family of proteins play critical role in development. The *Drosophila so* gene is crucial for the development of its visual system. Depending on the allele, *so* mutants show a range of phenotypes, from loss of ocelli to complete loss of the compound eye (Cheyette, 1994; Serikaku, 1994). When SIX3 is expressed ectopically in zebrafish, it results in the enlargements of the forebrain (Kobayashi et al., 1998). Ectopic expression of SIX1, together with another factor that has a role in eye development, called EYA2, activates myogenic genes in somite culture, suggesting a role in differentiation of the muscle tissue (Heanue, 1999). Ectopic expression of SIX6 in the retinal pigmented epithelial cells, on the other hand, results in their transformation towards neural fate (Toy et al., 1998).
Importantly, in humans, multiple SIX genes have been linked to genetic disorders, indicating a critical role of this gene family during development. Specifically, SIX1 has been implicated in tumor formation in the breast tissue (Ford et al., 1998). Mutations in SIX6 have been linked to holoprosencephaly (HPE), a congenital disorder characterized by abnormal prenatal development of the forebrain into two hemispheres (Roessler and Muenke, 1998). Finally, disrupted SIX5 gene function has been proposed to contribute to phenotypes of multiple disorders, which will be discussed in more detail below.

1.1.3.1 Potential involvement of SIX5 in multiple human genetic disorders

Several human disorders have been linked with SIX5 mutations. The Branchio-oto-renal syndrome (BOR) is an autosomal dominant disorder. It is characterized by the defects in the branchial arch, where patients develop branchial cleft cysts in the neck, which are sometimes connected to the surface of the neck through the formation of fistulae, the hearing loss associated with an abnormal development of the inner, middle and outer ear, and less frequently, renal abnormalities, including, hydronephrosis, collective system duplication and cystic dysplasia (Hoskins et al., 2007; Izzedine et al., 2004).

BOR is a heterogeneous genetic disorder. Mutations in SIX1, SIX5 and EYA1 have been identified to associate with BOR. Importantly EYA1 is an ortholog of the Drosophila gene Eyes absent. It codes for a transcription factor, which through GST pull-down and yeast-two-hybrid assays, has been found to interact with the SIX family members SIX1 and SIX2 (Buller et al., 2001). Furthermore, mutations in SIX1, associated with BOR syndrome, disrupt the protein interaction with EYA1 and its ability to bind DNA (Ruf et al., 2004). Finally, after analyses in C.
*C. elegans* that demonstrated a yeast two-hybrid interaction between the *C. elegans* homologue of SIX5, UNC-39/CEH-35, with the *C. elegans* EYA-1, a cohort of patients with BOR lacking mutations in EYA1 and SIX1, was tested for mutations in SIX5. Four unique heterozygous mutations that affect SIX5-EYA1 interaction and the translocation of the complex to the nucleus have been identified (Buller et al., 2001). Together, these results indicate that disruption of cooperative activation of target genes by SIX1-EYA1 and SIX5-EYA1 may underlie abnormal organ development seen in BOR syndrome.

*SIX5* has also been proposed to be responsible for some phenotypes of the Type 1 Human Myotonic Dystrophy Type 1 (DM1). DM1 patients exhibit a wide range of clinical features, including muscle weakness, mental impairments, cataracts, abnormalities in cardiac conduction and endocrine insufficiency (Harris et al., 1996). This disorder is associated with a CTG expansion, which serves as a recognition sequence for the CTCF protein responsible for inducing silenced chromatin in the region of DNA that covers the 3’UTR of the myotonic dystrophy protein kinase (*DMPK*) gene (Brooke, 1992), and also overlaps the promoter region of the *SIX5* gene (Boucher, 1995). Several lines of evidence indicate that at least some DM1 clinical features are likely attributed to the dysfunction of *SIX5*. The CTG expansion leads to a two to four times down-regulated expression of *SIX5* in DM1 patients (Klessert et al., 1997; Thomton et al., 1997). The fact that *SIX5* is involved in upregulation of myogenin in mice (Kawakami et al., 2000) suggests indirectly that dysfunctional *SIX5* likely contributes to the muscle phenotype in DM1 patients. Moreover, human *SIX5* is expressed in the lens, while *DMPK* is not, favoring a possibility that the cataract phenotype is also a result of the *SIX5* dysfunction (Winchester et al., 1999).
1.1.3.2 *unc-39*, the *C. elegans* homologue of *SIX5* is involved in specification of mesodermal tissues and anterior neurons

*unc-39*, previously named *ceh-35*, is the *C. elegans* member of the *Six4/5* family. UNC-39 is required for normal development and behaviors. Three loss-of-function alleles of *unc-39* (*e257, rh76* and *ct73*) lead to uncoordinated locomotion, as well as a wide range of defects in the development of the mesodermal tissues and anterior neurons (Yanowitch et al., 2004). In addition to protein sequence similarity, *unc-39* is a functional homologue of the human *SIX5* gene. Replacing the conserved domains, SD and HD, in the *unc-39* translational reporter, with those of the human *SIX5* gene, partially rescued the defects in *unc-39* mutants’ phenotype (Yanowitch et al., 2004).

Consistent with a putative role of human *SIX5* in muscle dystrophy, *C. elegans* UNC-39 is required for normal mesodermal development. *C. elegans* mesodermal tissues include coelomocytes, somatic cells of the gonad, head mesodermal cells, body wall muscles, pharyngeal muscles and vulval muscles, all of which were reported to be affected by *unc-39* mutations.

Coelomocytes are scavenger cells that take up molecules from the pseudocoelomic space, and have been proposed to function as a primitive immune system in invertebrates (Fares and Greenwald, 2001). *C. elegans* has three pairs of coelomocytes in the pseudocoelomic cavity. Two pairs reside on the ventral side, one in the anterior and posterior regions, respectively, and the other pair resides on the dorsal side of the posterior body. *unc-39* mutations result in abnormal migration of coelomocytes, sometimes leading to the accumulation of all 6 coelomocytes in the anterior region of the animals. *unc-39* also affects coelomocyte cell fate specification, with
the number of coelomocytes in \textit{unc-39} mutants ranging from one to seven (Yanowitch et al., 2004; Manser and Wood, 1990).

Another mesodermal defect in \textit{unc-39} mutant is the formation of an extra somatic gonad precursor cell, Z5, which in some cases resulted in the formation of a full gonad arm. In \textit{C. elegans} there are normally two somatic gonad precursor cell, Z1 and Z4. They have two sister cells, the right and left head mesodermal cells (hmcR and hmcL). Yanowitch et al. propose that the extra cell, Z5, comes from an abnormal division of the hmcR in \textit{unc-39} mutants into two daughter cells: the hmcR-like cell and a cell that undergoes a transformation towards its sister cell fate, becoming the Z5 cell. This suggests that UNC-39 normally functions to suppress this division in wild-type worms. hmcS have branched processes, and the authors report branching defects in these cells in \textit{unc-39} mutants, that are similar to neuronal branching abnormalities.

The M myoblast normally divides to produce the sex myoblasts, coelomocytes and the body wall muscles (Sulston and Horvitz, 1977). Abnormal M mesoblast migration in \textit{unc-39} mutants, reported by Manson and Wood in 1990, was found to be insignificant by a later study (Yanowitch et al. 2004). However, the differentiation defects in M mesoblast are manifested in the defects in specification of cells that it gives rise to, for example, the lost of the M-derived coelomocytes, abnormal number of vulval muscle cells (from none to extra), and a variable loss of the M-derived body wall muscle cells (Yanowitch et al., 2004). Together, these data suggest a role for \textit{unc-39} in the specification of mesodermal cell fate.

In addition to mesodermal cell fate specification, \textit{unc-39} also affects ectoderm development in \textit{C. elegans}, which is reflected in defects in CAN, GLR-1-expressing interneurons and amphids. CANL and CANR are two neurons, born in the anterior region, that then migrate to the mid-body and each of them extends two processes – one anterior that extends to the head and
one posterior that runs to the end of the body. The migration, axon branching and pathfinding
of the CAN neurons are all affected in unc-39 mutants. CAN cell bodies often do not reach their
normal destination, and in many cases their axons are not fully extended, show abnormal
branching, or wander in abnormal trajectories (Yanowitch et al., 2004). In GLR-1-expressing
interneurons, which are glutamatergic neurons located in the anterior ganglion and are involved
in locomotion, unc-39 affects the pathfinding of their processes. Normally, these processes
extend to the ventral and dorsal nerve cords, as well as the never ring. In unc-39 background,
some processes extended laterally and ran along the body wall (Yanowitch et al., 2004). Finally,
sensory amphid neurons, located anteriorly, are also affected by unc-39. Normally, amphids
extend their dendrites to the nose, and their axons posteriorly to the ventral midline. In unc-39
mutants, these processes are often misguided and abnormally branched (Yanowitch et al., 2004).
Unlike the case in mesodermal development, the number of amphid neurons, CAN and GLR-1-
positive interneurons was unaffected, suggesting that UNC-39 plays a role in ectoderm
development after the post-mitotic cell division.

Interestingly, affected neurons in unc-39 mutants are anteriorly-derived or positioned.
Neurons born in the posterior and middle parts of the body do not seem to be affected in unc-39
mutants. For example, no defects were observed in the phasmid neuron, HSN, which is born in
the posterior region and migrates to the anterior, and two posterior neurons, PDE and PVM
(Manser and Wood, 1990; Yanowitch et al., 2004). Collectively, these studies suggest that UNC-39
is involved in specification of some anteriorly-derived post-mitotic neurons.
1.2  *C. elegans* as a model to study cell fate determination

1.2.1  *C. elegans* as model organism for genetic studies.

*C. elegans* is a free-living nematode, about 1 mm in body length for adults. In the wild, it feeds on bacteria found mostly in rotten fruits and vegetables. The establishment of *C. elegans* as a model organism began with the work of Syndey Brenner in 1970s. To date, it is one of the most well-established genetic models that affords many advantages as an experimental system for fundamental development and neurobiology research.

*C. elegans* is easily maintained in large population under the laboratory conditions. It has a short three-day life cycle during which a large number of progeny (~300 in a wild-type animal) is produced. This allows genetic experiments to be performed in a timely fashion. The body of *C. elegans* is transparent. This allows researchers to express fluorescent markers, such as GFP, in the cells of interest for the purposes of visualization of cellular morphology, or attach these markers to specific proteins in order to study their expression pattern, subcellular localization, etc. *C. elegans* are mostly hermaphroditic, while males arise spontaneously at a low frequency (0.1%) by non-dysjunctional chromosomal segregation during germ cell differentiation. This feature facilitates the ease of genetic manipulations as homozygous lethal mutations can be easily maintained in a heterozygous background through self-fertilization, and genetic mutations from different background can be combined by mating males to hermaphrodites.

*C. elegans* is highly amendable to forward genetic screening. Various chemical mutagens, such as Ethyl Methyl Sulfonate (EMS) are used routinely to introduce random mutations into the worm’s genome. The progeny of these mutagenized animals carry heterozygous mutations, which can be homozygoused simply by allowing them to self-fertilize and screening the progeny
of these animals for the phenotype of interest. This way, in the next generation recessive mutations can be easily isolated.

