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Large-Scale RNAi Screen to Identify Genes Involved in Axon Guidance in *Caenorhabditis elegans*

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CONTENTS

ZUSAMMENFASSUNG

Diese Arbeit wurde durchgeführt, um Gene, die axonale Wegfindung im Nervensystem von *Caenorhabditis elegans* steuern, zu identifizieren. *C. elegans* stellt aufgrund seiner einzigartigen physiologischen Eigenschaften ein gutes Modellsystem für das Studium einer Vielzahl biologischer Prozesse dar. Das Nervensystem von *C. elegans* ist einfach strukturiert und umfasst 302 Neuronen. Diese Neuronen bilden stereotype Netzwerke mit ihren anterior-posterior und dorsal-ventral verlaufenden axonalen Fortsätzen aus. In dieser Arbeit nutzen wir die kürzlich beschriebene Methode der RNA Interferenz (RNAi) im Wurm zur Identifikation von neuen Genen der axonalen Wegfindung. Allerdings ist das Nervensystem von *C. elegans* resistent gegen systemische RNAi und Transport von doppelsträngigen RNA Molekülen in benachbarte nicht-neuronale Zellen veranlasst keine neuronale RNAi. Aus diesem Grund begannen wir mit der Identifizierung von *C. elegans* Mutanten, die eine erhöhte Empfindlichkeit für RNAi im Nervensystem aufweisen. Eine chemische Mutagenese wurde durchgeführt, gefolgt von einem Screen nach Mutanten mit effizienter RNAi im Nervensystem. Eine der Mutanten (*nre-1*, für neuronal RNAi efficient) zeigte starke Suppression der Genexpression im Nervensystem nach RNAi durch Füttern. Wir nutzten die *nre-1* supersensitive Mutante für einen revers genetischen Screen zur Idenfizierung von Genen der axonalen Wegfindung in *C. elegans*. Um die Fortsätze der Nervenzellen sichtbar zu machen, wurde ein transgener Stamm im *nre-1* Hintergrund erzeugt, in dem ein Teil der Inter- und Motoneurone durch gelb fluoreszierendes Protein (YFP) markiert ist. Dieser Stamm wurde für einen Screen von 2416 Genen auf Chromosom I verwendet. Dazu wurde eine library von Bakterienklonen, die einem bestimmten Gen entsprechende dsRNA exprimieren, an *C. elegans* verfüttert. Der Screen führte zur Identifizierung von 57 Kandidatengenen, die penetrante axonale Wegfindungsdefekte in Motoneuron-Kommissuren und Axonen des Ventralstrangs in *C. elegans* zur Folge haben. Die identifizierten Gene sind involviert in eine Vielzahl von biologischen Prozessen wie DNA-Metabolismus, Translation, Transkription und Signaltransduktion. Einige kodieren für Zelloberflächenmoleküle und Zytoskelettkomponenten. Zusätzlich zu neuen Genen konnten im Screen Gene identifiziert werden, die in andere biologische Prozesse involviert sind, aber bis jetzt nicht mit axonaler Wegfindung in Verbindung gebracht wurden. Beispielsweise führt Verlust von *pry-1*, einem Axin Homolog in *C. elegans*, zu axonalen Defekten. Axin ist ein assoziierter Faktor des ß-Catenin Komplexes und damit ein negativer Regulator in Wnt vermittelter Signaltransduktion. Weitere Studien an anderen, in diesem Screen identifizierten Kandidatengenen wie z.B. neuen Rezeptoren, Signalmolekülen, Kinasen und Transkriptionsfaktoren können uns in Zukunft einen weiteren Einblick in die molekularen Mechanismen der axonalen Wegfindung geben.

SUMMARY

This study was undertaken to identify genes involved in axon guidance in the nervous system of *Caenorabditis elegans*. Due to its unique physiological properties, the nematode worm *C. elegans* is a powerful genetic model system to study a variety of biological processes. The nervous system of *C. elegans* is a simple organ comprising 302 neurons. These neurons create stereotypic neuronal networks formed by their anterior-posterior and dorsal-ventral running axons. Here, we took advantage of the recently discovered phenomenon of RNA interference in the worm to identify axon guidance genes. However, the nervous system of *C. elegans* is refractory to the systemic RNA interference, and delivery of dsRNA molecules to the neighboring non-neuronal cells does not initiate RNAi in the neurons of the worm. Therefore, we started with the identification of mutants of *C. elegans* that are efficient for RNAi in the nervous system. A standard chemical mutagenesis screen was performed to identify mutants of the worm that showed enhanced RNAi efficiency in the nervous system. One of the mutants (*nre-1*, for neuronal RNAi efficient) showed marked suppression of gene expression in the nervous system by feeding RNAi approach. We used the *nre-1* supersensitive strain as a tool in a reverse genetic screen to identify genes required for axon guidance in *C. elegans*. A transgenic strain was constructed in the *nre-1* background, wherein a subset of interneurons and motor neurons were labeled with the yellow fluorescent protein to visualize axons of the neurons. We used this strain to screen 2416 gene of the worm located on chromosome I by feeding a library of bacterial clones expressing dsRNA fragments specific to the genes. This screen has identified 57 candidate genes that give rise to penetrant axon guidance defects in the commissural and ventral nerve cord axons in *C. elegans*. The genes identified include genes involved in various cellular processes such as DNA metabolism, translation, transcription, cell-surface molecules, signaling pathways and cytoskeletal molecules. In addition to novel genes, the screen has also identified genes that have been previously implicated in other cell biological processes, but their roles in axon guidance were not known. For example, this screen has identified a *C. elegans* axin homolog *pry-1,* a signaling molecule involved in a Wnt signaling pathway. Axin is an associated factor of the β-catenin complex and is a negative regulator of Wnt signals. Besides, further studies on other candidate genes, e.g. novel receptors, signaling molecules, kinases and transcription factors identified in this screen should provide us with more information on the molecular mechanisms employed by neurons to steer their axons.

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This thesis is dedicated to my mother.

1 INTRODUCTION

1.1 Introduction to the axon guidance problem

The function of the nervous system depends on the ability of neurons to connect with each other and their target cells. In a nervous system, the neurons form neuronal circuits or networks that are made by specialized neurites of the neurons called the axons and the dendrites. Axons transfer neural signals from the cell body or soma of the neuron to their targets, while dendrites are the processes that receive signals from the targets and send them to the soma of the neuron. Axons constitute a larger portion of the nervous system and their development is fundamentally important for the functioning of the nervous system. However, the study of axon development in mammals has been difficult due to the complexity of the mammalian nervous system (Kandel *et al.,* 2000).

During embryonic development, axons have to travel considerable distances to reach their final targets. The navigation of axons takes place in a highly complex environment with remarkable order and stereotypic manner. The growth cone at the tip of the growing axon is the site of all the dynamic activity that leads to navigation of the axon to their targets (Figure 1-1). The growth cone guides the axon towards its targets by sensing the molecular cues present in the environment and changing the direction of the growing axon. The cues involved in the process of axon guidance are of two types, attractive and repulsive (Tessier-Lavigne and Goodman, 1996). For example, the long-range attractive cues pull the axons towards their targets, while long-range repulsive cues push the axons from behind or side and prevent them from entering certain territories. The other two types of local contact-dependent attractive and repulsive cues finetune the movement of axons locally at the level of target selection and/or guidance of follower axons. The effect of attractive cues on axons is the growth of the axon towards their targets and stabilization of the interaction with the targets. On the other hand, repulsive cues cause diversion of axons from the area of presence of the cues or cause collapse and inhibition of the growth cone and synapse formation.

The repertoire of axon guidance molecules is classified on the basis of their structural and functional features (Kaprielian *et al.*, 2000). The first type includes the secreted molecules or ligands that are produced by the target cells. They diffuse and form a gradient that leads to either the attraction or repulsion of growing axons. The second type of molecules includes the receptors for the secreted ligands. The receptor molecules are mainly

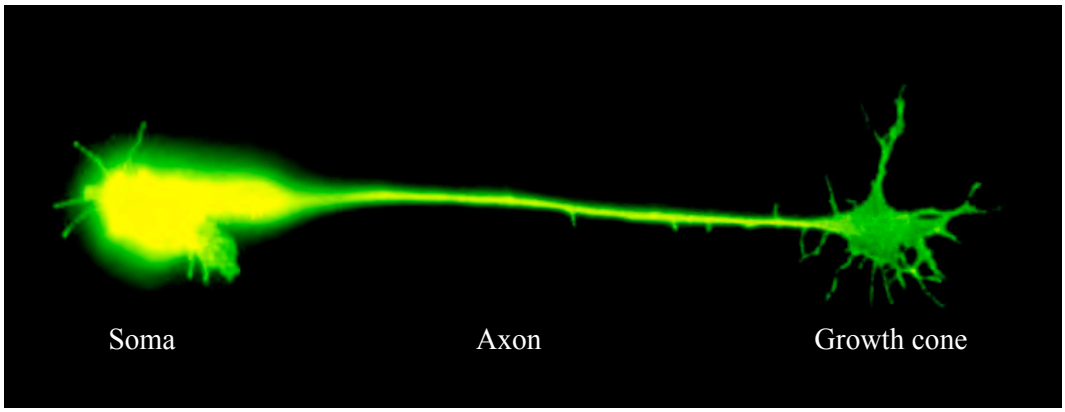

Figure 1-1: The anatomical features of a chick retinal ganglion neuron labeled with anti-NCAM antibody (courtesy of G. E. Pollerberg).

localized on axons and growth cones. In the presence of ligands receptor molecules activate intracellular signaling pathways and subsequently execute changes in growth or target selection by the axons. The third type includes the intracellular molecules that form the components of intracellular signaling networks responsive to the cell surface receptors. The signaling networks formed by the intracellular molecules are complex and poorly understood due to the involvement of these molecules in multiple signaling pathways. Besides the above types, many extracellular matrix molecules are also involved in the process of axon guidance. These extracellular matrix molecules are secreted and are integrated into the complex matrix surrounding the cells. However, the mechanisms involved in these processes are also poorly understood.

1.2 Why study axon guidance mechanisms?

The nervous system is a unique organ that not only contains a collection of neurons, but each of the neuron is connected to a variety of neuronal and non-neuronal targets by their neurites. These neuronal circuits form the basis of the nervous system. Defects in the information processing, connectivity and/or functioning of the nervous system cause many life threatening diseases and disorders in human. Recently, it has been shown that a variety of genetic neuropathies are manifestations of defective axon guidance mechanisms. For example, mutations in a human Robo gene lead to the disruption of hindbrain axon pathway crossing and defects in morphogenesis leading to a genetic disease called horizontal gaze palsy with progressive scoliosis (Jen *et al.*, 2004). While in the case of multigenic Charcot-Marie-Tooth disease that causes motor axonal neuropathy, mutations in a heat shock protein involved in axon guidance has been shown to be responsible for the final manifestation of the disease (Evgrafov *et al.*, 2004). In yet another case, a Kallman syndrome gene homolog has been shown to be required for axon branching (Bulow *et al.*, 2002; Rugarli *et al.*, 2002). Therefore, to study these biomedically important aspects of brain development, the elucidation of axon guidance mechanisms is fundamentally necessary. Secondly, studying the nature of mechanisms involved in axon guidance is also important for the understanding of the functioning of neuronal circuits and ultimately the way the nervous system works. It is also important for studies on medical conditions such as spinal cord injury, regenerative neuronal medicine and neuronal cell therapy (Clarke *et al.*, 2000).

To date, several studies on axon guidance mechanisms have been done in different model organisms. Due to simplicity of genetics, biochemical and anatomical advantages, invertebrate model organisms have been widely used for axon guidance research and they have provided valuable information on different biological processes required for the development of the nervous system. The invertebrate model organisms extensively studied are *Caenorabditis elegans* and *Drosophila melanogaster* (Dickson, 2002). These model organisms have provided much of our current knowledge of the molecular mechanisms of axon guidance and development of the nervous system. Many of the genes identified in these invertebrate organisms have direct homologs in vertebrates. These homologs also show functional homology as many of them interact with other molecules that are also conserved. From the experiments in invertebrates and vertebrates a set of common principles on dynamic regulation of axon guidance have emerged (Yu and Bargmann, 2001). However, still many gaps in these schemes remain and not all the mechanisms involved in axon guidance of different types of neurons are known. Therefore, as a first step to address the phenomenon of axon guidance at the molecular level the identification of new genes required for the mechanisms of axon guidance is needed.

1.3 Developmental events and the signaling pathways

The embryonic development is one the most complex processes in animals. After fertilization of the oocyte rapid cell division processes take place leading to the establishment of embryonic patterning. This is followed by the developmental events of gastrulation, neurulation and morphogenesis. The development of the nervous system starts in the neurulation stage of the embryonic development. All biological processes involved in the formation of an organ are driven by the internal genetic programs of the embryonic cells as well as the different cell-cell interactions taking place during the process of development. At the molecular level different signaling pathways play their roles generating complex networks of interactions. Over the last few decades, various studies on the signaling pathways have yielded surprising information on these pathways (reviewed by Pires-daSilva and Sommer, 2003). The studies on different model organisms have shown that a few classes of signaling pathways are sufficient to pattern a wide variety of cells, tissues and morphologies. How this complexity in the biological system is achieved by a few signaling mechanisms is the topic of intense research. Axon guidance is such a developmental event in which complex interactions of different signaling

molecules and pathways take place. Many molecules involved in the process of axon guidance have been identified and their mechanisms of action elucidated (Araujo and Tear, 2003; Guan and Rao, 2003). However, recent availability of complete genome sequences of different model organisms have greatly increased the repertoire of genes to be examined for their roles in axon guidance based on their structural features and/or information available through comparative genomics approaches (Hutter, 2000; Teichmann and Chothia, 2000).

1.4 Molecular mechanisms of axon guidance

As in all developmental biological phenomena, the guidance of axons to their targets is the result of the interplay of molecular factors inside and outside of the neurons. After differentiation of the cell into a neuron, it starts to grow its axons in the milieu of a complex tissue environment. The extracellular factors affecting axon guidance either are secreted molecules acting as ligands for specific receptors on the growing axons or are extracellular matrix components that interact with cell surface molecules on the axons. The following sections review the current information on axon guidance ligands, their receptors and mode of action. Table 1-1 briefly summarizes the known axon guidance molecules and their guidance functions identified in different model organisms with their mechanisms of action in the guidance of different types of axons. Figure 1-2 shows the domain structures of some axon guidance ligands and their receptors. Most of the axon guidance molecules are made of a small number of conserved peptide domains and these domains play important roles in the protein-protein interaction properties of these molecules (Dickson, 2002).