Identification of the mutations responsible for the mutant phenotype is also relatively easy, especially with the implementation of the whole genome sequencing (WGS) technology. The well annotated *C. elegans* genome sequence in the various genetic background, including the commonly used N2 background, used for the majority of mutant screens, and the CB4856 background, which differs from N2 by a number of SNPs and is utilized for mapping purposes, provide information for the reference genomes. Recent advances in combining fast mapping of *C. elegans* mutations and the whole-genome sequencing has exemplified the genetic advantage of the system. Briefly, the mutation is first mapped roughly to a region on a specific chromosome using SNP mapping and mapping with genetic markers (such as mutations that produce a visible and easily identifiable phenotype). Then, the entire genome of the animal is sequenced, the sequence is compared to the N2 background, revealing all mutations, and the candidates in the mapped region are then investigated to identify the causative mutation. The availability of the entire genomic sequence makes this approach possible.

*C. elegans* was the first multicellular organism whose genome was sequenced completely. For such a simple organism, *C. elegans* has a large number of protein-coding genes: 20,553 vs around 20,000 to 25,000 in humans (Wormbase WS232, 20012; International Human Genome Sequencing Consortium; 2004). Moreover, many genes have been conserved through evolution between the worm and the human. First insights into the function of many human genes have been made in *C. elegans*. For example, the first known conserved axon guidance cue Netrin was first discovered in *C. elegans* as UNC-6 (Hedgecock et al., 1990).
An important advantage of *C. elegans* system that is particularly relevant for my thesis work is the simplicity of its nervous system, which consists of only 302 neurons. The positions of the neurons and their connections are highly stereotypical. This allows for an accurate anatomic comparison between the wild type and a specific mutant that exhibits neuronal defects.

### 1.2.2 *C. elegans* nervous system development and organization

Of the 302 *C. elegans* neurons, 282 belong to the somatic nervous system and 20 form the pharyngeal nervous system. All neurons are divided into four categories, based on the general functions they perform. Sensory neurons receive information, such as temperature, chemical odorants, touch, etc. from the external environment and relay it to the interneurons. There are several “levels” of interneurons that ultimately synapse onto motorneurons to activate an appropriate behavioral response, such as backward movement upon touch to the nose. The last category of neurons, called polymodal, can perform functions of several categories. The majority of the sensory and interneurons are organized into several ganglia in the anterior of the worm, close to a synapse-rich region, termed the nerve ring, and in the tail. Motor neurons that drive locomotion are positioned along the ventral side of the animal and their processes are organized into two major tightly bundled fascicules called the ventral nerve cord (VNC) and the dorsal nerve cord (DNC). The synapses in *C. elegans* are formed *en passant*, meaning that they are organized in “swellings” along the axon, rather than at the axon terminals. The motor neurons are confined to the nerve cords and cannot extend to the muscles to form neuromuscular junctions. Instead, the muscles send their projections, called muscle arms, towards the neurons.

All *C. elegans* neurons have been grouped into 118 different classes, based on the topology and the patterns of the synaptic connections they make (White et al., 1986). Sulston et al.
performed cell lineage tracing by Normaski imaging of the developing *C. elegans* embryo and mapped its entire embryonic cell lineage (Sulston et al., 1983). *C. elegans* neurons are born at three developmental stages: during proliferation phase in embryogenesis, at the first larval stage, L1, and at the second larval stage, L2 (Sulston et al., 1983; Sulston and Horvitz, 1977). After the first several rounds of division of *C. elegans* zygote, six founder cells are produced: AB, MS, E, C, D and P4. Most worm neurons are derived from the AB founder cell. With each division, the daughter cell’s lineage name is given an additional letter, depending on the axis of division: a for anterior, p for posterior, etc.

1.3 The RID neuron as a model to study cell fate control

1.3.1 The cell lineage that gives rise to the RID neuron.

Apoptosis plays a critical role in specifying *C. elegans* cell fate. Multiple apoptosis events occur in the lineage that is of particular interest to my project, called Abalappa. The first apoptosis event occurs at ABalappaa. The second one occurs at ABalappaap, where the anterior daughter (ABalappaapa) becomes the RID neuron, whereas the posterior sister cell (ABalappaapp) dies shortly after the division of the precursor. The distinct fates of the RID and the RID sister cell arise from asymmetric cell divisions. This type of cell division, in which factors that control cell fate are distributed unevenly in the mother cell is common through development and serves as a way to introduce cellular diversity (Horwitz and Herskowitz, 1992).

1.3.2 RID neuron has a unique morphology and unknown functions

John White and his colleagues performed serial electron microscopy (EM) reconstruction of the *C. elegans* nervous system, and identified and mapped the connectivity of all neurons of the
nervous system (White et al., 1986). One neuron they called RID exhibits a unique feature: it is the only neuron that extends its neurite along the entire length of the DNC. With its cell body located on the dorsal side of the animal, in close proximity to the nerve ring, RID sends a ventral process that makes a loop back to the dorsal side of the animal, which then turns and extends posteriorly, all the way to the tail (Figure 2). This loop serves as the dendrite, where the RID neuron receives a number of chemical synaptic inputs from the several interneurons, such as PVC, AIN and RMG, and from a sensory neuron AVM. The same process makes a gap junctional connection with a very short process that originates from the RID cell body immediately before it turns posteriorly. Despite of extending along the entire length of the dorsal nerve cord, it was reported to make only a very small number of en passant synaptic connections with the hypodermal cells, muscle cells and the DD motor neuron (White et al., 1986). Based on the morphological observation, RID was proposed to be a unipolar neuron where a single process is compartmentalized into the dendritic and axonal regions.

**Figure 2: Schematic representation of the RID neuron.**

The initial EM reconstruction that was done by John White and his colleagues was incomplete, and the pattern of its synaptic connections was deduced from the partial reconstruction.
Despite its unique morphology and positioning of the axon, very little is known about the RID neuron, its function and the processes that govern its development.

1.3.3 HAM-1 regulates the developmental potential in asymmetric cell division of RID and its sister cell

One factor involved in routing the developmental potential to a surviving daughter cell through the asymmetric cell division of the RID precursor is HAM-1. HAM-1 is a novel protein, which has homologues of unknown functions in humans and mice, and a homologous stretch to the *Drosophila* Knockout protein, involved in innervations of muscles in the larvae (Frank et al., 2005; Hartmann et al., 1997). During development, HAM-1 affects cell division events that produce two daughter cells with distinct fates. There are five cell divisions known to be affected by HAM-1, namely the HSN/PHB, ADE/ADA, and PLM/ALN neuroblasts, as well as the precursors of RID and ADL neurons. Interestingly, in all five of these cell divisions, one of the daughter cells normally adopts a specific fate, while the other one undergoes apoptosis shortly after the division occurs (Sulston et al., 1984). For example, HSN/PHB neuroblast AB plapppap gives rises to a daughter cell ABplapppapa.that dies through apoptosis, and ABplappppapp that becomes the HSN/PHB precursor cell, which divides again to produce the HSN and PHB neurons. In *ham-1* mutants, 16-23% of worms produced additional HSN and 22-33% produced additional PHB neurons (Guenther and Carriga, 1996). These additional neurons result from transformation of the anterior sister of the HSN/PHB precursor, a neuron which dies in WT animals, into another HSN/PHB precursor in 21-33% of the embryos, which then produces additional HSN and PHB neurons (Guenther and Carriga, 1996). As mentioned previously, in addition to creating extra HSN and PHB neurons, loss of *ham-1* results in extra neurons in at least four other asymmetric cell divisions, including the lineage the gives rise to the RID neuron.
All of these cell divisions have two common features. First, these cells produce two daughter cells, one of which dies. Second, all HAM-1-affected divisions occur between 200 and 300 minutes post-fertilization. Consistent with a role HAM-1 promoting distinct fate of the daughter cells, HAM-1 is expressed in the dividing cells in a restricted pattern – it is localized to only one side of the cell in a crescent shape along the membrane. It begins expression in many cells at the onset of gastrulation and its expression ceases after 1.5-fold stage (Guenther and Carriga, 1996). Interestingly, blocking apoptosis is insufficient to recapitulate ham-1 phenotypes. Mutants defective in apoptosis fail to form additional HSN and PHB neurons: although the sister cell of the HSN/PHB precursor that is normally destined to die survived in these mutants, it does not proceed to the next division to differentiate into the HSN and PHB neurons. HAM-1 thus plays a role promoting the HSN/PHB precursor fate, instead of simply inhibiting apoptosis.

Together, these data suggest that ham-1 is responsible for distributing the developmental potential asymmetrically in a small subset of dividing neuroblasts, including the RID precursor cell. The authors propose that HAM-1 functions by tethering cell fate determinants asymmetrically to the surviving daughter cell.

1.3.4 Apoptosis in C. elegans

Programmed cell death, or apoptosis, is a highly controlled mechanism that maintains a balance between cell proliferation and death during metazoan development. There are many examples of apoptotic cell death regulating tissue morphogenesis, such as the removal of webbing between digits in the human embryo. C. elegans also uses apoptosis to eliminate specific cells, mostly neurons, during development. All cells that undergo apoptosis during development are known -
there are 131 of them, their identity is invariable, and the timing of cell death is highly stereotypical (Ellis and Horvitz, 1986). Apoptosis is divided into three recognized phases: the specification phase, when the cell receives a signal to undergo cell death; the killing phase, during which apoptosis is activated, and the execution phase, characterized by the disintegration of the cell, and the clearing of the cell corpses (Horwitz, 1999).

A core set of pro-apoptotic factors, ced-3, ced-4, and egl-1, are required for nearly all cell death events during *C. elegans* development (Ellis and Horwitz, 1986). Strong loss-of-function alleles of any of these genes result in survival of all cells that are normally destined to die (Ellis and Horwitz, 1986). In contrast, ced-9 is an anti-apoptotic gene, mutations in which result in embryonic lethality due to ectopic apoptosis in the cells that should have survived. egl-1 is the founding member of the pro-apoptotic Bcl-2 family; it contains a motif that mediates binding to the anti-apoptotic members of this family. ced-9, is a homologue of a human bcl-2 gene, which normally functions to prevent apoptosis (Hengartner, and Horvitz, 1994). ced-3 codes for a caspase (Alnemri et al., 1996), and ced-4, similar to the human Apaf-1, activates the caspase (Yuan and Hortwitz, 1992) to execute cell killing. In *C. elegans*, surviving cells do not express EGL-1, and CED-4 is held at the mitochondrial membrane by CED-9. When a cell is about to undergo apoptosis, egl-1 transcription is upregulated. EGL-1 binds to CED-9, which induces a conformational change in CED-9 and relieves CED-4 repression. CED-4 then relocates to the peri-nuclear membrane, where it oligomerizes and activates the CED-3 caspase.

Apoptosis is a tightly regulated process during embryogenesis. However, very little is known about the developmental cues and the mechanisms that are responsible for activating and inhibiting cell-division and lineage-specific apoptosis events. Dysregulation of apoptosis in humans leads to various disorders, including cancer and neurodegenerative diseases (Friedlander,
2003; Lowe and Lin, 2000). Therefore, research of developmental apoptosis in *C. elegans* could provide some insight into the mechanisms that underlie these various pathologies in humans.

In the sister cell of the M4 motor neuron, which is normally destined to undergo programmed cell death, the activation of *egl-1* transcription requires two transcriptional factors, EYA-1, a *C. elegans* ortholog of the *Drosophila* Eyes absent, and CEH-34, a member of the SIX family of proteins (Hirose et al. 2010). They form a protein complex that binds to the regulatory sequence in the *egl-1* promoter region, inducing its transcription and activating apoptosis in the M4 sister cell. Interestingly, in a genome-wide yeast two-hybrid screen (Li et al., 2004), a potential physical interaction between EYA-1 and the UNC-39/SIX5 transcription factor was reported. The physiological relevance of this interaction remains to be verified. However, given the involvement of EYA-1 and CEH-34 in co-activating apoptosis of the M4 sister cell, the EYA-1/UNC-39 interaction may suggest a possible function of EYA-1 and UNC-39 in activating UNC-39-lineage specific apoptosis events.

1.3.5 *lim-4* controls some, but not all aspects of RID terminal differentiation

Very little is known about the mechanisms that govern development of the surviving RID neuron. There are a handful of terminal differentiation genes that are expressed in the mature RID neuron, but most of them have a fairly broad expression pattern, and none is expressed exclusively by RID. Among them is *LIM-4*, a member of the LIM homeodomain LHX6/7 family transcription factor. *lim-4* is most homologous to the mouse *Lhx6* and *Lhx7*, as well as to the Drosophila *Arrowhead* genes.
Expressed in a subset of anteriorly located *C. elegans* neurons, including RID, LIM-4’s function appears to be cell context-dependent. For example, LIM-4 is expressed in a neuroblast that gives rise to two distinct sensory neurons AWB and ADF. In AWB, LIM-4 is required for all aspects of its terminal differentiation, such that in *lim-4* mutants, AWB cell fate is transformed to another sensory neuron AWC (Sagasti et al., 1999). However, in the ADF neuron, LIM-4 only affects specific aspect of its terminal differentiation, where it is only required for the expression of THP-1, an enzyme responsible for the biosynthesis of serotonin (Zheng et al., 2005).

LIM-4 is expressed in the RID neuron and controls certain aspects of RID terminal differentiation (Tsalik et al. 2003). In wild type animals, there are two splice forms of the serotonin receptor (*ser-2a* and *ser-2b*), which are also expressed by the RID neuron. In *lim-4* mutants, the expression of *ser-2b* is almost completely abolished, while the expression of the *ser-2a* splice form is largely unaffected in RID. Four other cell fate markers, *kal-1*, *zig-5*, *ceh-10* and novel putative neuropeptide receptor, C50F7.1, that are expressed in RID, also appear to be differentially regulated by LIM-4. KAL-1 and ZIG-5 markers exhibited either reduced or absent expression in *lim-4* mutants. CEH-10 expression was abolished completely. However, C50F7.1 expression was largely unaffected. It should be noted that the role of these RID markers in RID development and function is unknown. KAL-1 is required for axonal branching in AIY neurons (Bulow et al., 2002). ZIG-5, an immunoglobulin protein, affects the organization of the ventral nerve cord (Aurelio et al., 2003), while CEH-10 is a homeobox protein that regulates CAN and AIY neuron cell fate specification (Svendsen and McGhee, 1995). Taken together, LIM-4 controls only a subset of terminal differentiation features of the RID neuron. Based on the expression pattern of markers that are not affected, the morphology of the RID neuron cell body,
its axon and the presumptive synaptic patterns are unaffected in \textit{lim-4} mutants (Tsalik et al., 2003). This suggests that there are other, unexplored mechanisms that are higher in the hierarchy of the transcription factor cascade control the development of the RID neuron.
Results

1.4 Postembryonic laser ablation of the RID neuron affects, but does not abolish *C. elegans* locomotion

In order to visualize the RID neuron, I utilized a fluorescent reporter Pceh-10::GFP. Upon hatching, this marker shows bright and constitutive GFP expression in RID, CANL and CANR, AIY and an unidentified cell in the head (Figure 3). Except RID, none of these cells contribute their processes to the dorsal nerve cord, which allows for the unobstructed visualization of the RID neuron morphology.

![Visualization of the RID neuron morphology using a fluorescent Pceh-10::GFP reporter.](image_url)
To gain insight into the role of the RID neuron, Jyothsna Chitturi, another graduate student in the lab, performed ablations of the newly hatched L1 Pceh-10::GFP animals. She ablated the RID neuron with a focused laser beam, leaving all other cells intact. Mock ablations were also performed on the wild-type animals, where they were treated exactly the same way as ablated worms, without undergoing the actual ablations, as well as a control ablation of another neuron, AIY. I performed an automated, on-plate movement analysis of these ablated animals when they grew into adults. This analysis measured the velocity of animal’s forward and backward movements, as well as the propensity of movement by proportion of time the animal spent in forward or backward locomotion, or pausing.

RID-ablated animals showed a decrease in forward velocity, an increased number of pauses and backing episodes (Figure 4). Mock-ablated animals did not show this phenotype (Figure 4). Previously, Jyothsna analyzed the movement of the AIY-ablated animals in liquid using a thrashing assay, and showed that there were no movement defects (data not shown). This confirms that the process of ablation itself does not induce any locomotion defects and that the behavioral defects we see in RID-ablated animals in the on-plate movement assay were related to the functional loss of the RID neuron. These results suggest that while the RID neuron is not essential for locomotion, it modulates the mode and velocity of motor behavior. RID-ablated animals did not exhibit developmental or other obvious morphological defects (data not shown).
Figure 4: RID-ablated animals show a shift in velocity, compared to wild type. The histogram shows a decrease in forward velocity, increased instances of backing and pausing behaviour in RID ablated animals, compared to wild type and mock-ablated.

1.5 Identification of genes involved in the development of RID

1.5.1 A genetic screen for mutations affecting marker expression in RID

Together with Jyothsna Chitturi and Wesley Hung, we performed a forward genetics, visual screen for mutations that disrupt the development of the RID neuron, labeled by the $\text{Pceh-10}::\text{GFP}$. In total, we screened the progeny of ~6000 F1 animals, and isolated over 30 mutants that showed a full spectrum of defects in RID development, including mutants that completely
lack marker expression in RID, or have a faint GFP signal in this neuron, mutants with short or misguided RID process and with abnormal synapse morphology or number.

We grouped these mutants into five classes based on the RID phenotype (Figure 5). Class I consists of seven mutants that exhibit defective axon guidance of the RID neuron – short axon, no axon at all, or axon on the ventral side (Figure 5B). Class II includes two mutants in which the RID axon grows past its normal termination point and overgrows to the ventral side (Figure 5C). Figure 5D shows a zoomed image of a wild type RID axon with boutons. Class III has six mutants that have smaller boutons along the axon (Figure 5E). Six mutants from class IV have the opposite phenotype – enlarged boutons along the axon (Figure 5F). Class V has three mutants with reduced $\text{Pceh-10}\cdot\cdot\cdot$GFP fluorescence intensity in the RID neuron and three mutants that completely lack $\text{Pceh-10}\cdot\cdot\cdot$GFP expression in the RID neuron (Figure 5G).

After the screen, I focused on genetic analyses of Class I and V mutants. Through non-complementation tests among mutants with similar phenotypes, I determined that Class I and Class V mutants represent five and two genetic loci, respectively.
Figure 5: Phenotypic classes of mutants isolated from the screen.
A) Wild type animal, expressing Pceh-10::GFP.
B) Mutant Class I showing a mixture of axon guidance defects: no RID axon, short axon or ventral axon.
C) Mutant Class II with an axon that overgrows to the tip of the tail and to the ventral side.
D) Wild type RID axon, showing evenly spaced boutons.
E) Mutant Class III with a “few boutons” phenotype.
F) Mutant Class IV with enlarged boutons along the axon.
G) Mutant Class V with either no or reduced Pceh-10::GFP expression in RID.
1.5.2 Mapping and cloning of isolated mutations

I excluded severely paralyzed or uncoordinated animals from further investigation. Since RID ablated animals exhibit changes in locomotion pattern without abolishing the mobility, for mapping and cloning I selected three mutants among Class I and V - *hp701, hp689* and *hp724*, that exhibit similar locomotion phenotypes, hoping to uncover mutations that affect specifically the RID neuron. *hp701* has no visible *Pceh-10::GFP* expression in the RID neuron (Figure 5G); *hp689* has a very faint *Pceh-10::GFP* signal in RID (Figure 5G), while *hp724* has a short RID axon, which usually stops around mid-body (Figure 5B).

*hp701* is an allele of *unc-39*, a gene that codes for a homeodomain transcription factor in the Six4/5 family (Yanowitz et al., 2004). *hp701* was mapped to chromosome V between 5.84 and 6.87cM, where *unc-39* resides. A known allele of *unc-39*, *e257*, which harbors a R203Q missense mutation in *unc-39*, failed to complement *hp701*. The RID phenotype in *hp701* was fully rescued with a fosmid that contained a wild-type copy of *unc-39*, and a PCR fragment that contains only the *unc-39* genomic fragment. Finally, whole genome sequencing (WGS) identified a C to T substitution that leads to a P298L missense mutation in *unc-39* (Figure 6B). UNC-39 exhibits the highest sequence homology of its conserved SD and HD to the human SIX5 and Drosophila Six-4 proteins (Figure 6B).

*hp724* encodes *vab-8*, which codes for an atypical kinesin-like protein known to be involved in axon extension and migration of the CAN neuron (Wolf et al., 1998). I mapped *hp724* to chromosome V between 3.08 and 5.84cM, where *vab-8* resides. WGS revealed several mutations on chromosome V, one of which is an early stop codon in *vab-8* gene. A fosmid
harboring the \textit{vab-8} genomic sequence fully rescued \textit{hp724} defects, confirming that the nonsense mutation in \textit{vab-8} is the causative to \textit{hp724}'s RID defect.

\textit{hp689} encodes \textit{lim-4}, a LIM family transcription factor. \textit{hp689} exhibits a similar RID phenotype as reported for \textit{lim-4} mutants (Tsalik et al., 2003). Both \textit{hp689} and \textit{lim-4} reside on the X chromosome, and \textit{hp689} failed to complement \textit{lim-4(e257)}. Moreover, a fosmid bearing a wild-type copy of \textit{lim-4} rescued the mutant phenotype.

After identification of \textit{unc-39}, \textit{lim-4} and \textit{vab-8} as genes bearing mutations responsible for RID defects, I focused on \textit{hp701}, which does not have any visible expression of \textit{Pceh-10::GFP} in the RID neuron (Figure 5G) for further analyses. Based on the phenotype, \textit{unc-39} appears to function at a highest position in the regulatory hierarchy of the RID neuron among the three mutants.

1.6 UNC-39 specifies RID and other neurons in the ABalap lineage

1.6.1 UNC-39 is required for the expression of all known RID fate markers

To determine if \textit{unc-39} affects all, or a subset of RID fate markers, I crossed both alleles (\textit{hp701} and \textit{e257}) of \textit{unc-39} into strains carrying various fluorescent reporters known to be expressed in RID, driven by the \textit{kal-1}, \textit{zig-5}, \textit{mod-1} and \textit{ser-2} promoters (Tsalik et al., 2003). In addition to \textit{ceh-10}, all currently known RID cell fate markers fail to be expressed in \textit{unc-39} mutants, with nearly 100% penetrance (Figure 7).
Figure 6: Identity and functions of unc-39, lim-4 and vab-8 - genes involved in aspects of RID development.
A. Summary of hp701, hp689 and hp724 mapping, mutant phenotypes and known gene functions.
B. Protein structure ofUNC-39 and its alignment to the Drosophila and human homologues. Percentages indicate homology of the conserved domains between respective proteins. Percentages below the human Six-5 indicate the homology of its domains to C. elegans UNC-39.
Figure 7: Loss of RID cell fate markers in two alleles of unc-39. The positions of some unidentified neurons, expressing cell fate markers examined in this experiment, are changed. Arrows point to RID neuron in wild type animals, circles denote the position where RID normally resides, but is absent in the mutants.