1.4.1 Netrin signaling

Netrins were the first group of axon guidance molecules to be found in both invertebrate and vertebrate nervous systems (Hedgecock *et al.*, 1990; Ishii *et al.*, 1992; Kennedy *et al.*, 1994; Serafini *et al.*, 1994). Netrins are secreted proteins and they act as attractive or repellent guidance cues depending on the type of receptor complexes they bind that are present on the growth cones of axons. In *C. elegans* UNC-6, the prototypical netrin, was identified in genetic screens for uncoordinated mutants that showed defective body movement and later also in screens performed for axon guidance molecules (Brenner,

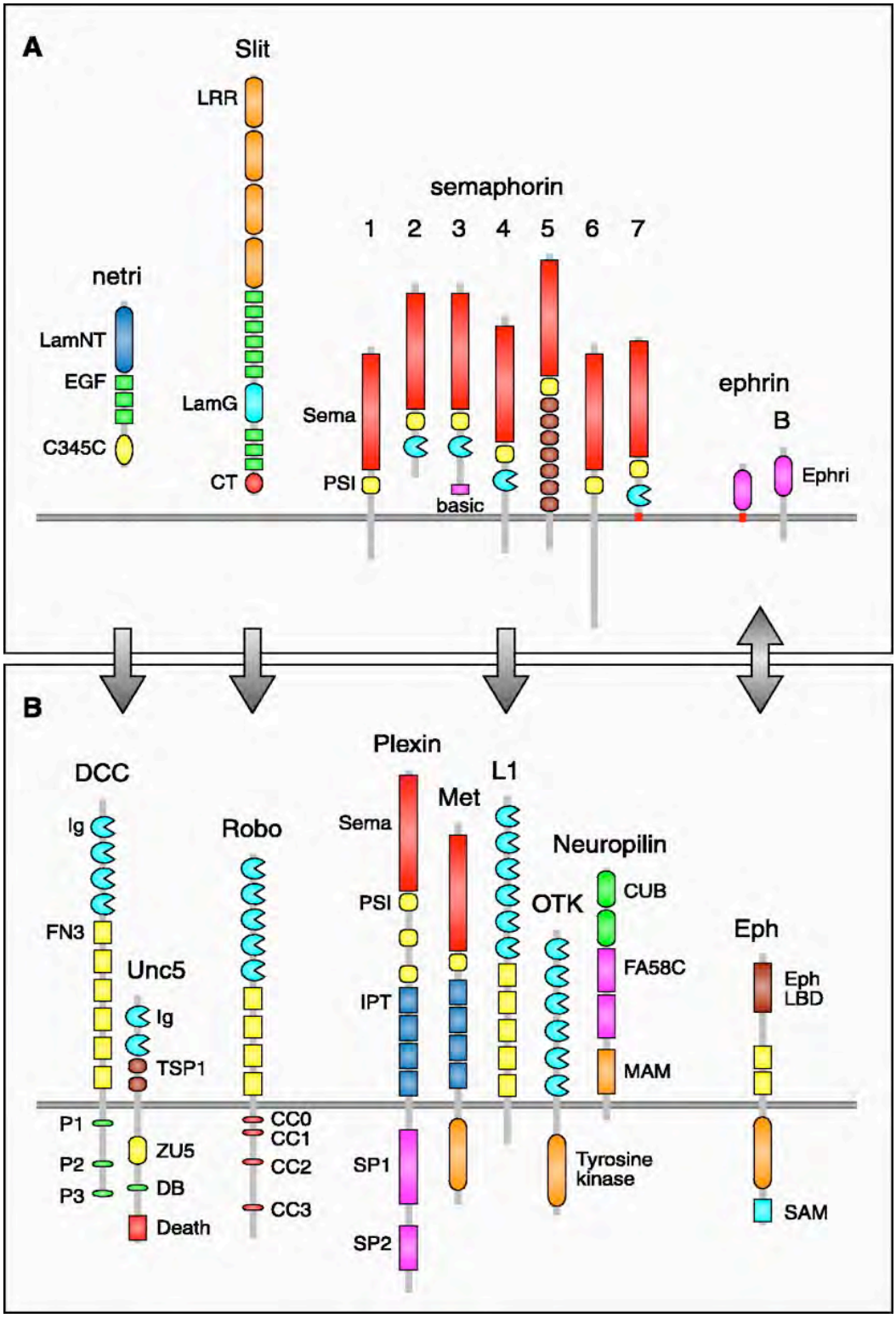

Figure 1-2: Domain structure of some conserved guidance molecules (A) and their receptors (B) (adapted form Dickson, 2001).

Table 1-1: A brief description of axon guidance molecules.[a]

Molecule	*C. elegans*	*Drosophila*	Vertebrate	Molecular Function	Guidance Function
Netrin	UNC-6	Netrin A/B	Netrin 1-4 β-netrin	Attractant, repellent	VNC axons (Ce, Dm, vertebrates)
Receptors	UNC-5	Dunc-5	Unc5h1-4	Attraction	-
	UNC-40	Frazzled	DCC, neogenin	Repulsion	-
Slit	SLT-1	Slit	Slit1-3	Repellent	Longitudinal axons (Ce)
Receptors	SAX-3	Robo1-3	Robo1-4	-	Retinal axons (zebrafish)
Sema-phorins	SMP-1-3[b]	Sema 1-2	Sema3-7, viral semaphorin	Attractant, repellent	Motor axons (Dm)
Receptors	PLX-1-2[b]	Plexin A-B	PlexinA1-4, B1-3, C, D	-	-
Ephrins	EFN-1-4	Ephrin	EphrinA1-6, B1-3	Forms morphogen Gradient	Retinotectal projections (mouse)
Eph	VAB-1	Eph	EphA1-8, B1-6	Ephrins Receptors	-
Wnts	n.r.[c]	Wnt5	Wnt4	Attractant	Midline crossing (mouse)
Receptors	n.r.	Derailed	Frizzled	-	-
TGFβ	UNC-129	n.r.	BMP	Repellent	Motor axons (Ce)
Receptors	n.r.	n.r.	BMPR	-	-
Insulin	n.r.	n.r.	n.r.	-	-
Receptors	n.r.	DInR	n.r.	-	Photo-receptor axons (Dm)
Intra-cellular Proteins	UNC-73	Trio	Trio[b]	Rho-GEF, signaling	Motor, longitudinal axons(Ce), photo-receptor axons (Dm)
	UNC-34	Enabled	Ena	Actin binding	Signaling
	UNC-115	n.r.	n.r.	Actin binding	Signaling
	UNC-44	n.r.	Ankyrin[b]	Binds actin receptors	Signaling
	MAX-1	n.r.	n.r.	Adaptor protein	Netrin repellent signaling (Ce)
Extra-cellular Matrix Proteins	CLE-1	n.r.	Collagen XV/XVIII	Cell-matrix interaction	Motor axons (Ce)
	NID-1	n.r.	Nidogen[b]	Cell-matrix interaction	Longitudinal axons (Ce)

Adhesion	PAT-3	n.r.	β-Integrin	Cell-cell interaction	Commissural axons (Ce)
	HMR-1	Hammer-head-1	N-cad[b]	Cell-cell interaction	Longitudinal axons (Ce)
IgCAM	SYG-1	IrreC	DM-GRASP	Cell-cell interaction	Synapse formation (Ce) RGC axons (chick)
	SYG-2	n.r.	n.r.	Cell-cell interaction	Receptor for SYG-1 (Ce)
	n.r.	n.r.	Axonin-1	Cell-cell interaction	Retinal axons (chick, mouse)
	LAD-1?	n.r.	NrCAM	Receptor for Axonin-1	-

[a]Signaling pathways and the genes having homologs in all three model organisms, namely *C. elegans* (Ce), *Drosophila* (Dm) and vertebrates are listed.
[b]Homologs present, but no function in axon guidance reported.
[c]n.r.; Homologs present, not yet functionally characterized.

1974; Hedgecock *et al.*, 1990). UNC-6 is involved in controlling the direction of both ventrally and dorsally projecting axons. In vertebrate, netrins were biochemically identified as molecules promoting axon outgrowth in chick spinal cord (Kennedy *et al.*, 1994). The identification of netrins in invertebrates and vertebrates has established the paradigm of conservation of guidance cues during the evolution of multicellular organisms (Culotti and Merz, 1998). Netrins act through two types of transmembrane receptors, namely UNC-40 and UNC-5 in *C. elegans* and their respective homologs in mammals (Table 1-1). In the worm, genetic studies have shown that the UNC-40 receptor is mainly required for ventral projections, while the UNC-5 receptor is required for dorsal projection (Hedgecock *et al.*, 1990). However, in a different type of guidance mechanism UNC-40 and UNC-5 alone can repel the distal tip cells in the gonads of *C. elegans* (Su *et al.*, 2000). It is also shown in mammals that an UNC-40/UNC-5 complex can mediate growth cone repulsion. This combinatorial association of different netrin receptors, receptor complexes and their cognate ligands give rise to diverse physiological responses. The signaling pathways downstream of the netrin receptors lead to the modulation of actin polymerization. In *C. elegans* genetic studies have shown that UNC-34 (Enabled homolog), UNC-115 (actin binding protein), CED-10 (Rac homolog) and MAX-1 (adaptor protein) act downstream of netrin receptors (Colavita and Culotti, 1998; Huang *et al.*, 2002; Struckhoff and Lundquist, 2003). These molecules either are associated with actin filaments or are intracellular components of signaling pathways

(Table 1-1). Besides, a netrin and its receptor have been implicated in the etiology of a tumor and the control of colorectal tumors by regulating apoptosis (Mazelin, 2004).

1.4.2 Slit/Robo signaling

The Slit gene was discovered in a genetic screen for genes involved in the embryonic patterning in *Drosophila* (Wieschaus *et al.*, 1984). Slits are secreted molecules and are members of the epidermal growth factor (EGF) repeats protein family (Figure 1-2). It was thought that they are required for the development of central nervous system and differentiation of glial cells (Rothberg, 1988). However, later they have been implicated in repulsion of commissural axons away from the midline in the fly and vertebrates (Brose *et al.*, 1999; Kidd *et al.*, 1999; Wang *et al.*, 1999). They are also involved in neuronal cell migration (Zhu, 1999). Slit molecules are ligands for Roundabout (Robo) class of cell surface receptors present on the growth cones of growing axons. They are involved in multiple pathways of axon guidance in retinal and commissural axons and neuronal migration (Table 1-1). Genetic studies in *Drosophila* and *C. elegans* have shown that Robo signaling is transduced by Abl tyrosine kinase to Enabled (Ena) and to Capulet, a homolog of adenylyl cyclase-associated protein (Guan and Rao, 2003). Some receptor tyrosine phosphatases are also shown to be involved in Slit/Robo signaling. However, the fine detail on different molecules required and mechanisms they control are not fully understood. In *C. elegans* a Slit homologue, SLT-1, was identified by a homology search and a deletion allele of the gene has been implicated in axon guidance at the midline and in dorsal-ventral and anterior-posterior directions (Hao, 2001). *C. elegans* also has a single Robo homolog called SAX-3, and SLT-1 acts through SAX-3 mediated signaling. However, it is unclear if Slit is the only ligand for the Robo or there are yet other unknown ligand(s). However, several aspects of the Robo pathway in flies show no direct conservation in the worm. The *C. elegans* genome does not encode any protein that is similar to Commissureless (Comm), which is involved in dynamic regulation of Robo levels in flies (Keleman *et al.*, 2002). But a second Comm protein exists in flies and has been implicated in functions at the midline, yet this protein has a low degree of similarity to the original Comm protein.

1.4.3 Semaphorin signaling

The semaphorins are a family of secreted and membrane associated proteins. The SemaI, the prototypic invertebrate semaphorin, was first discovered in *Drosophila* as Fascisclin IV, a protein implicated in axon fasciculation (Kolodkin, 1992). In vertebrates semaphorins were identified as factors required for growth cone collapse activity (Kapfhammer and Raper, 1987). Sema3A/collapsin-1 was the first member of this group (Luo *et al.*, 1993). Later it became clear that semaphorins are a family of conserved proteins involved in axon repulsion and growth cone inhibition or collapse in various regions. They are also present in several tissues and are implicated in cell migration and immune response (Pasterkamp and Kolodkin, 2003). The semaphorins bind to cell surface receptors called plexins (Figure 1-2). In vertebrates semaphorins also bind to the neuropilin receptors, which alone or in combination with plexins form receptor complexes. However, neuropilins do have small intracellular signaling domains of unknown functions. Plexins have a large conserved intracellular domain, which is essential for signal transduction (Pasterkamp and Kolodkin, 2003). Downstream of plexins are the Rac family of GTPases (Dalpe *et al.*, 2004). Rho activation is also shown to be downstream of plexin-B in vertebrates (Driessens *et al.*, 2001). In *C. elegans* three genes encoding proteins with a semaphorin domain in extracellular parts and two genes with plexin-like domains are known (Hutter, 2000). Recently, *C. elegans* plexin-1 (*plx-1*) and semaphorin-1 (*smp-1*) have been implicated in the conversion of cell movement responses from attraction to repulsion in cell migration during male tail ray positioning (Dalpe *et al.*, 2004; Ikegami *et al.*, 2004). The fact that the *C. elegans* genome contains only five genes for semaphorin signaling makes it a simple model system to study the interaction between different semaphorins and plexins to understand roles of these molecules in biological processes like axon guidance.

1.4.4 Ephrin signaling

Ephrins are membrane associated guidance molecules and have been divided into two classes based on their mechanism of membrane association (Kullander and Klein, 2002). A-class ephrins are tethered to the membrane by a GPI anchor; whereas B-class ephrins are type I transmembrane molecules with intracellular domains. The receptors for the ephrins are the Eph transmembrane proteins with an intracellular tyrosine kinase domain (Figure 1-2). Eph were originally defined as receptors for the ephrins A and B (Table 1-1).

However, the signaling mechanisms are complex and Eph can also act as ligands for the ephrins. The first ephrin was found in mammalian system by biochemical approaches (Cheng and Flanagan, 1994). It was later discovered that the ephrin is ligand for a receptor tyrosine kinase and the interaction between the ephrin and the receptor is important for the guidance of retinal axons during formation of retinotectal maps in vertebrates (Drescher *et al.*, 1995). In the developing nervous system ephrins cause collapse and repulsion of growth cones, while in the adult nervous system they have been implicated in synaptic plasticity (reviewed by Murai and Pasquale, 2004). The signal transduction pathways used by the Eph receptors share some commonality with the growth-factor receptors, but also have some unique features. Upon ligand binding the Eph receptors dimerise causing self-phosphorylation as well as phosphorylation of the downstream molecules initiating different signaling cascades. Various intracellular soluble signaling molecules have been shown to interact with the Eph receptors. However, it is likely that the Ephrin and Eph signaling system leads to changes in actin cytoskeletal organization (Yu *et al.*, 2001). One of the most striking features of the ephrin-Eph signaling is the reverse signaling mediated by intracellular domains of B-class ephrins. The A-class GPI anchored ephrins also mediate reverse signaling through the recruitment of integrins (Bruckner *et al.,* 1997; Holland *et al.*, 1996). These mechanisms have been implicated in axon or cell attraction and/or adhesion processes. In *C. elegans* four genes encode ephrin homologs, while one gene encodes for Eph receptor protein (Hutter, 2000). However, the ephrin-Eph system of the worm has been involved in epidermal morphogenesis and other developmental events, and its role in axon guidance is not clearly understood (Chin-Sang *et al.*, 2002; Miller *et al.*, 2003).