1.6.2 unc-39 functions at multiple points in the ABalap lineage

I examined the effect of unc-39 mutation in other neurons that express the Pceh-10::GFP marker. AIYL and AIYR share all known RID terminal differentiation markers, including ceh-10, kal-1, zig-5 and mod-1. Moreover, unc-39 is expressed in these neurons (discussed in the next section). However, in sharp contrast to the complete loss of these markers in RID, neither AIYL nor AIYR are affected at all by unc-39 mutations (data not shown). AIYs and RID are separated very early on in cell lineage: RID is derived from the ABa, and AIYL/AIYR the ABp lineage, respectively. This observation suggests that UNC-39 may differentially affect neurons of
different cell lineages. Using the $\text{P}_{\text{ceh-10}}::\text{GFP}$ and other markers, I examined whether the effect of UNC-39 on neurons correlates with their lineage distance from RID.

I first compared the morphology of three ABalap lineage neurons – RID, CANL and CANR. CANL is separated from RID and CANR upon the first round of cell division after ABalap, while RID and CANR are more closely related, with their lineages are separated after three rounds of division after ABalap (ABalappa) (Figure 8). In both $\text{unc-39}(hp701)$ and $\text{unc-39}(e257)$ mutants, only RID neuron completely lacked the $\text{P}_{\text{ceh-10}}::\text{GFP}$ marker expression. However, although both CANL and CANR neurons express the marker, their soma exhibited aberrant positions, and their axon morphology was aberrant. CANR had a more penetrant phenotype than that of CANL, and $e257$ had a greater effect than $hp701$. For CANL, roughly 50% of the animals in $e257$ allele, and in 10% of $hp701$ animals exhibited aberrant soma position and/or axon extension. For CANR, the penetrance was 90% in $e257$, and 40% in $hp701$ (Figure 9B). Interestingly, another set of neurons, RIAL and RIAR interneurons (ABalapaapaa and ABalaappppaa, respectively) are affected differently by the $\text{unc-39}$ mutation. In both neurons, $\text{unc-39}$ induces decreased expression of $\text{glr-3}::\text{GFP}$ reporter, which is expressed exclusively in these neurons. However, the morphology, position and the number of RIA interneurons seem to be unaffected (Figure 9C). These data support a notion that neurons that are more closely related to RID in lineage are likely more severely affected by $\text{unc-39}$ and that the effect of $\text{unc-39}$ is context dependent.

Lineage distance alone, however, does not fully explain the differential effect of $\text{unc-39}$. Two neurons that weakly expressed $\text{P}_{\text{ceh-10}}::\text{GFP}$, ALA and RMED, were unaffected by $\text{unc-39}$ mutations (Figure 9A). The precursor of both neurons is separated from the precursor of RID and CANR after two round cell division after ABalap (Figure 8). Additional markers for these
neurons, including inIs717 (Pida-1::GFP) that labels multiple neurons including ALA, and 
juIs76 (Punc-25::GFP), which labels all GABAergic neurons including RMED, were also 
normally expressed in ALA and RMED in unc-39 mutants. The significantly lower expression 
level of Pceh-10::GFP in these neurons when compared to RID, CANR and CANL may indicate 
a less prominent role of UNC-39 in their fate determination, and may contribute to a lack of 
phenotypes in unc-39 mutants.

Together, these data suggest that UNC-39’s effect may be lineage-dependent, most 
prominently at multiple points in the ABalap lineage to specify cell fate, establish proper 
migration and/or axon extension.
Figure 8: UNC-39 functions at multiple point in the ABalap lineage. Branches of the lineage and cells that are affected by unc-39 mutations are highlighted in red; those that have been shown to be unaffected are in green. Adapted from Sulston et al., 1983.
Figure 9: UNC-39 affects CAN and RIA neurons, but not ALA and RMED.
A: Two neurons, ALA and RMED, from the ABalappaa lineage are not affected by unc-39 mutations.
B: CANL from ABalapaaapa, and CANR from ABalappapp lineages are affected by unc-39 mutations.
C. RIA interneurons show decreased intensity of fluorescence of glr-1::GFP marker.
1.6.3  *unc-39* does not affect other UNC-39-expressing neurons

*unc-39* expression is not restricted to *Pceh-10::GFP* expressing neurons (Figure 11). To determine if UNC-39 plays a role in UNC-39-expressing neurons that do not express the *Pceh-10::GFP* marker, I examined the phenotype of the ventral cord motor neurons, since my transcriptional reporter of *unc-39* showed expression in these neurons (Figure 11D).

I crossed *unc-39(hp701)* into various markers for the ventral cord motor neurons. For GABAergic motor neurons, I used three markers, *juIs76* (*Punc-25::GFP*), which labels throughout the neuron to examine its general morphology, *juIs1* (*Punc-25::SNB-1::GFP*) and *hpIs61* (*Punc-25::UNC-10::GFP*), both labeling the presynaptic termini of these neurons to allow assessment of their synapse morphology. Expression patterns of these markers showed no qualitative differences between the wild type animals and *unc-39* mutants (Figure 10A). Quantitatively, the number and morphology of cell bodies and their commissures were also unaffected: both *juIs76* and *unc-39(hp701)*; *juIs76* animals had a correct number of GABAergic neurons (19, n=5), with comparable number of commissures (16.4±0.5 for *juIs76* versus 16.6±1.67 for *unc-39(hp701)*; *juIs76*, p=0.8, n=5) (Figure 10B). For cholinergic motor neurons, I used *nuIs152* (*Punc-129::SNB-1::GFP*), which labels the soma and presynaptic termini of the DA subclass cholinergic motor neurons. I did not observe any effect on the synapse morphology of these cholinergic neurons in both *unc-39(hp701)* and *unc-39(e257)* mutants (Figure 10C). These results further support that UNC-39’s function is cell-lineage dependent.
Figure 10: *unc-39* does not have an effect on GABAergic and cholinergic neurons.

A. Synaptic GABA marker is normal in *unc-39* mutant.

B. Wild type and *unc-39* worms had 19 cell bodies each (n=5), 16.4 and 16.6 commissures, respectively (n=5, p=0.8). p values were calculated using the Mann-Whitney U test.

C. DA type cholinergic synapses are unaffected by *unc-39*. 
1.6.4 UNC-39 is transiently expressed in RID

In order to gain insight into which tissues unc-39 functions in, I generated a fluorescent unc-39 transcriptional reporter. This construct contains a 5kb fragment upstream of unc-39 coding sequence, which includes the entire unc-39 promoter element from the minimal unc-39 genomic rescue fragment, and the first 29bp of the coding sequence fused in-frame with a GFP reporter. The number of cells expressing this reporter decreases with progression from embryonic to larval to adult stage. In embryos, it is expressed in many cells, including in the RID neuron (Figure 11). In newly hatched larvae, it is expressed in many anterior neurons including RID and AIY, body wall muscles, ventral cord neurons and posteriorly located CAN neurons. However, by the adult stage, many of these cells, including RID, lose UNC-39 expression. This suggests that unc-39 is likely required during early development of the RID neuron.

1.6.5 RID neuron is likely absent in unc-39 mutants

In order to test whether the absence of the RID cell fate marker expression is due to the absence of a neuron, or, a mis-specification of its cell fate, I performed electron microscopy analysis of the C. elegans dorsal nerve cord (DNC) (Figure 12). First, I analyzed a continuous series of 420 cross-sections of the wild type DNC sections (each section 70nm thick). Based on the positions of the neurons in the dorsal nerve cord, and the patterns of their synaptic connections, I identified two RID candidates that fit the described position of the RID neuron, and behave similarly (Figure 12B). Both run next to the hypodermis in close proximity to the DD motor neuron, and have a few synapses. The 3D reconstruction of both candidates shows a similar pattern of synapses that alternate with the synapses of the DD motor neuron (Figure
12C). In *unc-39(hp701)*, one of these candidates is absent (Figure 12D). I am currently performing immuno-EM experiments with wild-type animals that carry the *Pceh-10::GFP* marker to verify the identity of RID, and to confirm that the missing axon corresponds RID. My current results, however, tentatively indicate the loss of the RID neuron in *unc-39* mutants.
Figure 11: UNC-39 expression pattern.
A-C. UNC-39 is expressed in RID during embryogenesis and early larval stages, but not in adults. Other *unc-39*-expressing cells include unidentified anterior neurons, AIY, CAN, neurons in the ventral nerve cord (D) and muscle cells (E).
Figure 12: Identification of the RID neuron using electron microscopy analysis of the dorsal nerve cord.
A. Schematic representation of serial cross-section preparation. For wild type, 420 serial sections were prepared and analyzed, for *unc-39*, 150 sections were made.
B. Electron micrograph of the wild type DNC showing body wall muscle, hypodermis, and neuronal processes. RID candidates are labeled 1 and 2, DD is an inhibitory motorneuron.
C. Serial 3D reconstruction of the potential RID candidates and the DD motorneuron.
D. Electron micrograph of the DNC of *unc-39* mutant. DA/DB are cholinergic neurons, DD and VD are GABAergic, 1 behaves as one of the two RID candidates seen in wild type.
1.6.6  **unc-39** mutants exhibit locomotion defects similar to RID-ablated animals

If **unc-39** mutants lose the RID neuron (either anatomically, or functional wise), it should share at least some behavioral defects to RID-ablated animals. I compared the locomotion profiles between **unc-39** mutants, mock-ablated, wild-type, and RID-ablated ‘wild-type’ animals. Wild-type animals moved mostly forward with an average velocity of 7.8 pixels/sec. RID-ablated wild-type animals’ average forward velocity was significantly decreased (3.9 pixels/sec, p=0.0004 compared to wild-type animals). A similar decrease of forward velocity (3.96 pixels/sec) was observed in **unc-39**(*hp701*) animals (p=0.0004 versus wild-type animals) (Figure 13A). Moreover, both RID-ablated animals and **unc-39**(*hp701*) mutant spent a larger proportion of time moving backwards and pausing, when compared to wild type animals (Figure 13B). The striking similarity in the locomotion phenotypes of RID-ablated and **unc-39** animals suggests that at least some aspect of UNC-39’s role in locomotion may be mediated through its function in the RID neuron. This result also supports at least a functional loss of the RID neuron in **unc-39** mutants.

2.4.  **unc-39** does not regulate RID development through apoptosis

How does the loss of UNC-39 function lead to the loss of RID? One possibility is that UNC-39 may prevent ectopic apoptosis in RID upon the precursor giving rise to RID and its sister cell (which subsequently activates apoptosis). To test this hypothesis, I examined the status
of the RID neuron in *ced-3;unc-39* and *ced-4;unc-39* double mutants, in which all apoptosis is blocked. If the loss of RID neuron in *unc-39* mutant results from the ectopic apoptosis, RID should be restored in these double mutants.

With the *hpIs202 (Pceh-10::GFP* marker), I first showed that blocking apoptosis does lead to the survival of the RID sister cell. The “two RID” phenotype was observed in 95% of the *ced-4*, and 91% of *ced-3* mutants. Therefore, blocking apoptosis in the RID sister not only promoted its survival, but was also sufficient for it to exhibit the RID fate (Figure 14A and B). However, 100% of *ced-3;unc-39* and 90% of *ced-4;unc-39* double mutants still missed the marker expression in RID (n=10) (Figure 14A and B). This suggests that *unc-39* does not simply promote RID survival by blocking apoptosis.

### 2.4.1 The surviving RID sister in *ham-1* mutants requires UNC-39 to exhibit the RID fate

As reported, using the *hpIs202* marker, I found that 80% of *ham-1(n1438)* mutants also exhibit a “two-RID” phenotype (Figure 15). *ham-1* was hypothesized to regulate the asymmetric division of the RID precursor, allowing strict allocation of the ‘RID determinants’ to only its anterior progeny. In the *ham-1* mutants, an equal distribution of the ‘RID determinants’ was proposed to lead to the survival and expression of RID fate in the RID sister.