1.4.5 TGFβ signaling

Transforming growth factor (TGFβ) super family members are also implicated in axon guidance in vertebrates and invertebrates (Hogan, 1996). BMPs (bone morphogenic proteins) are members of this family, and they have been involved in the determination of dorsal-ventral patterning of the commissural axons (Augsburger *et al.*, 1999; Bagri *et al.,* 2003). BMPs are secreted from the roof plate of the vertebrate neural tube, and form a gradient that is important for the determination of the fate of dorsal commissural neurons. They have been shown to repel commissural axons, suggesting that they might initiate the ventral projection of commissural axons. The UNC-129 protein is a TGFβ

family member, and is required for guidance of motor axons along dorsal-ventral axis in *C. elegans* (Colavita *et al.,* 1998). Its role in repulsion of these axons is similar to that of UNC-6/netrin. However, the receptor for UNC-129 is not known in *C. elegans* and it is likely that the signaling pathway that mediates UNC-129 output uses a non-canonical mechanism, since mutants of the only canonical TGFβ receptor in *C. elegans*, DAF-4, have no axon guidance defects (Estevez *et al.*, 1993).

1.4.6 Wnt signaling

Wnts are members of a secreted glycoprotein family and are best characterized for their roles in embryonic patterning (Moon *et al.*, 2002). The Wnt pathways are used multiple times and at different places to influence very diverse types of cell fates. There are multiple homologs of Wnt molecules and their receptors in different model organisms. For example, the *C. elegans* genome contains five Wnt molecules, *Drosophila* four and the human genome has at least 17 molecules (Ruvkun and Hobert, 1998). The Wnt signal is transduced by a cytoplasmic multiprotein complex consisting of Dishevelled (Dsh), glycogen synthase kinase 3 (GSK-3), a scaffolding protein APC, axin and β-catenin (Korswagen, 2002). In the absence of Wnt signaling the complex targets β-catenin for proteasomal degradation, whereas in the presence of Wnt signaling β-catenin is stabilized and enters the nucleus where it binds to transcription factors leading to gene expression. Genetic studies in model systems have shown that Wnt signaling acts in a variety of biological processes from embryonic development to the development of the nervous system. They have been shown to control the anterior-posterior patterning during embryogenesis in mouse and other organisms (Lyuksyutova *et al.,* 2003). Recently, studies in *Drosophila* and mouse have also shown that Wnt signaling is necessary for guidance of commissural axons along the anterior-posterior axis (Table 1-1) (Lyuksyutova *et al.*, 2003; Yoshikawa *et al.,* 2003). In *C. elegans* the Wnt pathway has been extensively studied with respect to embryonic development and migrations of a group of neurons in postembryonic development. However, the role of Wnt signaling pathway in axon guidance in *C. elegans* has not yet been explored.

1.5 Extracellular matrix molecules

The extracellular matrix (ECM) or basement membranes form the immediate external environment of the neurons and their growing axons. The extracellular matrix in *C.*

elegans is mainly made up of proteoglycans, laminins, collagens and associated proteins and their sequences have been highly conserved in mammalian ECM molecules (Kramer, 1997). These proteins make complex yet dynamic sheets and are shown to be necessary for many developmental events. In *C. elegans*, ECM proteins like laminin family member UNC-6/netrin (Serafini *et al.*, 1994), collagen family member CLE-1/collagen XVIII (Ackley *et al.*, 2001), NID-1/nidogen (Kim and Wadsworth, 2000), and KAL-1/Kallmann syndrome protein (Bulow *et al.*, 2002; Rugarli *et al.*, 2002) have been identified as axon guidance molecules. However, except for UNC-6, the molecular mechanisms of action of other ECM proteins are not known as their receptors or interaction partners have not been identified. Besides many extracellular matrix factors are important in the embryonic development of the organism and mutations in these proteins are lethal at the early stages of development limiting the characterization of developmental contribution of these molecules in the nervous system.

On the other hand, receptors for extracellular matrix components that have been implicated in axon guidance are the integrins. Integrins couple assembly of various extracellular matrix and cytoskeletal polymers (Howe *et al.*, 1998). In *C. elegans*, there are two α-chain and two β-chain integrins, of these INA-1/α-integrin and PAT-3/β-integrin interact with each other and are shown to be involved in axon migrations (Baum and Garriga, 1997; Poinat *et al.,* 2002). In the case of PAT-3 the intracellular interaction pattern has also been identified (Poinat *et al.,* 2002). Several other extracellular matrix molecules of *C. elegans* are also conserved in other organisms; however, it is not known if some of them are involved in axon guidance mechanisms. For example, several members of metalloproteases families like adamalysin, astacin, matrixin and neprilysin are implicated in ECM interactions or neuropeptide processing. Recently, a *C. elegans* homolog of a disintegrin and metalloprotease (ADAM) protein, UNC-71, has been implicated in regulation of motor axon guidance (Huang *et al.*, 2003). It will be important to find out if these molecules are involved in axon guidance in *C. elegans*.

1.6 Cell adhesion molecules

Cell adhesion molecules (CAMs) play important roles during various developmental processes such as cell adhesion, cell migration, differentiation and axon guidance. They are classified based on their amino-terminal extracellular domains into different super families. Of all the CAMs, the immunoglobulin-like (Ig) domain superfamily forms an

important class and many IgCAMs are involved in axon guidance in different organisms. In *C. elegans* IgCAMs form a superfamily of ~26 predicted genes encoding transmembrane or GPI-anchored proteins with extracellular Ig modules (Hutter, 2000). Besides Ig domains a few IgCAMs also have one or more fibronectin, thrombosponsdin or epidermal growth factor-like domains. Some IgCAMs are secreted molecules and the *C. elegans* genome has at least six genes encoding for secreted IgCAMs. The intracellular domains of transmembrane IgCAMs have characteristic enzymatic and binding functions and some of the IgCAMs have intracellular protein tyrosine kinase or phosphatase domains. The IgCAMs are principal mediators of cell recognition and adhesion in the developing nervous system and other tissues (Walsh and Doherty, 1997). They act combinatorially among themselves and with receptors from other cell surface molecule families to pattern cell movements and attachments. Many of these CAMs act as receptors in signaling pathways and some of them have been described previously (Table 1-1).

In *C. elegans* many members of IgCAM family are involved in axon guidance, prominent among them are transmembrane factors like SAX-3/Robo (Zallen *et al.*, 1998), UNC-40/DCC (Chan *et al.,* 1996), UNC-5 (Leung-Hagesteijn *et al.*, 1992), CLR-1 (Chang *et al.*, 2004), and LAD-1/SAX-7/L1CAM (Zallen *et al.*, 1999), SYG-1/irreC (Shen and Bargmann, 2003) and its ligand SYG-2/nephrin (Shen *et al.*, 2004, Table 1-1). Some of these molecules have provided paradigms for molecular mechanisms of axon guidance, for example, SAX-3, UNC-40 and UNC-5 receptors (Figure 1-2). These important functions of IgCAMs point towards the need for the identification of the involvement of other IgCAM molecules in the process of axon guidance that might shed more light on the development of the nervous system.

LrrCAMs form the second super family of CAMs that are distinguished from others adhesion molecules by the presence of LR-repeat domains in their structures (Hutter, 2000). One member of this family, SLT-1/Slit, has been implicated in axon guidance and it is a ligand for SAX-3/Robo receptor (Figure 1-2; Hao *et al.*, 2001). Cadherins are also members of the CAMs group and one of them, HMR-1, is shown to have a function in axon fasciculation in *C. elegans* (Broadbent and Pettitt, 2002). Apart from these molecules many other cell adhesion molecules have interesting structures with multiple protein-protein interaction domains (Cox *et al.*, 2004). However, their functions in developmental events like axon guidance are not known.

The functional importance of CAMs to the development of the nervous system and axon guidance is well studied in vertebrate models due to the ease of experimental manipulation of these systems in vitro. In vertebrates CAMs have more homologs and due to the complexity of vertebrate nervous system they are involved in different aspects of development. With regard to axon guidance mechanisms vertebrates like mouse, zebrafish and chick systems have been used extensively. Several axon guidance molecules, identified in *C. elegans* and *Drosophila*, have been studied in vertebrate models either by gene knockouts (mouse), identification of mutants (zebrafish) or in vivo and in vitro biochemical studies (mouse, chick). In mouse system, neural CAMs like L1CAM, axonin-1, NCAM, Robos and FGFRs, have been widely studied to define their roles in axon guidance mechanisms in the spinal cord and retinotectal axon targeting (reviewed by Kenwrick and Doherty, 1998). Vertebrates have proved to be excellent systems for identification of downstream effectors of the signaling pathways and their links to cytoskeletal machinery that takes part in axonal growth cone movements (reviewed by Kiryushko, 2004). However, mutational approaches in higher organisms are laborious and technically challenging due to the limitations of current techniques to identify point mutations related to an observed phenotype. Here, the invertebrate models like *C. elegans* provide many advantages due to simplicity of genetics and the reduced number of homologs in the genome leading to ease in epistatic studies of the genes.

1.7 Intracellular signaling pathways and axon guidance

Research in the past decades has yielded important advances in identification of the molecules that are involved in an array of signaling pathways. However, unexpectedly genetic and biochemical studies have revealed that only a few classes of signaling pathways are sufficient to control a wide variety of biological processes. In the case of neuronal responses to guidance cues, many molecular components of signaling pathways have been identified and their specific role are defined (reviewed by Guan and Rao, 2003). It is increasingly understood that the interplay of different signaling pathways and biological reactions are necessary for the complex developmental events like axon guidance. For example, recent work by Yu *et al.* has shown the involvement of the UNC-40/DCC receptor in the SAX-3/Robo pathway leading to identification of a crosstalk between these molecules in *C. elegans* (Yu *et al.*, 2001). They have demonstrated that SAX-3 signaling involves UNC-34 and UNC-40. Here the role of UNC-40 is UNC-

6/netrin independent and it acts as a repulsive receptor in SAX-3 pathway. This study gives information on the combinatorial logic of axon guidance mechanisms wherein different components of two pathways interact with each other generating very different responses to the same cues. In yet another example, a receptor tyrosine phosphatase CLR-1, which is a negative regulator of FGF signaling in *C. elegans* (Kokel *et al.*, 1998), is shown to be involved in inhibition of UNC-6/netrin mediated axon attraction in some neurons (Chang *et al.*, 2004). These examples underline the need to find roles of already known genes in guidance mechanisms as well as identification of new molecules involved in the interplay of different signaling pathways. In *C. elegans* major signaling pathways involved in different biological processes have been identified (Bargmann, 1998; Ruvkun and Hobert, 1998) and many of the genes involved in these pathways have mutants available. This should facilitate identification of novel factors involved in processes like axon guidance provided that some prior information is available to test a large number of genes identified by genome sequencing projects. In the case of interplay of signaling pathway in axon guidance, *C. elegans* provides a powerful genetic system due to the availability of the genome sequence and mutations in a variety of genes.

However, in addition to the previously mentioned cell surface molecules and their ligands, the process of axon guidance is also dependent on a large number of intracellular signaling molecules and their crosstalk in different signaling pathways (Guan and Rao, 2003). The intracellular signaling molecules act differently in diverse cellular processes and their functionality changes depending on cell or tissue type in which they are expressed. They also take part in diverse reactions; for example, some molecules are components of different axon guidance signaling pathways, while others are components of cellular cytoskeletal machinery. Still other molecules regulate the expression and transportation of axon guidance molecules. Some of the well-studied intracellular molecules involved in axon guidance in *C. elegans* are UNC-73 (Steven *et al.*, 1998), UNC-34 (Yu *et al.*, 2001), UNC-44 (Otsuka *et al.*, 1995), UNC-115 (Gitai *et al.*, 2003) and MAX-1 (Huang *et al.*, 2002) (for details see Table 1-1).

The *unc-73* gene encodes two differentially spliced multidomain proteins. These proteins contain a single Dbl homology domain and are involved in axon guidance. Dbl domains have guanine nucleotide exchange activity and like other Rho family members may have a role in regulation of the actin cytoskeleton (Steven *et al.*, 1998). The other domains of UNC-73 proteins may help in formation of protein complexes capable of reorganizing

the actin cytoskeleton on receiving extracellular signals. The other intracellular proteins like UNC-34, UNC-44 and UNC-115 have actin binding domains and they may form a part of actin organization mechanisms. The *max-1* gene encodes a novel adaptor protein (MAX-1) required in UNC-6/netrin mediated axon repulsion function (Huang *et al.*, 2002). Although a few more intracellular guidance molecules are known, our understanding of intracellular mechanisms remains fragmented. One obvious reason for this is the involvement of intracellular factors in multiple pathways and mutations in these molecules lead to severe phenotypes or lethality. Nevertheless, identification of roles of intracellular factors is necessary to define the functioning and regulation of signaling pathways.

The vertebrate model systems and cell culture experiments have provided a detailed insight into the complex events of intracellular signaling pathways and crosstalk among different signaling pathways (reviewed by Huber *et al.*, 2003), especially the cytoskeletal dynamics that are regulated during the process of axon guidance in the growth cone (reviewed by Dent *et al.*, 2003). From these studies common themes on the guidance cues, their receptors, intracellular signaling intermediates and their link to cytoskeletal dynamics and subsequent alterations in neuronal growth cone behavior have emerged and they have supplemented the genetic approaches used in models like *C. elegans* and *Drosophila*. However, cell cultures and vertebrate models are not suitable for large-scale genetic screens due to the complexity or experimental limitations of those systems. Therefore, in this work we have tried to address the problem of identification of genes required for axon guidance in a genetically tractable model organism *C. elegans*. The nematode *C. elegans* due to its biological and experimental advantages over other models is very attractive system for genetically addressing a biological problem like axon guidance towards their targets in a complex tissue environment in vivo.