To test whether in *ham-1* mutants RID and the survived RID sister require UNC-39 for expressing the RID fate, I examined the phenotype of *ham-1;unc-39* double mutants. As the case for *ced-3* and *ced-4*, *ham-1* failed to suppress *unc-39* phenotype – there was still no visible RID
in 100% of the double mutants (Figure 15). To ensure that the RID sister cell was completely prevented from activating apoptosis, I further examined the phenotype of *unc-39; ham-1; ced-4* triple mutants, where apoptosis is blocked and the cell fate determinants are distributed into both daughter cells. 100% *unc-39; ham-1; ced-4; hpIs202* triple mutant still did not have any visible RID neuron (n=10) (Figure 15). These results strongly argue against the hypothesis that the absence of RID neuron in *unc-39* mutants is due to aberrant cell death, either caused by ectopic apoptosis, or aberrant cell division of the RID precursor.
Figure 13: *unc-39* animals exhibit locomotion defects similar to RID-ablated worms.

A. Histogram, showing a shift in forward velocity of RID ablated and *unc-39*(hp701) animals.

B. Average forward velocity is decreased in RID ablated and *unc-39*(hp701) animals (n=10, p\(<0.005")). p values were calculated using the Mann-Whitney U test.

C. RID ablated and *unc-39* (hp701) worms spend a larger proportion of time paused and moving backwards, than wild type (n=10).
Figure 14: UNC-39 is not involved in apoptosis.
A. *ced-3* and *ced-4* mutants do not suppress *unc-39* RID phenotype. Arrows point to RID cell bodies, circles denote absence of RID.
B. Percentage of animals having no, 1, or 2 RID neurons. 95% of *ced-4* and 91% of *ced-3* animals had 2 RID neurons. 90% of *ced-4;unc-39* and 100% of *ced-3;unc-39* worms were missing RID (n=10).
Figure 15: UNC-39 is required for differentiation of RID neuron in ham-1 mutant.
A. ham-1 mutant does not suppress unc-39 RID phenotype. Arrows point to RID cell bodies, circles denote absence of RID.
B. Percentage of animals having no, 1, or 2 RID neurons. 80% of ham-1 animals had 2 RID neurons. 100% unc-39 animals were missing RID and 100% of ham-1;unc-39 worms were missing RID (n=10).
2.4.2 Does UNC-39 function in RID precursors to specify RID?

Most transcription factors function cell autonomously to determine the cell fate. To determine where UNC-39 is functionally required in the RID lineage to allow the expression of the RID fate, I performed tissue-specific rescue experiments. Because all known RID reporters require UNC-39 for expression, I could not specifically express UNC-39 using any known RID-lineage specific promoter to drive its expression. Alternatively, I generated a panneuronally expressed \textit{unc-39} cDNA expression construct (\textit{Punc-119-UNC-39::GFP}), and tested its ability to rescue of the RID phenotype.

This construct was toxic when injected at regularly used concentration (~40ng/uL). I was only able to obtain several transgenic lines once the concentration of the plasmid was reduced to ~2ng/uL. No rescue of \textit{unc-39} phenotype was observed in these transgenic lines. But, at this concentration, UNC-39::GFP signal from the transgene was so faint that I was unable to determine if it was properly expressed in the RID lineage.

This panneuronal promoter is constitutively expressed during development. Because \textit{unc-39} transcriptional reporter is transiently expressed in embryos and newly hatched larvae, and is rapidly suppressed in later stages, the toxicity of this construct indicates the necessity to suppress UNC-39 expression during late larvae and adult stages.
2.4.3 Ectopic expression of *unc-39* in mature neurons is insufficient to induce activation of the RID fate markers.

Since preventing apoptosis does not suppress *unc-39*, the absence of RID and all RID terminal differentiation markers suggest that RID neurons are either not born, or the derived ‘neuron’ fails to activate all terminal selector genes for RID in *unc-39* mutants. The latter possibility would imply that UNC-39 acts as a master control gene to specify all aspects of RID cell fate through activating downstream effectors.

An approach to test the master gene hypothesis is to examine whether an ectopic expression of UNC-39 in cells that do not express the RID terminal differentiation markers is sufficient to convert fate. The success of this approach was demonstrated in a previous study (Altun-Gultekin et al. 2001), where an ectopically expressed transcriptional factor *ttx-3* by a constitutive panneuronal promoter (*Punc-119*) led to the ectopic activation of two AIY fate markers, *ceh-23::GFP* and *sra-11::GFP*, in the RID neuron.

Because the constitutive panneuronal expression of UNC-39 is toxic to transgenic animals, I induced the expression of the *unc-39* cDNA, tagged with an RFP reporter, in six pairs of mechanosensory neurons (*Pmec-4*), AVM, PVM, ALMR, ALML, PLMR, PLML, which mediate the touch response in *C. elegans*. *Pmec-4-UNC-39::RFP* is robustly expressed in these neurons without causing lethality to transgenic animals. Three RID markers, *Pceh-10::GFP*, *kal-1::GFP* and *mod-1::GFP*, when crossed into the transgenic animals, all failed to exhibit any obvious ectopic expression in these neurons (data not shown).

It is possible that the lineage of *Pmec-4* expressing neuron is too distant from *unc-39-* and *ceh-10*--expressing neurons to prevent UNC-39-mediated fate transformation. *Pmec-4-*
expressing neurons arise from a lineage that separates very early on from the RID lineage after the AB and ABa division. If UNC-39 indeed functions as a component of the protein complex to activate transcription of all target genes, these touch neurons may not express the appropriate partners of UNC-39 to induce fate transformation. To test this possibility, I used the Prig-3 promoter, which is expressed in AVAL and AVAR and a small number of anterior neurons, to ectopically express UNC-39. The AVA interneurons are more closely related to RID lineage-wise: AVAR separates from that of RID after ABala division, whereas AVAL separates from RID after ABal. There was still not ectopic activation of RID markers in AVAs or other neurons that express Prig-3-UNC-39::RFP.

These results, while are not entirely conclusive, would favor the possibility that UNC-39 may not function as a master control gene for RID fate determination, but instead, may affect the birth of the RID, or its precursors.
2 Discussion

2.1 A genetic screen for mutations affecting RID neuron development

We have chosen the RID neuron as a model to gain insights into the mechanisms that control the terminal neuronal differentiation for several reasons. First, despite of being the only neuron that extends a process along the entire length of the dorsal nerve cord, virtually nothing is known about its identity, development and function. Because RID was observed to make a few synapses with body wall muscles, it has been proposed to be a motor neuron. However, these synapses are fairly small and sporadic. Its reconstruction, in particular the synaptic output pattern, was partial, and its identity and physiological functions remain to be determined.

Second, RID has quite a unique and interesting morphology. It has a single process that becomes compartmentalized, and functions both as a dendrite and an axon. Intrigued by such an unusual morphology and the fact that so little is known about its development, we set out to uncover new mechanisms responsible for this process.

There is no known marker expressed exclusively in RID, but the hplIs202 (Pceh-10::GFP) marker that we generated for this study is sufficiently restricted to allow the morphological analyses of the RID neuron, since none of the other neurons that express this marker are positioned in close proximity to RID cell body or its process. The expression of hplIs202 is activated in the RID precursor, ABalappaap, after the 200-cell stage embryos (unpublished result from our collaborator, Ralf Schnabel’s laboratory), and is maintained in the RID neuron throughout development. RID axon is fully extended by the L1 stage and grows with the animal.
In addition to RID and its precursors, the marker is activated in ALA neuron at the L1 stage, whose cell body is in very close proximity to RID, however its expression dims at later developmental stages, making visualization of RID unobstructed.

Using this marker, we screened mutagenized animals at the young adult stage in order to catch mutations that affect both early and later stage of its development. Upon the completion of the screen, we selected mutants that did not have any apparent behavioral phenotypes, such as severely uncoordinated animals. Since our RID ablation experiments have demonstrated that a specific loss of the RID neuron impairs, but does not abolish locomotion, by selecting mutants with similar behavioral phenotype as ablated animals, we were more likely to identify specific regulators of RID development, rather than genes that have pleiotropic effects.

2.2 RID neuron is likely missing in *unc-39(hp701)* mutants

The absence of a terminal differentiation marker expression may indicate the absence of the cell, or a loss of its unique identity. Current results from my studies favor the possibility that RID neuron is missing in *unc-39* mutants.

First, I have examined all known RID makers in *unc-39* mutants, and none of them were expressed in RID. I attempted to use a panneuronal marker to determine whether the RID neuron is still present in *unc-39* mutants. The result of this experiment was inconclusive, since too many neurons are positioned close to where RID should be, obstructing the identification of RID. Indirect evidence that RID might be missing in *unc-39* mutant comes from the analysis of LIM-4 expression pattern. Normally found in RID, our *lim-4* reporter is not observed in *unc-39*
background. Since LIM-4 is responsible for turning on multiple terminal differentiation markers in RID, its absence suggests that either the neuron is not there, or its fate is dramatically transformed. Another transcription factor that regulates RID development is UNC-3, which has a domain homologous to immunoglobulin (Ig). unc-3 mutant was recently shown by our collaborator to have a ‘two-RID’ phenotype through preventing apoptosis in the RID sister cell (Wang and Xun, unpublished). An unc-3 fosmid reporter suggests that UNC-3 expression is turned on prior to the division of the RID precursor (ABalappaap) and fades after the death of the RID sister (Wang et al., unpublished). I am currently using this fluorescent unc-3 fosmid reporter to monitor cell division of the RID precursors, and whether this survival-regulating transcription factor is expressed in RID in unc-3 background.

Second, my ongoing EM analysis points towards the possibility of a complete absence of RID. Because the initial EM reconstruction of the RID neuron and the described synapse pattern was incomplete (White et al., 1986; our unpublished data), first I needed to identify reliable landmarks of the RID axon in wild-type animals. Through tracing a series of the dorsal nerve cord cross sections of wild-type animals, I identified two candidates that fit the description of the RID axon. Both run along the hypodermis, close to the DD motor neuron and have few synapses. In parallel, my structural EM analyses for unc-39(hp701) animals showed that one of the RID candidates was missing throughout the serial sections. To definitively conclude that RID neuron is missing in unc-39 mutants, I need to further distinguish the RID axon from the two candidates in wild-type animals. To this end, I am in the process of immuno-EM studies using transgenic animals expressing the Pceh-10-GFP marker. Results from this experiment will allow me to determine if the missing axon in unc-39 corresponds to the RID axon.
2.3 The missing RID neuron in *unc-39* does not result from ectopic apoptosis

A potential loss of the RID neuron in *unc-39* mutants raises a possibility that it undergoes cell death in the absence of UNC-39. In the cell lineage that gives rise to the RID neuron, two apoptosis events occur: one in the sister cell of the immediate RID precursor (ABalappaaa), and the other in the RID sister (ABalappaapp) soon after either is born. Indeed, when apoptosis is blocked in *ced-3* and *ced-4* mutants, I observed two RID neurons, both expressing the Pceh-10::GFP marker, with proper RID soma morphology and position. Normal axon morphology of the second RID neuron was also observed in at least 30% of *ced-3* and *ced-4* mutants. This value might be an underestimation, since due to their close positions, it is often difficult to discern the morphology of both neurons clearly. The activation of apoptosis in the RID sister requires the UNC-3 transcription. In *unc-3* mutants, two-RIDs with normal soma and axon morphologies were also observed (Wang and Xun, unpublished communications). Therefore, the RID sister (ABalappaapp) has an equal potential to differentiate as RID, and whether to turn on apoptosis is the key process that differentiates their destinies.

The presence of two properly differentiated RID neurons in *ced-3* and *ced-4* mutants raises doubts on HAM-1’s proposed role in asymmetric division of the RID precursor. If HAM-1 is truly responsible for diverting developmental potential to the surviving RID cell, as hypothesized in a previous study (Guenther and Garriga, 1996), then in apoptosis-defective mutants, the second RID neuron should not differentiate properly, presumably because all the cell fate factors have been directed to the normally surviving cell.
Because RID and its sister, which undergoes apoptosis, have equal potentials, the simplest explanation for the absence of RID is that the default program for both RID and its sister cell is apoptosis, but UNC-39 actively suppresses apoptosis in the surviving RID. However, RID is not restored in \textit{ced-3;unc-39}, \textit{ced-4;unc-39}, \textit{ham-1;unc-39}, and \textit{ced-3;ham-1;unc-39} mutants. Moreover, the loss of function of UNC-3, a transcription factor that is specifically required to activate apoptosis in RID sister (Wang and Huang, unpublished results), also failed to bring back RID in \textit{unc-39} mutants. Together, my current results strongly argue against a role of UNC-39 in protecting RID from ectopic activation of apoptosis.

These negative results raise an alternative possibility that UNC-39 may function even earlier during RID differentiation, for an example, during precursor cell division to give rise to the RID precursor, prior to the terminal differentiation of the RID neuron. If the RID precursor is not even born in \textit{unc-39} mutants, \textit{ham-1}, \textit{unc-3}, \textit{ced-3} and \textit{ced-4}, all of which only manipulate the fate of its progenies, would not be able to restore RID. How each cell lineage determines how many rounds of cell division prior to activating terminal differentiation is not understood. In this case, UNC-39 may function as a lineage-specific regulator for cell division. This hypothesis also explains why an ectopic expression of UNC-39 in both lineage-distant (P\textit{mec-4}-expressing) or more closely related (P\textit{rig-3}-expressing) neurons, was insufficient to induce cell fate changes. If UNC-39 promotes precursor cell division, it would not result in induction of cell fate determinants in already programmed neurons.

If UNC-39 functions as a master control gene to activate downstream transcription factors that control specific aspects of RID terminal differentiation, a restored expression of these downstream factors should bypass the requirement of UNC-39, and be sufficient to at least partially restore RID differentiation.
Currently, I am examining whether restoring LIM-4 in *unc-39* mutants is sufficient to partially restore RID fate. LIM-4 is required for multiple aspects of RID fate differentiation. If RID is born and present, but unable to express the RID fate in *unc-39* mutants, *Punc-39*-driven LIM-4 should allow this neuron to express some RID specific markers in *unc-39* mutants. If restoring LIM-4 expression in the RID precursor cells is sufficient to activate *Pceh-10*, or other RID terminal markers, it would support the hypothesis that UNC-39 has a direct role in establishing RID cell fate through activating transcription of terminal differentiation markers. If not, it supports the role of UNC-39 in giving rise to RID precursor via division. I am also trying to identify lineage markers that give rise to RID precursor, and does not depend on UNC-39 for expression. This will allow me to directly trace the RID lineage in *unc-39* mutants. Lastly, I can test mutations that induce cell division, such as *let-60/ras*, for the ability to suppress the missing RID phenotype in *unc-39* mutants.

2.4 UNC-39 does not affect the development of motor neurons

If UNC-39 is required for cell division and/or differentiation that gives rise to the RID neuron, it may function as a general regulator in all UNC-39 expressing cells, or a lineage-specific regulator. My *unc-39* transcriptional reporter analysis has revealed its expression in multiple motor neurons in the ventral nerve cord, indicating its potential presence in both GABAergic and cholinergic motor neurons. However, *unc-39* mutants exhibit normal number and morphology of the VD and DD type GABAergic motor neurons, and the DA type cholinergic neurons. I need to further examine the morphology of remaining cholinergic motor neurons, including the DB, AS, VC, VA and VB classes. Together with the specific loss of RID
among multiple Pceh-10-GFP expressing neurons, my current results favor the possibility that UNC-39 functions in a lineage-specific fashion.

2.5 *unc-39* expression needs to be tightly regulated

The dynamic expression pattern of *unc-39* supports its critical role during early development. The number of cells expressing the *P.unc-39::GFP* transcriptional reporter decreases drastically from embryos to adults. Many cells only transiently express the marker during embryogenesis. The expression of the marker in RID neuron is maintained in newly hatched L1 larvae, but gradually diminished afterwards. This is consistent with both the master control gene hypothesis – UNC-39 activates a cascade of terminal selectors during early development, and the downstream terminal selectors are sufficient to determine and maintain the specific cell fate, as well as the cell cycle regulator hypothesis – UNC-39 promotes precursor division, and prevents premature activation of terminal differentiation.

Several lines of indirect evidence suggest that UNC-39 expression needs to be actively repressed after its initial activation to ensure proper development. First, *C. elegans* is very sensitive to UNC-39 overexpression. An overexpression of either a fosmid harboring the *unc-39* gene, or a plasmid harboring only the *unc-39* genomic fragment, induced lethality in transgenic animals. Only when I diluted them to ~2ng/uL (about 1/20 of the standard concentration for standard transgenic studies), I was able to get a very small number of transgenic lines and they fully rescued the RID phenotype in *unc-39* mutants. Interestingly, in the translational reporter rescuing line, an ectopic expression of the *P.ceh-10::RFP* marker was observed in body wall muscles, a tissue that also expressed *unc-39*. In wild-type animals, body wall muscles do not
express the Pceh-10-GFP reporter during any developmental stages. Muscles and neurons are separated from the very first zygotic division, yet other Punc-39 positive neurons do not turn on the Pceh-10-GFP reporter. These results suggest that the lethality caused by an overexpression of UNC-39 may result from neuronal lineages. A sustained UNC-39 overexpression in the muscle lineage is sufficient to induce ectopic ceh-10 marker expression, but is less toxic to the animal.

Another indication of the necessity of temporal control of unc-39 expression came from the failure to rescue the RID phenotype in unc-39 mutants using a constitutive panneuronal promoter (Punc-119). As the case for the endogenous unc-39 genomic fragment, I had difficulty obtaining transgenic lines with this construct. Even when I reduced the DNA concentration of the construct by 50 fold to obtain a rare transgenic line, it did not restore Pceh-10-GFP expression in unc-39 mutants. Because the DNA concentration was so low, I could no longer determine if the transgenic array was even expressed in RID. These results suggest that the viability of the animal and the proper development of the RID neuron require a tight temporal control of UNC-39 activity. This would be in line with the hypothesis that the presence of high dose of UNC-39 promotes precursor division and prevents their terminal differentiation.
2.6 Does UNC-39 function cell-autonomously?

Many, but not all transcription factors act cell-autonomously. To determine if UNC-39 functions in the RID lineage to regulate RID development, I have performed both tissue and cell-specific rescue experiments in unc-39 animals. Unexpectedly, C. elegans’ sensitivity towards the level and temporal regulation of UNC-39 activation has made this supposedly standard experiment challenging.

To further investigate where unc-39 is required for normal RID development, I plan to perform mosaic analysis using a fosmid-based unc-39 translational reporter (unc-39fosGFP). Fosmid-based reporters are expressed in lower copy number, which might help reduce toxicity associated with unc-39 overexpression. Since fosmid reporters include more of the regulatory elements than my plasmid-based transcriptional and translational unc-39 reporters, they will also reflect more physiological gene expression pattern with proper temporal control. I have obtained a C-terminal-tagged unc-39::GFP fosmid reporter that includes around 9kbp upstream and 21kbp downstream of the unc-39 gene. I will inject the unc-39fosGFP fosmid reporter into unc-39 mutant that carries a Pceh-10-RFP RID reporter, and establish a transgenic line that restores RFP expression in the RID neuron. Because the non-integrated C. elegans transgenic array is subjected to random loss during each somatic cell division, a fully rescued mother will give rise to mixed populations of transgenic progenies with, or without rescued RID. If unc-39 acts cell-autonomously, this transgenic array must be present in RID in all rescued animals.
3.7 UNC-39 may play diverse roles in multiple cell lineages

My results that *unc-39* functions at multiple points in a lineage-dependent manner are in line with an earlier study, which showed that UNC-39 is required for the development of M mesoblast cell and all six coelomocytes. In *C. elegans*, two coelomocytes arise during embryogenesis from the MSpp lineage. Two more coelomocytes and the M mesoblast cell are derived from the MSap lineage, also during embryogenesis. The M cell then undergoes post-embryonic divisions and produces the remaining two coelomocytes. Interestingly, the M cell had less severe defects than coelomocytes that it gives rise to (21% vs. 56%), so that in the cases when the M mesoblast is unaffected, its daughter cells may still show defects (Yanowitch et al., 2004). Therefore, UNC-39 is activated at multiple time points in the same lineage and the severity of the defects seen in the affected cells may vary.

How does UNC-39 achieve separate roles in different lineages? One possibility is that the partners of UNC-39 may vary, depending on the context. By forming complexes with different partners, UNC-39 can activate different sets of target genes and execute various functions. This may explain why the phenotype in *unc-39* mutants differs from one affected cell type to another. This also explains that UNC-39 is expressed more broadly than cells that express the *Pceh-10* marker.

Various transcription factors act in this manner. For example, a LIM homeodomain transcription factor Lhx2 controls the development of multiple tissues. It is essential for ascribing cortical cell fate identity in the developing mouse brain (Mangale et al., 2008). The same factor is expressed in hair follicles and regulates skin regeneration after injury through modulating the activity of stem cell regulators Sox9, Tcf4 and Lgr5 (Mardaryev et al., 2011).
Our data thus supports the growing body of evidence that the functions of transcription factors are highly context-dependent.

### 2.8 The physiological roles of the RID neuron

The physiological roles of the RID neuron have not been examined in previous studies. Our ablation studies suggest that RID has a role in modulating *C. elegans* locomotion. Although RID-ablated animals maintain the ability to generate movements and propagate sinusoidal waves, the forward velocity and the propensity in directional movement and pausing were both affected. Similar locomotion defects were observed in *unc-39(hp701)* mutant. This suggests that the lack of RID neuron contributes prominently to the locomotion defects of the *unc-39* mutants.

The connectivity of the RID neuron also supports its role in modulating locomotion. The loop structure receives synaptic inputs from the PVC command interneurons, and forms a gap junction connection with AVB neurons. Both PVC and AVB regulate the activity of B type motor neurons, which are responsible for driving forward locomotion. Moreover, based on the incomplete reconstruction data, RID makes a few synapses onto the dorsal body wall muscles, as well as the excitatory DA and AS class motor neurons and inhibitory VD and DD motor neurons. Finally, RID also receives synaptic inputs from a mechanosensory neuron AVM. Therefore, although it is not essential for movement, RID might have a function in fine-tuning locomotion in *C. elegans*, perhaps in response to some environmental stimuli, such as touch. Further investigation is needed to dissect the function of RID.
3 Methods

3.1 Strains

*C. elegans* strains were cultured and maintained at 22°C on the NGM plates using standard protocols (Brenner, 1974). New stocks of the widely used laboratory reference strain N2 were thawed annually to prevent accumulation of spontaneous background mutations. A complete list of strains used in this study is listed in Table 1. The transgenic strains were generated by microinjection of a plasmid DNA mixed with a co-injection marker that produces an easily identifiable phenotype, into the gonads of young adult animals using standard procedures (Mello et al., 1991).