1.8 The model organism *Caenorhabditis elegans*

C. elegans is a small free-living soil nematode worm and can be grown either in Petri plates on lawns of bacteria or in liquid cultures if large amounts are required. The life cycle of worm has four postembryonic larval stages, namely L1-L4. It has a four and half-day generation time at 20°C and the strains can be stored as frozen stocks (Riddle *et al.*, 1997). *C. elegans* is a self-fertilizing hermaphrodite, which produces both sperm and oocytes. Males are produced at a low frequency and can be mated with hermaphrodites

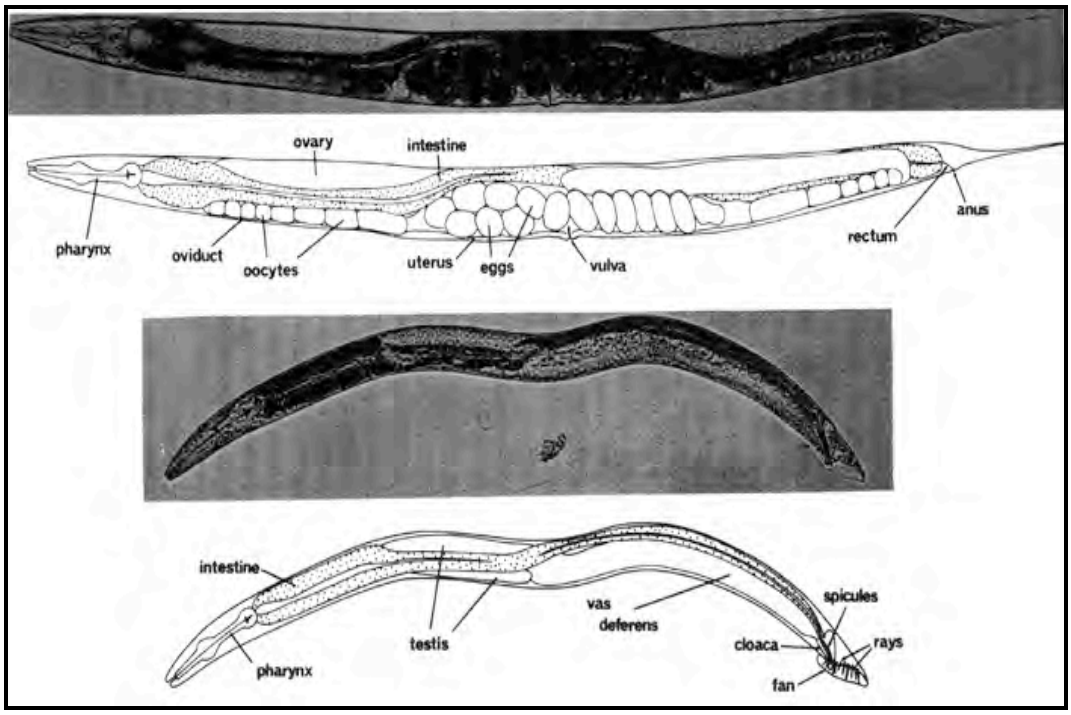

Figure 1-3: The anatomical features of the *C. elegans* adult hermaphrodite (top) and the male animal (bottom) (adapted from Wood, 1997).

to produce cross progeny (Figure 1-3). A single hermaphrodite can produce up to 300 progeny. The worm embryo develops through a series of invariant cell divisions that occur during the first 8 h of embryonic development at 20°C after the fertilization of oocyte. After 18 h of development in the egg, the larva hatches from the eggshell. The animal then passes through four larval stages (L1-L4) that are separated by molting, during which the animal sheds its old cuticle. In food limiting conditions L2 larva can adapt an alternative developmental program called dauer stage. In dauer stage the animal can survive for months under unfavorable conditions and can develop into L4 stage when favorable conditions are restored. The hermaphrodite nature of the worm is very useful while performing large-scale genetic screens as self-fertilization omits requirement of males and simplifies the cloning of animals. It is also helpful while maintaining the mutant strains that are compromised for sexual mating. However, the presence of males allows crossing between mutant strains and is useful for mapping of the mutations. The genetics of *C. elegans* is well established and the complete genome is sequenced, greatly facilitating the genetic characterization of biological processes in this simple organism. The genome of *C. elegans* is 97 Mb in size and contains ~19000 genes and some 50% genes of the worm have homologs in mammals (*C. elegans* Sequencing Consortium, 1998). One other important feature of *C. elegans* biology is its invariant cell lineage, which is of tremendous help while analyzing the biological processes at the resolution of single cells (Sulston and Horvitz, 1977; Sulston *et al.*, 1983). These advantages make *C. elegans* an excellent system in which to investigate the genetics of basic biological processes such as the development of the nervous system.

1.8.1 General anatomy

All nematodes are built on the same basic body plan, which is made of two concentric tubes separated by a fluid-filled space called pseudocoelom. The outer tube is covered by the collagenous, extracellular cuticle, which is secreted by the underlying hypodermis. *C. elegans* moves on surfaces by contraction of the two subventral muscle strips with relaxation of the subdorsal strips, and vice versa, which generates sinusoidal movement in the dorsal-ventral plane. On the agar plate, animals move forward or backward on either lateral side and are confined to the surface by the surface tension of water in the medium. The nervous system, gonad, coelomocytes, and excretory system are the other components of the outer tube. The inner tube is composed of the muscular pharynx with

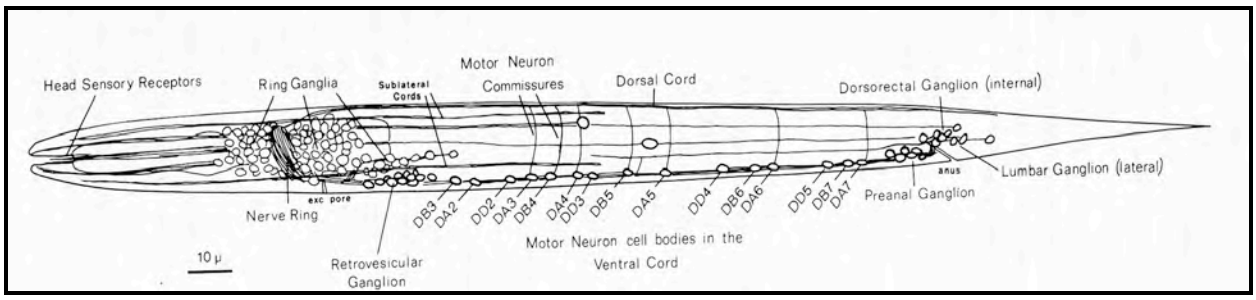

Figure 1-4: Structure of the nervous system of *C. elegans* L1 larva (adapted from Antebi, 1997).

its own autonomous nervous system and the intestine. The conserved nematode anatomy is generated by conserved developmental patterns. The early blastomeres, called founder cells, are generated by a series of asymmetric, asynchronous cleavages in which the germline precursor cell sequentially gives rise to the four founder cells of somatic lineages and one cell of germline lineage (reviewed by Schnabel and Priess, 1997). The embryonic lineages generate 671 cells, but 113 of these undergo apoptosis (Sulston and Horvitz, 1977). By the time larva hatches from the egg it has 558 cells. Some 10% of these cells are somatic blast cells that divide further to generate additional somatic tissues in the adult animal.

The complete anatomy of the worm is known at electron microscopic resolution (White *et al.*, 1986). In addition to the complete wiring diagram of the nervous system, knowledge of neurophysiological function has been gained from neurotransmitter analysis and electrophysiological studies on the nervous system of the worm (Strange, 2003). Besides a large body of data on the genetic mapping, cloning, mutants and the sequence of entire genome provide easy and powerful tools to characterize new genes in this organism (WormBase, www.wormbase.org). The transparency of the worm has greatly increased the suitability of this model system for analysis of biological processes in live animals. This has also facilitated the expression of different fluorescent proteins and their variants for easy labeling of various cells and tissues in the intact animal (Chalfie *et al.,* 1994, Hutter, 2004). For example, the dynamic biological processes like axonal growth cone migration through the complex tissue environment can be studied easily due to the transparent nature of *C. elegans* (Knobel *et al.*, 1999).

1.8.2 Anatomy of the nervous system

The nervous system of *C. elegans* is one of the simplest and elaborately described nervous systems of all the metazoans. The *C. elegans* nervous system consists of 302 neurons that are each uniquely recognizable in different individuals. All the neurons and the circuits they form have been reconstructed from serial section electron microscopy (White *et al.*, 1986). These electron microscopic studies have defined the morphology of each neuron, its chemical synapses, and its gap junctions. The basic positions and morphologies of some of the neurons are shown in Figure 1-4. In *C. elegans* there are 118 classes of neurons with 1 to 13 members in each class. Sensory and interneurons are mostly bilaterally symmetric pairs, with homologs on the left and the right side of the animal.

About half of the neurons, including most sensory neurons, are found in the head surrounding the posterior part of the pharynx forming the brain of the worm. The nerve ring, a part of the brain, is formed of a bundle of axons of sensory neurons and interneurons. The somas of most interneurons are located in the head or tail ganglia. Some interneurons have long axons that project from the head to the tail of the animal.
The motor neurons are located along the ventral midline of the body. The pharyngeal nervous system contains 20 neurons and is connected to the somatic nervous system with a single pair of interneurons. The entire nervous system has a total of 5000 chemical synapses, 700 gap junctions, and 2000 neuromuscular junctions (Durbin, 1987). Each neuron only synapses onto about 15% of the neurons it contacts, and it can have as few as 1 to as many as 30 synapses with other neurons (Durbin, 1987). Two thirds of the neurons of *C. elegans* (198 out of 302) are present as bilaterally symmetrical pairs of neurons (Hobert *et al.*, 2002). Most of the remaining neurons including 75 ventral nerve cord motor neurons that are located on or very close to the ventral midline, and have no contralateral analogue. The connectivity of the nervous system of *C. elegans* is over 75% reproducible between different animals (Durbin, 1987). The other major advantage of the *C. elegans* nervous system is simple anatomy of the neurons as they have only 1 to 5 neurites with distinct morphologies (White *et al.*, 1986).
The neurons of *C. elegans* have been classified in different types based on various criteria like the function of the neuron (namely sensory, motor or interneuron), the location in the body or the nature of neurotransmitter used (White *et al.*, 1986). However, morphological features are standard norms for identification of neurons in *C. elegans*. For example, sensory neurons have special endings associated with openings at the cuticle of nose of the animal, while motor neurons have characterized neuromuscular junctions. In addition to the above criteria, neurons have been defined by direct analysis of their function with cell-killing experiments (Bargmann and Avery, 1995). A single neuron in the transparent animal can be killed with a laser micro-beam focused through the objective of a microscope, and the operated animal can subsequently be tested for behavioral or developmental phenotypes caused by the removal of the neuron and hence defining the type of the neuron (Bargmann and Horvitz, 1991). For example, the AVG neuron is classified as a pioneer neuron for axon guidance of some of the VNC neurons due to the fact that its ablation leads to defective axon guidance in the neurons that develop after the AVG axon outgrowth (Durbin, 1987; Hutter, 2003).

1.8.3 Structure of the ventral nerve cord

Most of the *C. elegans* nervous system is situated in the head and is organized around a large pharynx. The head is rich for sensory neurons sending their sensory processes to the tip of the nose (Figure 1-4). The sensory neurons are of different types and their axons have receptors for mechano-, chemo-, osmo- and thermo-sensations. The soma of the sensory neurons, together with the soma of interneurons and some motor neurons are located between the two bulbs of the pharynx forming the brain of the worm. These cells send out processes that run circumferentially round the pharynx as a fiber bundle forming the nerve ring, which is the major region of the nervous system in the worm (White *et al.*, 1986). A large proportion of the processes in the ring enters and leaves on the ventral side forming the ventral nerve cord (VNC) of the animal (White *et al.*, 1976; White *et al.*, 1983; Chalfie and White, 1988). The VNC is formed of the major longitudinal axon tracts and it contains the axons of the neurons situated in the brain, in the lateral and tail ganglia, some neurons located in lateral sides of the animal. The VNC also contains axons of 75 motor neurons located very close to the ventral midline becoming a major part of the cord. These neurons innervate the body wall muscles on both the ventral and dorsal sides. All the motor neurons send their axons longitudinally as well as circumferentially (Figure 1-4). The circumferential axons are also known as commissures.

The *C. elegans* VNC is similar to the ventral or spinal nerve cord of vertebrate or insect systems, which consists of two axon bundles separated by a ventral midline structure called hypodermal ridge. The motor neuron somas are located very close to the ventral midline. The left and right fascicles of the VNC are asymmetric and most of the axons derived from the head or tail neurons are located on the right side of the VNC (Figure 1-5). The right axon tract is the major part of the VNC and some 50 axons passing through the VNC are bundled in this tract. On the other hand, the left VNC tract contains axons of only four neurons and is many times thinner than the right tract. The axons running through these tracts do not cross the tracts and are always fasciculate with each other in their own tracts, except at the beginning and end of the VNC where some axons cross the tracts and fasciculate in the opposite tract with respect to their soma positions. The VNC of *C. elegans* forms the major longitudinal nerve circuit along anterior-posterior axis of the animal (White *et al.*, 1976).

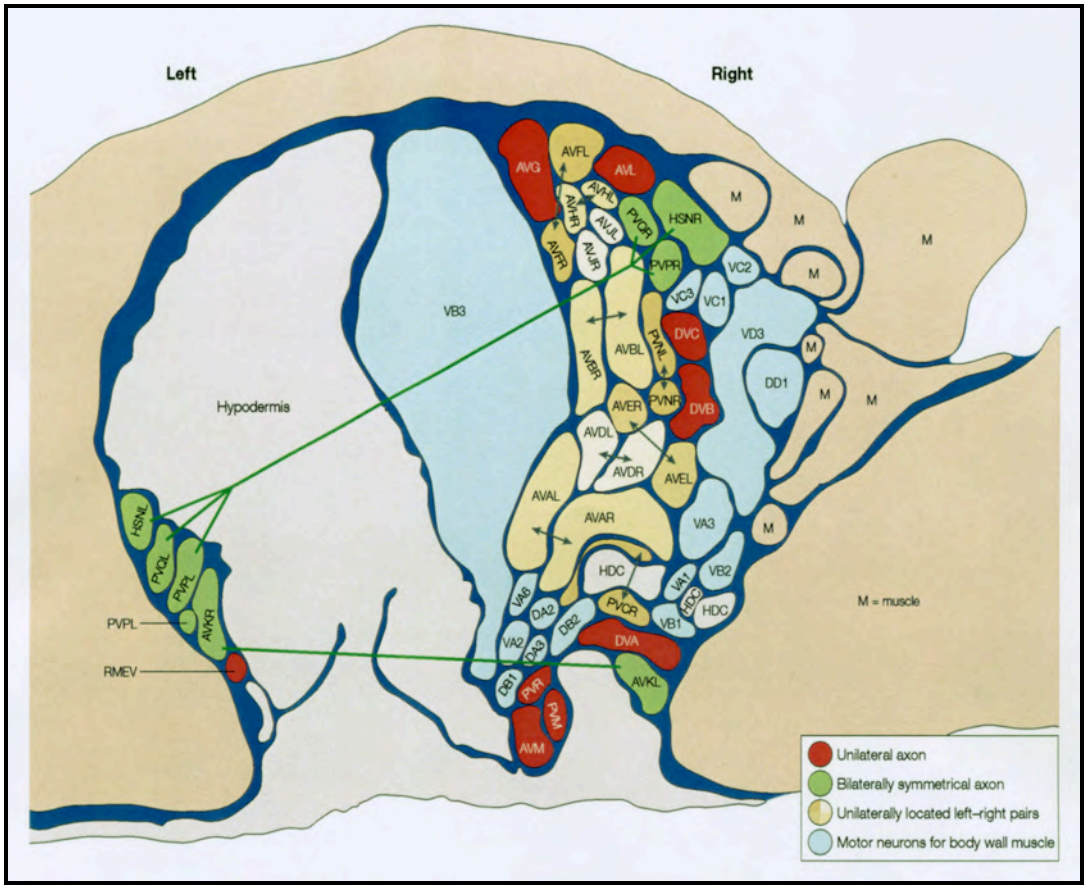

Figure 1-5: The Structure of the ventral nerve cord (VNC) of *C. elegans*. The right side of the VNC contains most of the axon. The left side contains four axons. All the axons in both tracts are tightly fasciculated and they do not cross the tracts (adapted from Hobert, 2002).

The motor neurons situated in the VNC are classified into six types based on their functions. The motor neurons VA, VB and VD innervate the ventral body muscles, while DA, DB and DD innervate the dorsal body muscles. The D-type (DD/VD) motor neurons are GABAergic inhibitory neurons, while all others are cholinergic excitatory neurons. Of the six types, DD, VD, DA and DB send their axons to dorsal cord and have commissures that run around the animals to reach dorsal nerve cord (Chalfie and White, 1988). All commissures of DD/VD motor neurons reach the dorsal cord from the right side of animal, while the commissures of DA/DB reach the dorsal side alternatively from left and right side of the animal forming the circumferential nerve circuits in the worm (Figure 1-4). Upon reaching the dorsal midline these axons form a tight fascicle of the dorsal nerve cord (Figure 1-4). Taken together, the neuronal circuits of *C. elegans* are stereotypic, simple and reproducible in different animals. This simplicity of the nervous system of *C. elegans* makes it a very good system to study the genetics of axon guidance mechanisms.

1.9 Solving the axon guidance problem

Typically the genetic analysis of biological processes in model organisms, for example in *C. elegans*, has been performed by forward genetic approaches (Herman, 1988; Jorgensen and Mango, 2002). The advantage of this is that the mutants are identified in a biological process by their interesting phenotypes (Chalfie and Jorgensen, 1998). Then the mutants are further studied to identify the underlying genes responsible for the observed phenotypes. The nematode *C. elegans* was chosen for forward genetic studies due to the fact that its biological traits are very well suited for the phenotype to genotype based genetic approach. *C. elegans* was introduced by Sydney Brenner primarily to study the embryogenesis and development of the nervous system. Brenner carried out his early chemical mutagenesis screens in *C. elegans* in 1967 and identified ~100 genes with interesting visible behavioral and morphological phenotypes such as Unc (uncoordinated movement), Dpy (dumpy body shape), Rol (roller movement), etc (Brenner, 1974). Of all the mutations identified by Brenner, the mutant class Unc proved to be very important to identify the genes involved in the nervous system development as many *unc* genes function in the neurons. Later several chemical mutagenesis screens were performed to identify genes giving phenotypes in different parts of the nervous system of *C. elegans* (Hedgecock *et al.*, 1990; McIntire *et al.*, 1992; Forrester and Garriga, 1997; reviewed by

Antebi *et al.*, 1997). Those screens were based on visible phenotypes like Unc movement or in some cases drugs affecting the nervous system functions were used to identify the drug resistant mutants (Brenner, 1974; Lewis *et al.*, 1980). On availability of green fluorescent protein (GFP) to tag the proteins present in different tissues in *C. elegans* (Chalfie *et al.*, 1994), GFP transgenic worms have been widely used to identify defects in axon guidance of a variety of different neurons in *C. elegans* (Zallen *et al.*, 1999, Wacker *et al.,* 2003). To date ~30 genes with an axon guidance phenotype have been characterized in *C. elegans* by the forward genetic approaches (Table 1-1). Many of these genes have been conserved across phyla and they have provided much insight into the axon guidance mechanisms in *C. elegans* as well as in other organisms. However, assigning the mutations isolated by forward genetics to the genes that give rise to phenotypes is cumbersome and lengthy process, which makes the large-scale genetic analysis of a biological process by this approach impractical. Secondly, forward genetic screens have repetitively identified mutations in already known genes.

Recently, upon availability of the complete sequence of the *C. elegans* genome large-scale reserve genetic approaches have been employed to identify genes involved in different biological processes (Jorgensen and Mango, 2002). In reverse genetic screens, the putative or known genes are studied for their roles in a biological process of interest by knocking out the function of the genes. The advantages of this strategy are that the nature of gene is known and by removing the activity of the gene product the possible function of the gene in a biological process can be deduced. They are also faster than the forward genetic screens and one also gets a choice of selecting a group of genes to identify their involvement in a biological process. After availability of the genome sequence of *C. elegans* a gene knockout approach based on chemical mutagenesis and identification of deletions in genes was developed for large-scale applications, but genome-wide application of this strategy is also limited (Jansen *et al.*, 1997). However, the genetic analysis of *C. elegans* and many other model organisms have been dramatically changed after discovery of the double strand RNA-mediated interference (RNAi) of gene expression in *C. elegans* (Fire *et al.*, 1998).

1.10 RNA interference and reverse genetic screens

The RNA interference is a biological phenomenon in which a cell-autonomous mechanism is activated in the presence of exogenously introduced or endogenously

produced dsRNA in the cell leading to degradation of the cognate mRNA (Figure 1-6). The exogenous dsRNA induces homology-dependent degradation of cognate mRNA leading to knockdown of the gene product by a conserved mechanism (reviewed by Hannon, 2002). The RNAi phenomenon was first discovered in *C. elegans* (Fire *et al.*, 1998) and subsequently shown to be present in many other organisms (Hannon, 2002). RNAi works by processing of dsRNA introduced in the cell by a complex of proteins called RNA induced silencing complex (RISC). The RISC complex consists of a dsRNA-specific RNase (DICER) along with other factors that cut the dsRNA into ~21 nucleotide dsRNA fragments called small interfering RNA (siRNA) molecules. These siRNA molecules are then primed to the cognate mRNA leading to degradation of mRNA by RISC. The RNAi technology has been adapted for different biological systems and has profoundly affected the way genetic screens are performed in post-genomic large-scale screens (Montgomery, 2004). In *C. elegans* RNAi is a particularly important technology due to the fact that it is very efficient in the worms and the delivery of dsRNA for knocking down of a gene function is easy compared to the other biological systems. In *C. elegans* RNAi is performed by different methods, for example, by injecting dsRNA (Fire, 1998) or by expressing dsRNA from a transgene in the worm (Tavernarakis *et al.*, 2000) or by feeding worms with bacteria expressing dsRNA (Timmons *et al.*, 2001). The later technique of feeding RNAi in *C. elegans* is particularly interesting due to the fact that it is simple, easily repeatable and scaleable for large-scale reverse genetic studies (Fraser *et al.*, 2000). However, RNAi shows variability and it depends on the nature of the genes under consideration. It also shows tissue specific variation, for example, the nervous system of *C. elegans* is refractory to the feeding RNAi technique (Tavernarakis *et al.*, 2000; Kennedy *et al.*, 2004). Nonetheless, RNAi is a very attractive method for reverse genetic screens in model organisms as well as in organisms in which genetic studies are difficult to perform. In *C. elegans* biology RNAi has become a standard reserve genetic approach for identification of genes in various biological events. To date several reverse genetic RNAi screens have been performed to identify genes involved in embryonic development (Zipperlen *et al.*, 2001), fat regulation (Ashrafi *et al.*, 2003), aging and longevity (Lee *et al.*, 2003) and more recently to understand cell division (Sonnichsen *et al.*, 2005) in *C. elegans*.

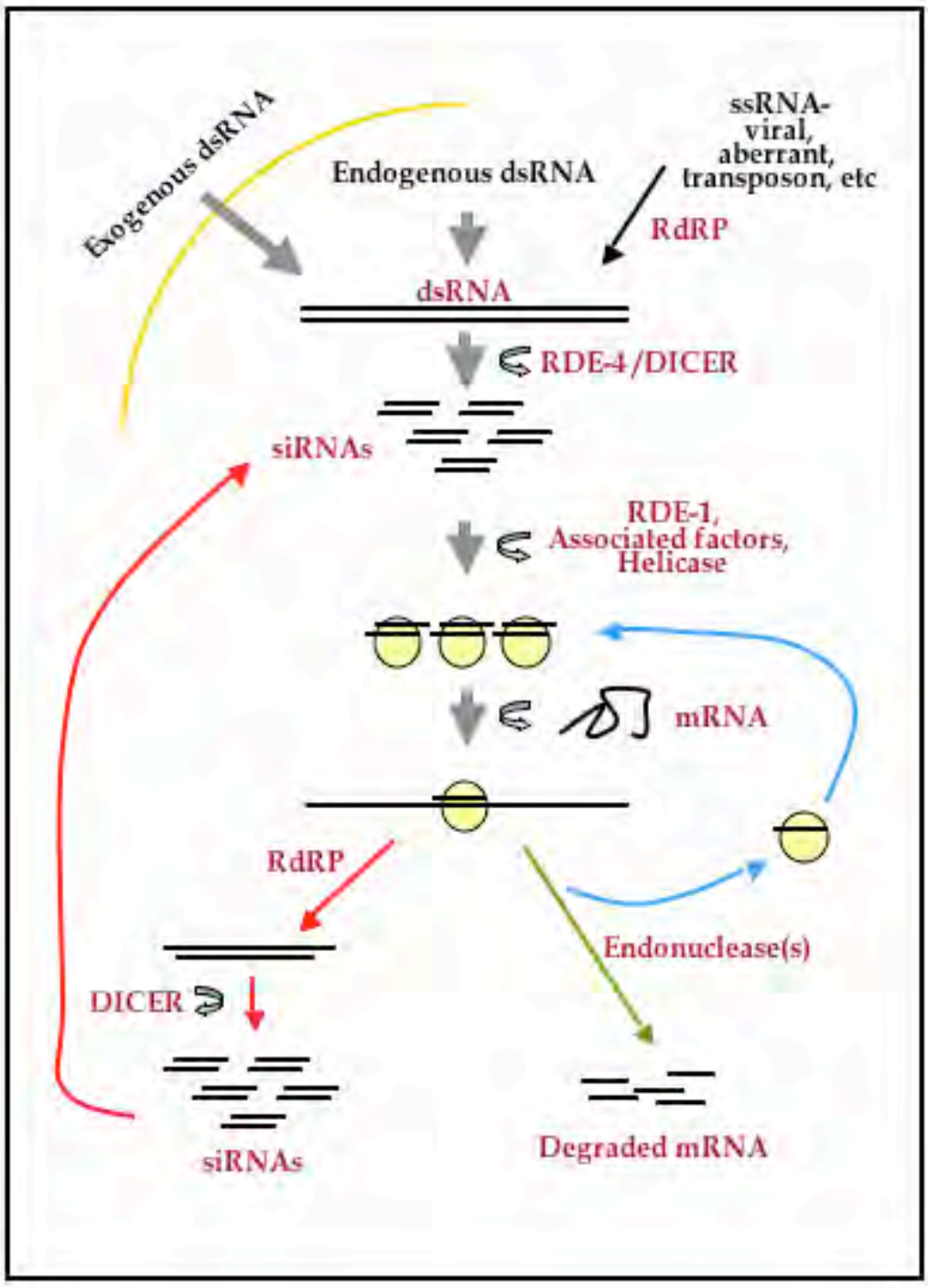

Figure 1-6: The mechanism of gene silencing by RNA interference in *C. elegans*. In the first step, RNase *dcr-1* acts upon the dsRNAs either introduced or produced in the cell by cleaving them into siRNAs. In next steps, siRNAs along with associated factors form the RISC complex that targets cognate mRNA for degradation by endonuclease(s) (green arrow). The RISC complex can also incorporate some of the degraded product of mRNA for a new round of RNAi cycle (blue arrows). The specificity of RISC complex is provided by the siRNAs that prime the cognate mRNA. siRNAs can also work as RNA-primers for RdRP-mediated dsRNAs generation from cognate mRNA, which are then subjected to a new amplifying around of RNAi cycle (red arrows).

1.11 Purpose of this work

Although several genes are known in *C. elegans* that are involved in axon guidance in various neurons (Table 1-1), they do not describe the complexity of neuronal networks observed in the nervous system of the worm. The goal of this work was to find out the missing and new components of the molecular machinery of axon guidance in a simple model organism. To this end, an RNAi-mediated reserve genetic screen was initiated to identify genes involved in axon guidance required in a subset of motor and interneurons of *C. elegans*. However, it has been previously reported that the neurons of *C. elegans* are refractory to the feeding or systemic RNAi technique (Tavernarakis *et al.*, 2000; Timmons *et al.*, 2001; Kennedy *et al.*, 2004). Therefore, we first had to solve the problem of inefficiency of systemic RNAi in the nervous system of *C. elegans* by a forward genetic screen to identify mutants of *C. elegans* that are efficient for RNAi in the nervous system. One of the RNAi efficient mutants was then used as a screening strain in a transgenic background for RNAi-mediated screening of genes located on chromosome I of the worm for novel axon guidance genes.

2 RESULTS

2.1 Overview of the work

In this study, we have performed a reserve genetic screen to identify genes involved in axon guidance mechanisms in *C. elegans*. Recently, the RNA interference (RNAi) phenomenon has been used in several genome-wide screens in *C. elegans* to identify genes involved in various biological processes like embryonic development (Zipperlen *et al.*, 2001), fat regulation (Ashrafi et *al.,* 2003) and aging and longevity (Lee *et al.*, 2003). All these screens have used the feeding method of dsRNA delivery to the cells of living animals to study the effect of down regulation of the targeted gene product on the development and behavior of the animals. However, the nervous system of *C. elegans* has not been subject to any feeding RNAi-mediated genetic screen, as the neurons of the worm are refractory to the uptake of the systemically delivered dsRNA molecules (Tavernarakis *et al.*, 2000; Timmons *et al.,* 2001; Kamath *et al.*, 2003).

To overcome the problem of lack of efficiency of the feeding RNAi in the nervous system, and to use the RNAi mechanism for genetic screening in *C. elegans*, we have performed this work in two stages. In the first stage, we have taken advantage of the simple genetics in *C. elegans* to do a chemical mutagenesis screen to isolate mutants of *C. elegans* that showed robust suppression of gene expression in the nervous system of the worm, when dsRNA molecules were delivered by the feeding method. The mutants we have isolated for feeding RNAi-mediated gene knockdown in the nervous system of *C. elegans* define a class of genes that are recently identified as RNAi supersensitive genes, as some of the phenotypes shown by our mutants are similar to the previously identified RNAi hypersensitive mutants (Simmer *et al.,* 2003; Kennedy *et al.*, 2004). These supersensitive mutants are useful tools for the identification of genes involved in a biological process in organs like the nervous system, which are less sensitive to the feeding RNAi-mediated gene knockdown process.