3.2 Molecular biology

A complete list of plasmids generated in this study is in Table 2. Primers used for constructing the plasmids and genotyping *C. elegans* mutants are shown in Table 3. The list of fosmids used for the rescue experiments is listed in Table 4. The transcriptional UNC-39 reporter included 29 base pairs of the coding sequence, in addition to the promoter.

3.3 EMS mutagenesis and forward genetic screen

5-6 plates with a large number of well-fed animals at L4 stage were washed with the M9 buffer three times, and placed into 47mM ethane methyl sulfonate (EMS) in M9 for 4 hours at room temperature (20-25°C). These animals were washed thoroughly with M9 buffer after the
mutagenesis, and allowed to recover for 2 hours on OP50-seeded NMG plates. Young adult (P0) animals were picked onto fresh plates (3-4 P0s per plate) and allowed to lay eggs for 3 days. Progenies (F1 animals) were transferred to fresh plates (1/plate) and allowed to propagate. Their progenies (F2) was screened using the fluorescent compound microscope for defects in the morphology of the RID neuron. Animals with putative morphology defects were isolated and propagated for one more generation (F3s) to verify the presence of the phenotypes.

3.4 Non-complementation tests

Mutant with a specific RID phenotype was crossed into N2 males, and resulting male progeny were crossed into another mutant with the same/similar RID phenotype. If the mutations were in the same gene, the progeny from this cross would be homozygous for the same mutation and show the mutant phenotype. If the mutations were in different genes, the resulting progeny would be heterozygous for both mutations, and not show the mutant phenotype.

3.5 Single nucleotide polymorphism and genetic mapping

All isolated mutants were backcrossed against N2 background 3-4 times before mapping. Isolated mutations were first rough-mapped to a region on a specific chromosome using single nucleotide polymorphism (SNP) mapping (Davis et al., 2005). A specific mutant in the N2 background was crossed into the Hawaiian mapping strain CB4856, which differs from N2 by a number of SNPs distributed over all six chromosomes. These SNPs introduce sites for various restriction enzymes such that when short regions around these SNPs were amplified by PCR and
digested with an appropriate enzyme, the N2 and Hawaiian digestion pattern looked different. 50 mutant animals from the F2 generation of these crosses were isolated and propagated as separate lines that have the N2/CB recombination events at different positions on the chromosomes. The closer the mutation was to a particular SNP, the less recombination with the Hawaiian strain occurred, and the more it was enriched for the N2 digest pattern. Very close to the mutation site, the digest pattern was strictly N2.

When the mutation region could not be narrowed further due to the absence of appropriate SNPs, standard mapping with genetic markers, in which the position of the mutation in relation to mutations in some known genes that produce a visible phenotype, was deduced based on the recombination frequency. Once the region containing the causative mutation was narrowed down to about 1 map unit, fosmid rescue experiments were performed. Overlapping fosmids, containing wild type genomic sequences spanning the region of interest, were put into groups, injected, and the progeny was screened for the rescue of the RID phenotype. Once a specific fosmid group rescued the mutant phenotype, the fosmids from that group were injected individually, and the causative gene from the rescuing fosmid was indentified.

3.6 Whole genome sequencing-assisted cloning

In parallel with standard SNP and genetic mapping, whole genome sequencing (WGS) of two isolated mutants (hp701 and hp724) was performed. WGS was performed on the same 50 N2/CB mutant lines that were previously used for standard mapping. The F3 and F4 populations for all 50 lines were pooled and the genomic DNA was isolated, and sent to the Genome Sciences Centre, BC Cancer Agency, Vancouver, BC. Sequencing was done on the HiSeq 2000
platform, and data analysis was done using the MAQGene software by our collaborator Stephane Flibotte.

N2 differs from CB4856 by roughly 100,000 SNPS. Based on the ratio of N2 to CB SNP variants, a region enriched for the N2 pattern was identified and all mutations in this region were reported (Figure 16). These results were confirmed with our own mapping data. Overlapping fosmids covering the identified region were injected and tested for the rescue of RID phenotype in order to identify the causative mutation in the region. After the fosmid rescue, re-sequencing was performed to confirm the presence of SNPs called by WGS.
Figure 16: Outline of combined SNP mapping and Whole Genome Sequencing (SNP-WGS) approach. A mutant is crossed into a polymorphic Hawaiian strain. From the F2 progeny, animals exhibiting the mutant phenotype are picked, their progenies pooled and their genomes are sequenced. The number of recombination events between the Bristol and the Hawaiian strains, and hence the density of the Hawaiian SNPs decreases at the positions closer to the mutation. This allows to narrow down the mutation-bearing region to a small segment on the chromosome, and of all mutations in the genome, identify the lesion that results in the phenotype of interest. Adapted from Doitsidou et al., 2010.
3.7 Locomotion analysis

The movement of individual young adult animals on 10cm NGM plates, lightly seeded with OP50 *Escherichia coli*, was recorded at 16x microscope magnification, and a fully zoomed Fujifilm Finepix digital camera, mounted on the Celestron Digital Camera adapter for 2 minutes. Prior to recording, the animal was allowed to move on the plate for 1 minute. The plate was shifted when necessary to avoid losing the worm out of the area being recorded. The movies were processed in ImageJ with a plugin written by Dr. Kawano in the lab (Kawano et al., 2011). This program tracks the movement of the animal by automatically subtracting it from the background. All empty frames (when the plate was shifted) were removed from the analysis. The data was graphed using a plugin in R. The two-tailed Mann-Whitney U test was performed to determine the differences in velocity between strains.

3.8 Electron microscopy

15-20 young adult animals were packed into 6mm in diameter aluminum carriers filled with OP50, and instantly frozen using a Leica EM HP100 high-pressure freezer in liquid N2. The frozen carriers were transferred to a tube of pre-frozen (in liquid N2) fixative solution, composed of 0.1% tannic acid (TA) and 0.5% gluteraldehyde (GA) in anhydrous acetone. The freeze substitution was performed using a Leica EM AFS2 Freeze Substitution and Low Temperature Embedding System. The following freeze substitution program was used:

1. -90C for 96 hours in 0.1% TA and 0.5%GA
2. -90C for 4 hours in anhydrous acetone, changing acetone every 30 minutes
3. -90C for 4 hours in 2% osmium tetroxide (OsO4) in anhydropus acetone
4. -90C to -20C at 5C/hour, total of 14 hours
5. -20C for 16 hours
6. -20C to 4C at 6C/hour, total of 4 hours
7. 4C for 4 hours
8. 4C for 2 hours in anhydropus acetone, changing acetone every 30 minutes
9. 4C to 20C over 1 hour

The samples were infiltrated and embedded in Spurr resin. After that, serial sections 70nm in thickness were prepared by Douglas Holmyard, and imaged using the FEi Techai 20 transmission electron microscope at 80 kV (x25,000) with a Gatan digital camera. Individual serial images were rotated and aligned using the Photoshop software. For the serial reconstruction of the RID candidates and the DD motor neuron, the outline of the neurons was first manually traced using a Wacom Bamboo Fun CT-450 digital drawing tablet, and a 3D image was constructed from the traced images using a program called Reconstruct.
### Table 1: Strains used in this study.

**Experiment: Mutants analyzed from the screen**

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Description/phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZM5488</td>
<td>hpIs202 IV</td>
<td>Pceh-10::GFP, RID marker</td>
</tr>
<tr>
<td>ZM5967</td>
<td>unc-39(hp701)V; hpIs202 IV</td>
<td>No Pceh-10::GFP expression in RID; CAN defects</td>
</tr>
<tr>
<td>ZM7019</td>
<td>unc-39(e257) V; hpIs202 IV</td>
<td>No Pceh-10::GFP expression in RID; CAN defects</td>
</tr>
<tr>
<td>ZH6905</td>
<td>hpIs292</td>
<td>Pceh-10::RFP</td>
</tr>
<tr>
<td>ZM7019</td>
<td>unc-39(hp701)V; hpIs292 IV</td>
<td>No Pceh-10::RFP expression in RID; CAN defects</td>
</tr>
<tr>
<td>ZM5982</td>
<td>vab-8(hp724) V; hpIs202 IV</td>
<td>Short RID axon</td>
</tr>
<tr>
<td>ZM5870</td>
<td>lim-4(hp689) X; hpIs202IV</td>
<td>Faint expression of Pceh-10::GFP in RID</td>
</tr>
<tr>
<td>CX3937</td>
<td>lim-4 (ky403) X</td>
<td>Faint expression of Pceh-10::GFP in RID</td>
</tr>
</tbody>
</table>

**Experiment: Mapping**

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Description/phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB4856</td>
<td>Wild isolate</td>
<td>Hawaiian strain for SNP mapping</td>
</tr>
<tr>
<td>CB2065</td>
<td>dpy-11(e224); unc-76(e911)V</td>
<td>Dumpy and uncoordinated; used for genetic mapping</td>
</tr>
</tbody>
</table>
## Experiment: RID marker expression

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Description/phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>OH904</td>
<td>otls33 IV</td>
<td>Pkal-1::GFP</td>
</tr>
<tr>
<td>ZM7139</td>
<td>unc-39(hp701)V; otls33 IV</td>
<td>No Pkal-1::GFP expression in RID</td>
</tr>
<tr>
<td>ZM7149</td>
<td>unc-39(e257)V; otls33 IV</td>
<td>No Pkal-1::GFP expression in RID</td>
</tr>
<tr>
<td>OH4837</td>
<td>lim-6(nr2073) X; otls11</td>
<td>zig-5::GFP + pRF4(rol-6)</td>
</tr>
<tr>
<td>ZM7488</td>
<td>unc-39(hp701)V; otls11</td>
<td>No zig-5::GFP expression in RID</td>
</tr>
<tr>
<td>ZM7483</td>
<td>unc-39(e257)V; otls11</td>
<td>No zig-5::GFP expression in RID</td>
</tr>
<tr>
<td>OH2246</td>
<td>otls107</td>
<td>ser-2::GFP</td>
</tr>
<tr>
<td>ZM7489</td>
<td>unc-39(hp701)V; otls107</td>
<td>No ser-2::GFP expression in RID</td>
</tr>
<tr>
<td>ZM7147</td>
<td>unc-39(e257)V; otls107</td>
<td>No ser-2::GFP expression in RID</td>
</tr>
<tr>
<td>ZM6658</td>
<td>hpIs274</td>
<td>Pmod-1::mitoGFP</td>
</tr>
<tr>
<td>ZM7105</td>
<td>unc-39(hp701)V; hpIs274</td>
<td>No Pmod-1::mitoGFP expression in RID</td>
</tr>
<tr>
<td>ZM7146</td>
<td>unc-39(e257)V; hpIs274</td>
<td>No Pmod-1::mitoGFP expression in RID</td>
</tr>
<tr>
<td>ZM7100</td>
<td>hpEx3035</td>
<td>lim-4::GFP</td>
</tr>
<tr>
<td>ZM7152</td>
<td>unc-39(hp701);hpIs292; hpEx3058</td>
<td>No lim-4::GFP expression in RID</td>
</tr>
<tr>
<td>ZM7150</td>
<td>hpIs328</td>
<td>Punc-39::GFP, transcriptional reporter</td>
</tr>
</tbody>
</table>
Experiment: *unc-39* involvement in apoptosis