In the second stage of this work, we have used one of the mutants of *C. elegans* that showed marked increase in the efficiency of RNAi in the nervous system as a supersensitive screening strain for searching the genes located on chromosome I for axon guidance phenotypes. We used the supersensitive mutant in combination with a fluorescent transgene that labels the major neuronal circuits formed by interneurons and motor neurons of *C. elegans* by expressing a fluorescent protein in the axons. The major

aim of this work was to take advantage of the efficiency of RNAi-mediated rapid genetic screens in combination with the supersensitive mutant that allows feeding RNA interference in the nervous system of *C. elegans,* to identify genes involved in axon guidance.

2.2 Feeding RNAi in the nervous system of *C. elegans*

To access the efficiency of RNAi in the nervous system we performed feeding RNAi experiments for the loss of GFP fluorescence in neuronal and non-neuronal cells in *C. elegans*. We started with comparative experiments where the feeding RNAi efficiency in the nervous system was compared to that of the muscle cells. It has been reported that the non-neuronal cells of *C. elegans* like the muscle cells are more sensitive to the feeding RNAi–mediated gene knockdown compared to the neurons of the worm (Winston *et al.*, 2002). Moreover, different feeding RNAi protocols have given varying results for RNAi in the nervous system due to the sensitivity of RNAi to experimental conditions like the temperature and induction time used for feeding bacteria to produce dsRNAs prior to feeding RNAi in the worm (Kamath *et al.*, 2000).

To test the feeding RNAi efficiency in the nervous system in our feeding RNAi experimental setup (for details see Materials and Methods), we compared the feeding RNAi efficiency in muscle cells and neurons of *C. elegans*. To this end, we used two worm strains: VH288 (*him-4::gfp*) expresses GFP under the control of the *him-4* promoter only in the body wall muscle cells but not in the neurons (Vogel and Hedgecock, 2001), and VH41 (*unc-119::gfp*) expresses GFP under the control of the *unc-119* promoter in all the neurons as well as some head muscle cells in *C. elegans* (Maduro and Pilgrim, 1995; Table 2-1). It has been previously reported that the muscle cells of *C. elegans* are strongly sensitive to the RNAi-mediated gene knockdown compared to the neurons (Timmons *et al.,* 2001). When VH288 animals were fed bacteria expressing *GFP* dsRNA, more than 95% of the treated animals showed loss of GFP in all the labeled muscle cells within 24 h of treatment compared to the control animals fed on normal bacterial food (Table 2-1). However, in the case of VH41 animals the same treatment only eliminated the expression of GFP in the head muscle cells without any effect on the expression of GFP in the neurons or their axons that are also refractory to the treatment (Figure 2-1, Table 2-1). The suppression of GFP expression in the muscle cells was rapid and reversible. Treating the VH41 worms for longer period of time did not show any change in the expression

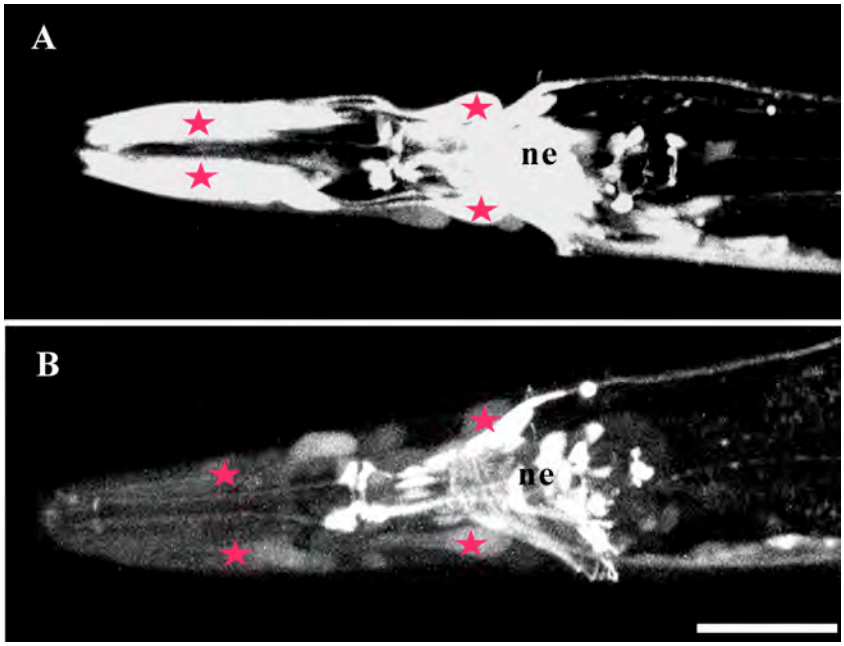

Figure 2-1: Inefficient RNAi in the nervous system of *C. elegans*. **A)** VH41 animals fed with the control bacteria do not show any reduction in GFP fluorescence in head muscle cells (asterisks) or neurons and the nerve ring (ne). **B)** After feeding VH41 animals with the bacteria producing *GFP* dsRNA show marked reduction of GFP fluorescence only in head muscle cells (asterisks) but not in neurons and the nerve ring (ne). Scale bar, 20 μM.

Table 2-1: Effect of RNAi on GFP fluorescence in muscle cells and neurons of *C. elegans* (n > 100).

Strain	Genotype	GFP expressing cells	Feeding vector	% Animals without GFP in	
				Muscle cells	Neurons
VH288	*him-4::gfp*	Body wall muscle cells	Control[a]	0	-
VH288	*him-4::gfp*	″	*GFP* dsRNA	>95[b]	-
VH41	*unc-119::gfp*	All neurons and head muscle cells	Control	0	0
VH41	*unc-119::gfp*	″	*GFP* dsRNA	95	0
VH624	*nre-1, unc-119::gfp*	″	Control	0	0
VH624	*nre-1, unc-119::gfp*	″	*GFP* dsRNA	95	~90

[a] Control feeding bacteria contained the empty feeding RNAi vector L4440.
[b] About 5% animals showed incomplete RNAi as observed by fluorescence in a few muscle cells in the treated animals.

level of GFP in the nervous system. These experiments showed that RNAi is restricted to the non-neuronal cells in *C. elegans* and is prevented in neurons by unknown mechanisms. Based on these and previously reported observations (Tavernarakis *et al.*, 2000), we concluded that the nervous system of *C. elegans* must have some genetic factors that prevent the RNAi in the neurons when dsRNA is delivered by the feeding RNAi protocol. We reasoned that this deficiency could be overcome by a forward genetic approach by the identification of mutants that show RNAi-mediated gene expression down regulation in the nervous system of *C. elegans*.

2.3 Isolation of neuronal RNAi efficient mutants of *C. elegans*

To address the inefficiency of the nervous system of *C. elegans* to the feeding RNAi method, we performed a forward genetic screen to isolate mutants that are efficient for feeding RNAi in the nervous system of the worm. We started with the worm strain VH41 (*unc-119::gfp*) as a parental strain for a chemical mutagenesis screen. The integrated *unc-119::gfp* transgene shows strong expression of GFP in all parts of the nervous system including the nerve ring, the ventral and dorsal nerve cords and axons of laterally located neurons in *C. elegans* (for details see Figures 2-1A, 2-3A-B; Maduro and Pilgrim, 1995). It is also expressed in non-neuronal cells like head muscle cells located to the anterior of pharynx (Figure 2-1A, asterisks).

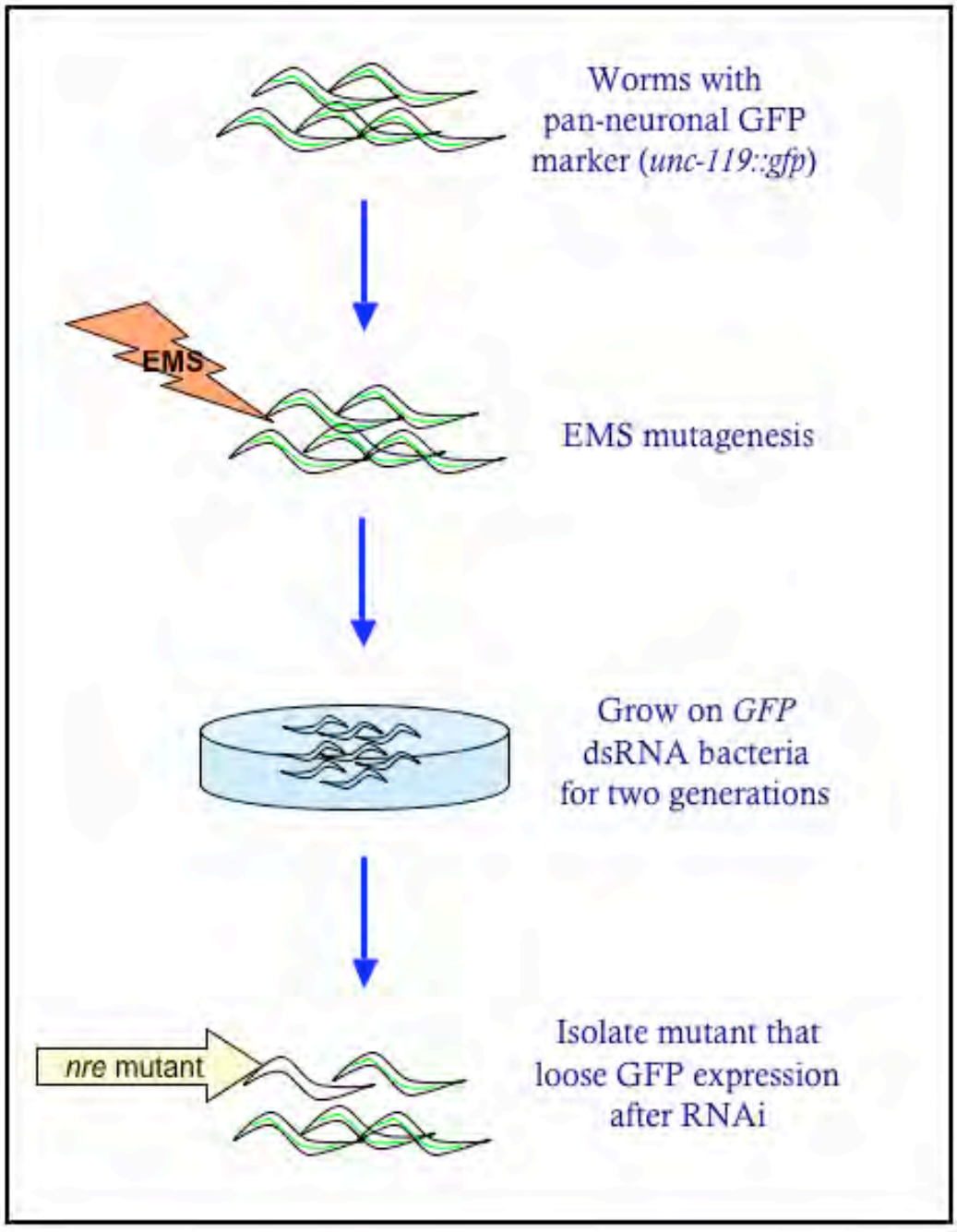

Figure 2-2: Scheme of genetic screen to isolate *n*euronal *R*NAi *e*fficient (*nre*) mutants of *C. elegans*. The VH41 (*unc-119::gfp*) hermaphrodites were mutagenized with EMS and mutants that showed loss of GFP in the nervous system upon feeding the bacteria expressing *GFP* dsRNA were isolated as *nre* mutants.

We used the VH41 strain in an ethyl-methane-sulfonate (EMS) mutagenesis screen to identify mutants that show marked loss of GFP expression on feeding RNAi in the nervous system. Figure 2-2 shows the scheme used for the identification of mutants that are efficient for feeding RNAi-mediated gene knockdown in the nervous system of *C. elegans* (for details see Materials and Methods). In brief, about 1000 young adult hermaphrodites of the VH41 strain were treated with 50 mM of EMS in M9 buffer for 4 h and allowed to recover for another 4 h on NGM plates with normal bacteria food. Subsequently 5-7 healthy looking and normally scrawling animals per plate were transferred onto 10 NGM plates with the bacteria expressing *GFP* dsRNA. They were allowed to produce self-progeny for two generations leading to the generation of ~20000 mutagenized genomes in total. In the next step, F2 progeny of the mutagenized animals were screened in the presence of sufficient *GFP* dsRNA bacteria food for the mutants that showed no GFP fluorescence in the nervous system. We reasoned that growing the animals immediately after mutagenesis on the *GFP* dsRNA plates would lead to isolation of mutants that rapidly show the RNAi-mediated suppression of neuronal genes like the GFP transgene (*unc-119::gfp*) used in the parental screening strain.

Two independent mutants (*hd20, hd21*) were isolated that showed strong suppression of GFP fluorescence in the nervous system upon RNAi by the feeding method. These mutants were tested several times alternatively on normal and *GFP* dsRNA bacteria food to confirm that the loss of GFP in the nervous system is a result of the presence of *GFP* dsRNA in the bateria food. We selected the mutant *hd20* for further studies as it gave consistent and strongest Nre (for neuronal RNAi efficient) phenotype, and the gene defined by *hd20* was named *nre-1*. The *nre-1* mutant was backcrossed three times to the wild-type animals to remove background mutations, however, the original pan-neuronal GFP marker (*unc-119::gfp*) was maintained in the new strain for ease of identification of the Nre phenotype. The new strain was called VH624 (*nre-1(hd20); unc-119::gfp*) and was used for further experimentation. This strain does not show any change in the expression of *unc-119::gfp* marker compared to the VH41 strain indicating no effect of the *nre-1* mutation on the expression of neuronal genes.

2.4 Primary characterization of the *nre-1* mutant

To facilitate further use of the *nre-1* mutant in genetic experiments, we performed primary studies on this mutant with respect to its Nre and temperature sensitive sterile

Table 2-2: The *nre-1* mutants have a reduced brood size at 25°C.

Genotype	Average brood size	
	20°C	**25°C**
Wild-type (N2)	270 ± 18[a]	169 ± 10[a]
VH41 (*unc-119::gfp*)	241 ± 18[a]	147 ± 20[a]
VH624 (*nre-1; unc-119::gfp*)	159 ± 18[a]	7 ± 2[b]

[a]n = 4, [b]n = 10

phenotypes. The temperature sensitive sterility of the *nre-1* mutant was observed during the experiments performed at 25°C as described below. The *nre-1* animals grew normally at 15° and 20°C and did not show any morphological or behavioral defects at these temperatures. The fecundity of hermaphrodites was also normal when compared to wild-type animals. However, when the number of eggs laid per animal (brood size) at 20°C was counted, the *nre-1* animals showed a decrease of about 41% in the brood size compared to wild-type animals (Table 2-2). At 20°C wild-type animals had an average brood size of 270 eggs, while the *nre-1* animals had an average brood size of 159 eggs. This decrease in the brood size pointed out that the *nre-1* mutation may have a role in germline development in *C. elegans* as previous mutations isolated in screens for RNAi supersensitive genes also have roles in germline development that affect the brood size of the mutant worms (Simmer *et al.,* 2003; Kennedy *et al.*, 2004).