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Description/phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZM7148</td>
<td><em>ced-4(n1162)</em> III; <em>hpIs202</em> IV</td>
<td>Two RID neurons</td>
</tr>
<tr>
<td>ZM7154</td>
<td><em>ced-4(n1162)</em> III; <em>unc-39</em> (e257) V; <em>hpIs202</em> IV</td>
<td>No RID</td>
</tr>
<tr>
<td>ZM7474</td>
<td><em>ced-4(n1162)</em> III; <em>unc-39</em> (e257) V; <em>ham-1</em> (n1438) IV; <em>hpIs202</em> IV</td>
<td>No RID</td>
</tr>
<tr>
<td>ZM7145</td>
<td><em>ced-3(n717)</em> IV; <em>hpIs202</em> IV</td>
<td>Two RID neurons</td>
</tr>
<tr>
<td>ZM7144</td>
<td><em>ced-3(n717)</em> IV; <em>unc-39</em> (hp701) V; <em>hpIs162</em>; <em>hpIs202</em> IV</td>
<td>No RID</td>
</tr>
<tr>
<td>ZM7140</td>
<td><em>ham-1(n1438)</em> IV; <em>hpIs202</em> IV</td>
<td>Two RID neurons</td>
</tr>
<tr>
<td>ZM7480</td>
<td><em>ham-1(n1438)</em> IV; <em>unc-39</em> (e257) V; <em>hpIs202</em> IV</td>
<td>No RID</td>
</tr>
</tbody>
</table>

Experiment: *unc-39* effect on neurons in ABalap lineage

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Description/phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL5717</td>
<td><em>inIs179</em> II</td>
<td>ALA marker</td>
</tr>
<tr>
<td>ZM7478</td>
<td><em>unc-39</em> (e257) V; <em>inIs179II</em></td>
<td>ALA marker expression normal</td>
</tr>
<tr>
<td>IK705</td>
<td><em>njIs10</em></td>
<td>RIA interneuron marker</td>
</tr>
</tbody>
</table>
Experiment: Induction of RID markers by ectopic *unc-39* expression

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZM7479</td>
<td><em>hpEx3149</em></td>
<td>Prig-3-UNC-39(cDNA)::RFP</td>
</tr>
<tr>
<td>ZM7487</td>
<td><em>hpEx3151; hpIs274</em></td>
<td>Prig-3-UNC-39(cDNA)::RFP; Pmod-1::GFP</td>
</tr>
<tr>
<td>ZM7486</td>
<td><em>hpEx3150; hpIs202</em></td>
<td>Prig-3-UNC-39(cDNA); Pceh-10::GFP</td>
</tr>
<tr>
<td>ZM7477</td>
<td><em>hpEx3152</em></td>
<td>Pmec-4-UNC-39(cDNA)::RFP</td>
</tr>
<tr>
<td>ZM7475</td>
<td><em>hpEx3152; hpIs202</em></td>
<td>Pmec-4-UNC-39(cDNA)::RFP; Pceh-10::GFP</td>
</tr>
<tr>
<td>ZM7481</td>
<td><em>hpEx3152; hpIs274</em></td>
<td>Pmec-4-UNC-39(cDNA)::RFP; Pmod-1::GFP</td>
</tr>
<tr>
<td>ZM7476</td>
<td><em>hpEx3152; otIs33</em></td>
<td>Pmec-4-UNC-39(cDNA)::RFP; Pkal-1::GFP</td>
</tr>
</tbody>
</table>

Experiment: *unc-39* effect on cholinergic, GABAergic neurons and muscles

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CZ1200</td>
<td><em>julS76</em> [Punc-25::GFP]</td>
<td>GABA axon marker</td>
</tr>
<tr>
<td>ZM7101</td>
<td><em>unc-39 (hp701)N</em>; <em>julS76</em> [Punc-25::GFP]</td>
<td>GABA axon marker normal</td>
</tr>
<tr>
<td>ZM7101</td>
<td><em>unc-39 (e257)N</em>; <em>julS76</em> [Punc-25::GFP]</td>
<td>GABA axon marker normal</td>
</tr>
<tr>
<td>CZ333</td>
<td>*juls1[Punc-25::SNB-1::GFP]</td>
<td>GABA vesicle marker</td>
</tr>
<tr>
<td>ZM7142</td>
<td><em>unc-39 (hp701)N</em>; *juls1[Punc-25::SNB-1::GFP]</td>
<td>GABA vesicle marker normal</td>
</tr>
<tr>
<td>Name</td>
<td>Description</td>
<td>Marker</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>ZM7142</td>
<td>unc-39 (e257)V; julIs1[Punc-25::SNB-1::GFP]</td>
<td>GABA vesicle marker normal</td>
</tr>
<tr>
<td>ZM1331</td>
<td>hpIS61 [Punc-25::UNC-10::GFP]</td>
<td>GABA synapse marker</td>
</tr>
<tr>
<td>ZM7016</td>
<td>unc-39 (hp701)V; hpIS61 [Punc-25::UNC-10::GFP]</td>
<td>GABA synapse marker normal</td>
</tr>
<tr>
<td>ZM7099</td>
<td>unc-39 (e257)V; hpIS61 [Punc-25::UNC-10::GFP]</td>
<td>GABA synapse marker normal</td>
</tr>
<tr>
<td>ZM3030</td>
<td>nuIS152[Punc-129::SNB-1::GFP]</td>
<td>DA (cholinergic) marker</td>
</tr>
<tr>
<td>ZM7015</td>
<td>unc-39 (hp701)V; nuIS152[Punc-129::SNB-1::GFP]</td>
<td>DA (cholinergic) marker normal</td>
</tr>
</tbody>
</table>
Table 2: Constructs used in this study.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Transgene</th>
<th>Description/expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>PJH2765</td>
<td>$\text{Plim-4::LIM-4 ::GFP}$</td>
<td>Expressed in RID, some anterior neurons</td>
</tr>
<tr>
<td>PJH3084</td>
<td>$\text{Punc-39-LIM-4 (cDNA)::GFP}$</td>
<td>Does not rescue RID phenotype</td>
</tr>
<tr>
<td>PJH2798</td>
<td>Genomic $\text{unc-39}$ in PSI1180 vector</td>
<td>Rescues $\text{unc-39}$ phenotype</td>
</tr>
<tr>
<td>PJH2811</td>
<td>$\text{Punc-39-UNC-39 ::GFP}$</td>
<td>Translational reporter</td>
</tr>
<tr>
<td>PJH2839</td>
<td>$\text{Punc-39::GFP}$</td>
<td>Transcriptional reporter</td>
</tr>
<tr>
<td>PJH2923</td>
<td>$\text{Prgef-1-UNC-39 (cDNA)::GFP}$</td>
<td>Panneuronal $\text{unc-39::GFP}$</td>
</tr>
<tr>
<td>PJH2890</td>
<td>$\text{Pmec-4-UNC-39 (cDNA)::RFP}$</td>
<td>Expressed in touch receptor neurons</td>
</tr>
<tr>
<td>PJH3059</td>
<td>$\text{Prig-3::UNC-39 (cDNA)::mCherry}$</td>
<td>Expressed in AVA, anterior neurons and tail neurons</td>
</tr>
</tbody>
</table>
Table 3: Primers used in this study.

### Purpose: Genotyping mutants

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer: sequence 5’-3’</th>
<th>Description/notes</th>
<th>Fragment sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ced-4(n1162)</em></td>
<td>OZM3202: atgtcttgcaaatcgaatgc&lt;br&gt; OZM3481: ttactgatattcttgacagcagtc</td>
<td>MseI digest</td>
<td>WT: 502bp&lt;br&gt;Mutant: 266bp + 236bp</td>
</tr>
<tr>
<td><em>ham-1(n1483)</em></td>
<td>OZM3656: tgctcctaggtacgagtgccttgata&lt;br&gt; OZM3657: gggcgtatgtaagcccatgtaagtga</td>
<td>Deletion allele</td>
<td>WT: 966bp&lt;br&gt;Mutant: 728bp</td>
</tr>
</tbody>
</table>

### Purpose: Plasmid construction

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
<th>Description</th>
<th>Restriction sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>OZM3698</td>
<td>taatggtaccataaaacttttgtcagcc&lt;br&gt;tatggtacatcctgcttacagcc</td>
<td>Amplification of <em>lim-4</em> cDNA</td>
<td>KpnI</td>
</tr>
<tr>
<td>OZM3699</td>
<td>tataggtacatcctgtcagctaacatgc&lt;br&gt;natggtacatcctgtcagctaacatgc</td>
<td>Amplification of <em>unc-39</em> promoter</td>
<td>SalI, SphI</td>
</tr>
<tr>
<td>OZM3246</td>
<td>tatggtacatcctgtcagctaacatgc&lt;br&gt;gataggtacaitcctgtcagctaacatgc</td>
<td>Amplification of full <em>unc-39</em> gene</td>
<td>SalI, SphI</td>
</tr>
<tr>
<td>OZM3219</td>
<td>atgacagaccatcgcgaatt</td>
<td>unc-39 RT-PCR</td>
<td>N/A</td>
</tr>
<tr>
<td>OZM3220</td>
<td>ttacggattgaagtgaatgcattatcag</td>
<td>unc-39 RT-PCR</td>
<td>N/A</td>
</tr>
<tr>
<td>OZM3439</td>
<td>atatccgggatgacagacatcgcgaatt</td>
<td>unc-39 RT-PCR</td>
<td>XmaI, KpnI</td>
</tr>
<tr>
<td>OZM3440</td>
<td>gtcggattgacggattgaagtgaatgcattatcag</td>
<td>unc-39 RT-PCR</td>
<td>BamHI, KpnI</td>
</tr>
<tr>
<td>OZM3441</td>
<td>ataggttcatgacagacatcgcgaatt</td>
<td>unc-39 cDNA sequencing</td>
<td>N/A</td>
</tr>
<tr>
<td>OZM3442</td>
<td>ggccgtgacatcggattgaagtgaatgcattatcag</td>
<td>unc-39 cDNA sequencing</td>
<td>N/A</td>
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<tr>
<td>OZM3462</td>
<td>gtatcagctagcctagcatatgcc</td>
<td>unc-39 cDNA sequencing</td>
<td>N/A</td>
</tr>
<tr>
<td>OZM3461</td>
<td>tgccagcatctagaacttce</td>
<td>unc-39 cDNA sequencing</td>
<td>N/A</td>
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</table>
Table 4: Fosmids used for mutant rescue experiments.

Fosmids tested for *hp701* rescue:

<table>
<thead>
<tr>
<th></th>
<th>Fosmid Code 1</th>
<th>Fosmid Code 2</th>
<th>Fosmid Code 3</th>
<th>Fosmid Code 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WRM0624cC12</td>
<td>13 WRM0636bA09</td>
<td>25 WRM0637cE04</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>WRM0624aA04</td>
<td>14 WRM0626dC07</td>
<td>26 WRM0641aB08</td>
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</tr>
<tr>
<td>3</td>
<td>WRM0611bA09</td>
<td>15 WRM0616aA05</td>
<td>27 WRM0632dA11</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>WRM0610aC04</td>
<td>16 WRM0619aC10</td>
<td>28 WRM0640bF07</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>WRM0634bF01</td>
<td>17 WRM0636cG07*</td>
<td>29 WRM0618dB12</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>WRM0632aC03</td>
<td>18 WRM0614dC12</td>
<td>30 WRM0666bC12</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>WRM0641dG04</td>
<td>19 WRM0630cG04</td>
<td>31 WRM0626dD02</td>
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<tr>
<td>8</td>
<td>WRM0641aH02</td>
<td>20 WRM0615bG11</td>
<td>32 WRM069dE09</td>
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</tr>
<tr>
<td>9</td>
<td>WRM0618dF08</td>
<td>21 WRM068dH02</td>
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<tr>
<td>10</td>
<td>WRM0625dC12</td>
<td>22 WRM061aB03</td>
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Fosmids tested for *hp724* rescue:

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Fosmids tested for *hp689* rescue:

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* rescued mutant phenotype.
References