To test if the elevated temperature enhanced the germline phenotype of *nre-1* animals, we examined the brood size of *nre-1* mutants at 25°C. When the *nre-1* L4 larvae were grown at 25°C for more than 48 h no obvious behavioral defects were observed. However, the brood size of the mutant worms was drastically reduced to about 7 eggs compared to wild-type animals (Table 2-2). In many cases, the *nre-1* worms due to their temperature sensitive sterile phenotype produced no progeny at all. The *nre-1* embryos that were able to hatch, however, later arrested at different developmental stages with no further growth even when they were shifted to the normal growth temperatures. These results confirmed that the *nre-1* mutation caused unknown defects in the germline development in *C. elegans*. These results are also similar to the observations made for other RNAi supersensitive strains of *C. elegans* (Simmer *et al.,* 2003; Kennedy *et al.*, 2004), showing

that the genes involved in RNAi in the worm are involved in biological events in the germline and larval development, besides being required for RNAi in the nervous system.

2.5 Inhibition of GFP expression in the nervous system of *nre-1* mutant

To demonstrate that the *nre-1* mutant shows specific suppression of GFP expression in the nervous system by the feeding RNAi method, we fed VH624 animals either with control bacteria food or *GFP* dsRNA bacteria food and allowed them to growth for 3 d at 20°C. The control bacteria contained the original empty feeding RNAi plasmid vector (L4440) used to create the RNAi library without any dsRNA transcribing element in its multicloning site (Timmons and Fire, 1998). The F1 progeny of treated animals were scored for the loss of GFP fluorescence in the nervous system by fluorescence microscopy. Control bacteria food had no effect on the expression level of GFP in the nervous system of VH624 animals (Figure 2-3A-B). The GFP expression level in these animals was comparable to that of the wild-type VH41 animals (Figures 2-1A and 2-3A). However, the *nre-1* mutant fed on *GFP* dsRNA bacteria showed marked suppression of GFP expression in the nervous system (Figure 2-3C-D). More than 90% *nre-1* animals showed no GFP in the nervous system at all (Table 2-1, shaded rows). However, a few animals had incomplete RNAi in the nervous system and they expressed low levels of GFP in some of the neurons (Figure 2-3C-D). The RNAi effect was reversible and returning the *nre-1* animals grown on *GFP* dsRNA bacteria food to control bacteria food released the suppression of GFP expression in the nervous system and restored the fluorescence level to the normal after 24 h. The *nre-1* animals grown on *GFP* dsRNA producing bacteria for several days did not show any other obvious phenotype. The incomplete suppression of GFP expression in a few neurons of some *nre-1* animals was used in further experiment as an indicator of the working of RNAi in the nervous system as the animals that are affected by the feeding of *GFP* dsRNA can be easily identified by the presence of GFP fluorescence in those few neurons of their nervous systems. This is particularly helpful when a fluorescent transgene is present as a hemizymous copy. These experiments showed that the *nre-1* mutation defines a gene in *C. elegans* that is part of the RNAi machinery in the nervous system. In this study we used the *nre-1* mutant as a tool to identify genes involved in axon guidance of a subset of neurons of *C. elegans*.

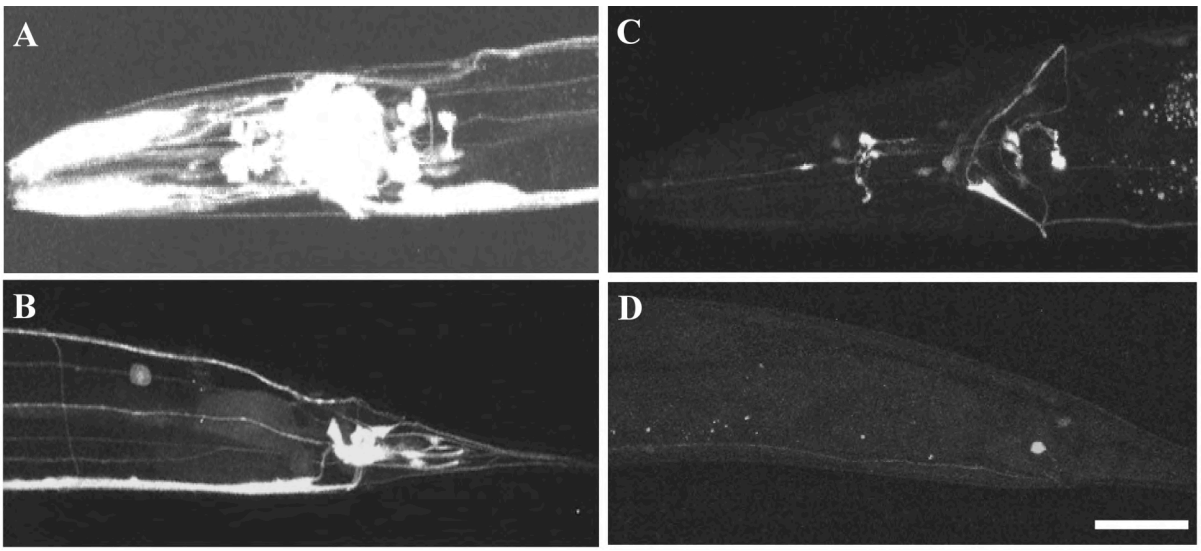

Figure 2-3: Phenotype of the *nre-1* mutant. **A-B)** The *nre-1* animals when fed with control bacteria did not show any change in the expression level of GFP in the nervous system. The GFP was robustly expressed in the head (A) and tail (B) neurons and their axons forming the ventral and dorsal nerve cords. **C-D)** After feeding the *nre-1* animals with the bacteria expressing dsRNA for GFP, the expression of GFP in the nervous system was robustly suppressed in all but a few head (C) and tail (D) neurons. The nervous system was visualized by the *unc-119::gfp* marker that is expressed in all neurons of the worm. Scale bar, 20 μM.

2.6 Mapping of the *nre-1* mutation

To identify the genomic position of the *nre-1* locus we used two-factor analysis to establish the linkage group of the mutation. At first, we used standard *C. elegans* mapping strains with mutations in *dpy* genes. In *C. elegans dpy* genes are required for the cuticle and epidermis formation, and lesions in these genes lead to small and thick or dumpy worms (Brenner, 1974). The *dpy* genes have been extensively used as chromosome and map position markers in genetic mapping experiments in the worm as they are very easy to distinguish and to score based on their unique Dpy phenotypes. To map the chromosomal location of the *nre-1* mutation, VH624 hermaphrodites were crossed to wild-type males to obtain heterozygous males of the genotype (*nre-1*/+; *gfp*/+). These heterozygous males were then crossed to hermaphrodites of various Dpy homozygous (*dpy*/*dpy*) mutant strains representing linkage groups (LG) I to V and were allowed to grow on control bacteria food plates.

In the next step, the GFP positive F1 hermaphrodites of genotypes (*nre-1*/+, *dpy*/+, *gfp*/+) or (+/+, *dpy*/+, *gfp*/+) were selected and fed with bacteria expressing *GFP* dsRNA. For further analysis we selected only those animals some of whose F2 progeny showed the loss of GFP expression in the nervous system on the plates for feeding GFP RNAi, that is, we selected the animals of genotype (*nre-1*/+, *dpy*/+, *gfp*/+). Next, in the F2 generation Dpy progeny were scored for the loss of GFP fluorescence in the nervous system to identify animals with genotypes of (*dpy*/*dpy; gfp*/*gfp; nre-1*/*nre-1*) and (*dpy*/*dpy; gfp*/*gfp; nre-1*/+). The animals homozygous for the *nre-1* mutation, that is, the animals with the genotype (*dpy*/*dpy; gfp*/*gfp; nre-1*/*nre-1*) showed marked reduction in GFP fluorescence in the nervous system (Table 2-3, shaded column). In this case, if the *nre-1* mutation is in the same linkage group as anyone of the five *dpy* genes, then F2 worms with that *dpy* gene should not have the *nre-1* mutation and hence will not show any reduction in GFP fluorescence in the nervous system after feeding RNAi treatment. We observed that the LGI-V did not represent the linkage group of the *nre-1* mutation, as all homozygous *dpy* mutants also had the *nre-1* mutation causing RNAi-mediated GFP loss in the nervous system. This data pointed to the absence of the *nre-1* locus onto chromosomes I to V, indicated its linkage to the X chromosome. Table 2-3 summarizes the two-factor analysis data for the five linkage groups representing chromosomes I to V of *C. elegans*.

Table 2-3: Two factor analysis of the *nre-1* mutant.

Chromosome (Gene)	**F2 *dpy*, green animals**	**Reduction in neuronal GFP[a]**	**No- Reduction in neuronal GFP[b]**
I (*dpy-5*)	11	8	3
II (*dpy-10*)	19	12	7
III (*dpy-17*)	16	9	7
IV (*dpy-13*)	8	8	0
V (*dpy-11*)	15	9	6

[a]Indicative of the presence of the *nre-1* mutation.
[b]Indicative of the absence of the *nre-1* mutation.

In *C. elegans*, male animals have one copy of the X chromosome, while hermaphrodites possess two copies. Hence, the male cross progeny of a male and hermaphrodite mating event always get their one copy of X chromosome from their hermaphrodite parent worm. This asymmetric distribution of X chromosome comes as an advantage for identification of the X-linked mutations, because they appear in all F1 male progeny of any mating event between a wild-type male and X-linked homozygous mutant hermaphrodite. During mating experiments we observed that the number of males expected from the crosses between wild-type males and *nre-1* hermaphrodites was unexpectedly low compared to normal situation when 50% of the cross progeny of any male-hermaphrodite mating is male progeny. To verify the presence of *nre-1* on the X chromosome, we carried out a two-factor experiment in which a X-chromosomally integrated GFP transgene (*oxIs12*) was used. The males heterozygous for *oxIs12* were crossed to *nre-1* hermaphrodites and the GFP positive F1 hermaphrodites of the genotype (*nre-1*/+, *gfp*/+) were allowed to grow at 25°C. As the *nre-1* mutation is temperature sensitive sterile, at 25°C homozygous animals do not produce any progeny (see Section 2.4). We used GFP-labeled X chromosome to trace the green heterozygous animals that would appear as F2 progeny of the animals with the genotype (*nre-1*/+, *gfp*/+). At 25°C all the F2 animals (hemizygous for both *nre-1* and *oxIs12*) were GFP positive confirming the location of the *nre-1* mutation on X chromosome. In similar experiments using GFP-labeled chromosome III and IV, we showed that the *nre-1* locus is not linked to these chromosomes (Table 2-4). These data confirmed the location of the *nre-1* mutation on X chromosome of *C. elegans*.

Table 2-4: Two factor analysis of the *nre-1* mutant.

Chromosome (integrated GFP transgene)	F1 animals	F2 animals all green	F2 animals green + non-green
X (*oxIs12*)	9	9	0
IV (*hdIs14*)	10	3	7
III (*rhIs4*)	11	3	8

2.7 RNAi in the nervous system of *nre-1* mutant

The *nre-1* mutant is efficient for RNAi in the nervous system, as shown by significant reduction of GFP fluorescence in the nervous system after feeding the mutant with bacteria expressing *GFP* dsRNA (see Section 2.5). However, to determine the efficacy of *nre-1* mutant to phenocopy the neuronal phenotypes of know genes that are cell-autonomously required for neuronal development and/or axon guidance in *C. elegans,* we tested a set of neuronal genes by the feeding RNAi method. To this end, we created a fluorescent transgenic strain VH715 that contained the *nre-1* mutation and a chromosomally integrated transgenic array *hdIs17*. The *hdIs17* array consists of three yellow fluorescent protein (YFP) fusion genes, which are under the control of promoters for three different neuronal genes, namely *glr-1, unc-47* and *unc-129*, labeling axons of major interneurons and motor neurons of *C. elegans* that run along the anterior-posterior and dorsal-ventral axes of the animal (detailed in Section 2.8).

To test the *nre-1* mutant in feeding RNAi assays we selected five neuronally expressed genes as listed in Table 2-5. Many genetic mutations are available in these genes, they function cell-autonomously and they define many aspects of conserved genetic pathways involved in the development of the nervous system in *C. elegans* (see Table 1-1). Mutations of the *unc-13* gene result in severe uncoordinated movement due to defective neurotransmission. It is a novel conserved protein involved in vesicle priming events at the synapses and is expressed in many types of neurons (Richmond *et al.,* 1999). The *unc-14* gene encodes a novel protein expressed in the cell bodies and axons of almost all the neurons. It is proposed that *unc-14* is a regulator of the *unc-51* protein kinase and acts in axon elongation and guidance (Ogura *et al.,* 1997). *unc-40* is a netrin receptor and is expressed in many neurons including all motor neurons that send their commissures circumferentially towards the dorsal side in *C. elegans* (Chan *et al.*, 1996). The *unc-73* gene encodes a guanine nucleotide exchange factor similar to the Trio protein

Table 2-5: Characterization of the *nre-1*mutant.[a]

Gene	Description	Mutant[b]	RNAi in[c]	
			wt	*nre-1*
unc-13	C1 domain	40	0	30
unc-14	Novel	30	0	30
unc-40	Netrin receptor	>90	0	>80
unc-73	GTPase	>90	0	>80
lin-11	Transcription Factor	50	0	55

[a]Axons were visualized with the *hdIs17* marker. The % nervous system defects were scored (n >100).
[b]Alleles used were: *unc-13(e450); unc-14(e57); unc-40(e271); unc-73(e936);* and *lin-11(n566).*
[c]Each gene was subjected to at least three independent RNAi experiments.

in the nervous system, and is required for outgrowth of commissures of D type motor neurons and axons of amphid neurons (Steven *et al.*, 1998). While *lin-11* is a LIM homeodomain transcription factor that affects neuronal development and fate specification of many neurons in *C. elegans* (Sarafi-Reinach *et al.,* 2001).

To test whether the *nre-1* mutant shows RNAi phenotypes that are similar to the phenotypes observed in genetic mutants of the abovementioned genes, VH715 animals were fed with bacteria expressing dsRNA for the genes described in Table 2-5. They were allowed to grow at 20°C for 5-7 days and their F1/F2 progeny were examined for phenotypes in the nervous system by fluorescence microscopy. We scored for gross defects in axon guidance and nervous system morphology and found that feeding RNAi caused moderate to severe defects in the treated *nre-1* animals for the genes tested (Table 2-5). The RNAi experiments were repeated at least three times for each of the genes. The penetrance of RNAi phenotypes was between 30% to >80% compared to wild-type experiments and were similar to the same defects present in the genetic mutations of the five genes tested. The genetic mutations that were used as positive controls are null alleles of the genes and showed the strongest phenotype among their other alleles. These experiments demonstrated that the *nre-1* mutation we isolated is capable of RNAi-mediated gene knockdown in the nervous system and is able to phenocopy the loss-of-function phenotypes of known genes that function in the nervous system of *C. elegans*.

2.8 Visualization of axons in *C. elegans*

In *C. elegans* the complete set of GABA neurons, DA/DB excitatory motor neurons, and a number of interneurons can be labeled with fluorescent proteins expressed under the

Table 2-6. Genes and their neuronal expression patterns

Gene	Major neurons	Number of labeled neurons	Location of axons
unc-47	DD1-6, VD1-13	19	VNC, commissures
unc-129	DA1-7, DB1-7	14	VNC, commissures
glr-1	AVA, AVB, AVD, AVE, AVJ	5	VNC, nerve ring

control of the promoters of *unc-47* (GABA transporter; McIntire *et al.,* 1997), *unc-129,* (TGFβ ligand; Colavita *et al.*, 1998), and *glr-1* (glutamate receptor subunit; Hart *et al.*, 1995) genes, respectively. Previously, the promoters of these genes have been used to express fluorescent proteins to perform genetic screens to identify mutants that caused phenotypes in the nervous system of *C. elegans* (for example, Zallen *et al.*, 1999; Hutter, 2003). Here we selected the promoters of *unc-47, unc-129* and *glr-1* genes to drive the expression of the YFP in a subset of neurons because: (i) they label the neurons that send their axons along the anterior-posterior and dorsal-ventral axes of the animal; (ii) the majority of axons that are labeled by these promoters are long compared to the axons of other neurons providing an advantage in identification of subtle defects in axon guidance; and (iii) these promoters are strongly expressed and their expression do not overlap leading to a consistent lighting up of major parts of the nervous system of *C. elegans* (Figure 2-4).

We created a transgenic strain VH477 that contained the chromosomally integrated transgenic array *hdIs17* (*unc-47::yfp, unc-129::yfp, glr-1::yfp, rol-6(su1006)*) having the promoters of *unc-47, unc-129* and *glr-1* genes fused to the YFP coding sequence in three independent fusion genes (for details see Materials and Methods). The semi-dominant *rol-6(su1006)* allele was included in the array as it causes rolling movement in transgenic animals providing the convenience of access to the different sides of the animal to observe the anatomy of the nervous system. The marker *hdIs17* is robustly expressed in the nervous system of *C. elegans* labeling the major tract of the VNC (Figure 2-4A), the dorsal nerve cord (Figure 2-4B) and commissures of motor neurons that form the circumferential axonal circuits in *C. elegans* (Figure 2-4C-D). In total the *hdIs17* marker prominently labels about 38 neurons that send long axons. Table 2-6 summarizes the types of neurons in which the three genes, *unc-47, unc-129* and *glr-1,* are expressed as well

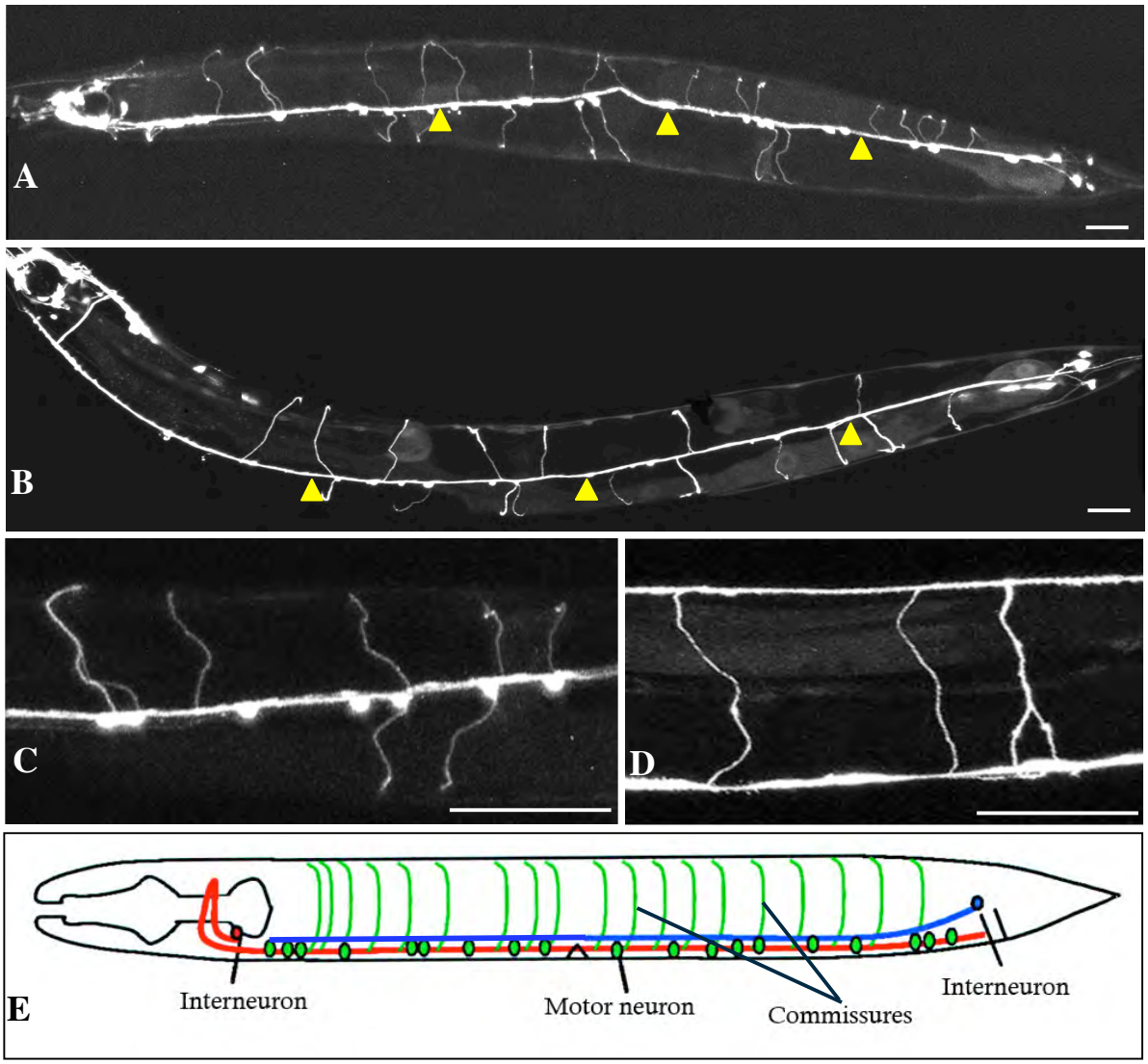

Figure 2-4: Expression pattern of the *hdIs17* marker in the nervous system of *C. elegans* (in the strain VH477). **A)** Ventral view showing the ventral nerve cord (arrowheads), the axons of interneurons and motor neurons are labeled. **B)** Dorsal view, commissures of motor neurons come together to form the dorsal nerve cord (arrowheads). **C)** Ventral close-up view showing a part of the VNC, somas of motor neurons are roughly placed equidistance with unbranched axons traveling towards the dorsal side. **D)** Side close-up view showing unbranched commissures of *C. elegans*. **E)** A representative diagram depicting the trajectories formed by axons of interneurons and motor neurons labeled by *hdIs17*. In all images, anterior is to the left. Scale bars, 20 μM.

as the location of their axons in the body of the worm. The expression of YFP was restricted to the nervous system and no non-neuronal cell was observed with YFP fluorescence. Figure 2-4E shows the sketch of the patterns formed by the axons of some of the interneurons and motor neurons that are labeled the *hdIs17* marker transgene. For the RNAi mediated screen we created a new worm strain, VH715, that combined the *nre-1* mutation and the *hdIs17* maker together giving a strain sensitive for neuronal RNAi with part of its nervous system labeled with YFPs. The expression pattern of the *hdIs17* marker in VH715 did not show any change compared to VH477 showing no effect of the *nre-1* mutation on the expression of the YFP transgenes.

2.9 Strategy for a feeding RNAi axon guidance screen

To perform rapid screening of the genes on chromosome I to identify genes involved in axon guidance in the worm we employed the *nre-1* strain as a tool in a feeding-RNAi-mediated genetic screen. Since the *nre-1* strain is supersensitive to RNAi-mediated gene knockdown in the nervous system (Section 2.7), we reasoned that it should be possible to identify genes that affect the nervous system function like axon guidance in this strain, when it is used as a background strain in the screening procedure that uses feeding as a method of dsRNA delivery in *C. elegans*. To this end, a screening strategy was developed in which the *nre-1* mutation was used as a supersensitive background in a transgenic worm strain that expressed the YFP fusion genes in a subset of interneurons and motor neurons under the control of the promoters of three neuronal genes. A new worm strain VH715 was created for the purpose this screen (Section 2.8).

As schematically depicted in Figure 2-5, we fed VH715 animals with the library of bacteria expressing dsRNA for ~86% of the genes on chromosome I. The individual bacterial clones from the library were grown in 96-well bacterial culture plates and were seeded onto 3.5-cm diameter NMG plates containing IPTG for the induction of dsRNA production in the bacteria. Then about 5 L3-L4 stage animals of strain VH715 were transferred to each plate and were allowed to grow for 5-7 days at 20°C. The progeny of the treated animals were washed off from the plates, mounted on the object slides with agar pads, and were examined for visible defects in their fluorescently labeled axons by fluorescence microscopy (for details see Materials and Methods). We screened 2425 clones representing 2416 genes located on chromosome I of *C. elegans*. Using a criterion of >30-50% gross defects in the axons of labeled neurons we identified a number of candidate genes for axon guidance phenotypes. These genes were rigorously retested in

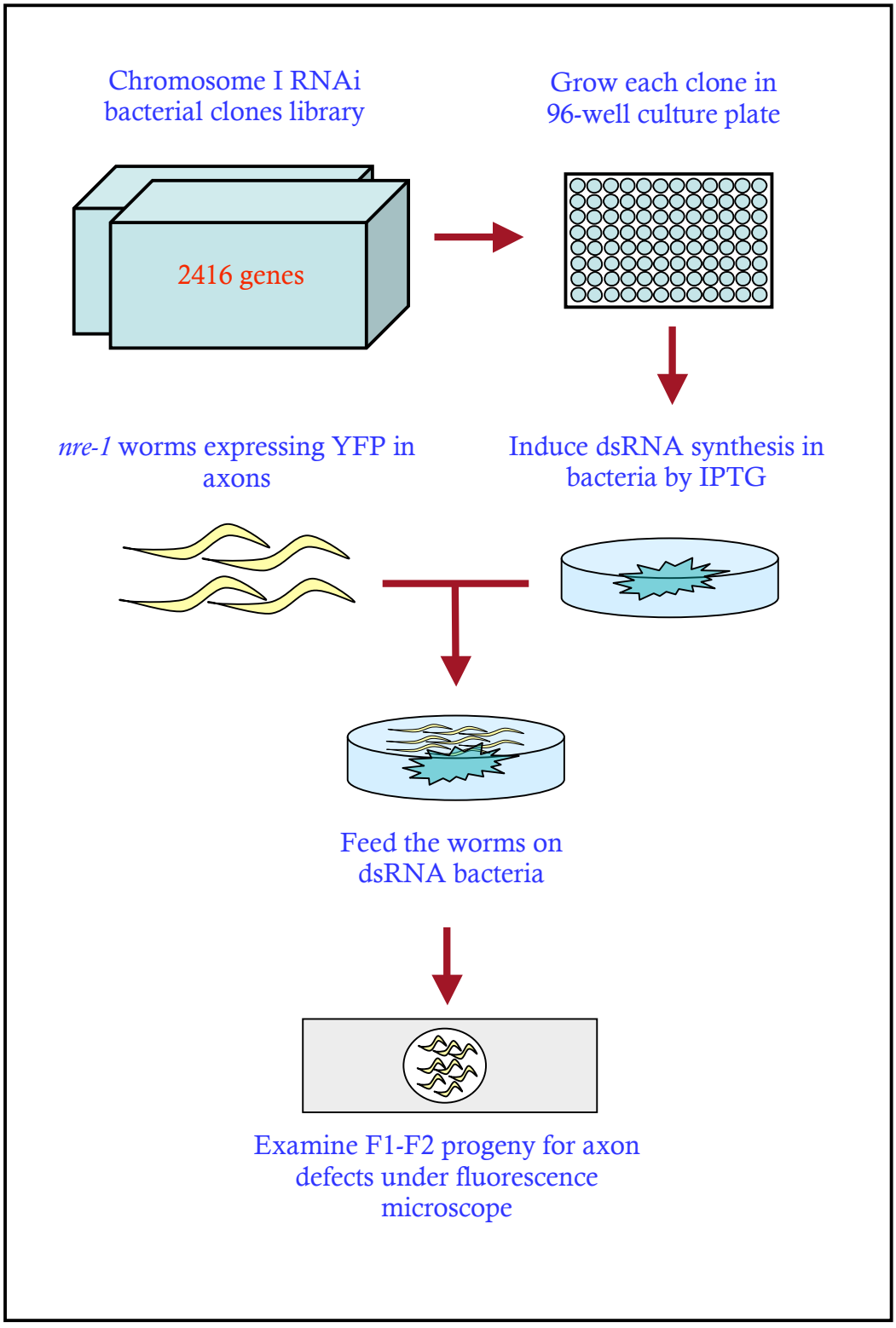

Figure 2-5: Schematic representation of the strategy used for feeding RNAi-mediated genetic screen for axon guidance genes in *C. elegans*.

further RNAi experiments to confirm their roles in axon guidance mechanisms (see Section 2.10).

In this screening protocol we selectively labeled axons of some interneurons and all the motor neurons located in the VNC of *C. elegans* as the circuitry made of these axons forms the major neuronal network and their stereotypic patterns provide ease in the identification of axon guidance defects in the network. The screening strategy used here is of general utility to any RNAi-mediated genetic screens in the nervous system of *C. elegans*. Besides the approach is high-throughput and repeatable for several times in different experimental settings; for example, the fluorescent protein can be expressed in different classes of neurons to identify genes involved in the development of those neurons.

2.10 Identification of genes involved in axon guidance on chromosome I

To identify genes on chromosome I that are involved for axon guidance in *C. elegans*, we started with the feeding-RNAi bacterial library available for chromosome I genes created by Fraser *et al* (2000). Chromosome I of *C. elegans* contains a total of 2856 genes spread along the length of the chromosome with higher density at the central region (*C. elegans* Sequencing Consortium, 1998; www.wormbase.org). The chromosome I RNAi library contains bacterial clones expressing dsRNA targeted for 2416 genes (Fraser *et al.*, 2000). We fed the transgenic strain VH715 with each clone separately for at least one-generation time. In the first round, we identified ~150 candidate clones that showed overall axonal morphology defects in 30-50% of about 50 animals observed. These clones were isolated and were retested for their ability to consistently give the observed axon morphology defects in repeat feeding RNAi experiments to identify the clones that show true RNAi-mediated axon guidance or outgrowth defects. Of the 2416 genes tested we identified 63 candidate genes that showed a variety of defects in the axonal morphology as represented in Figure 2-6. To confirm the identity of the clones that gave the RNAi phenotypes we sequenced the feeding RNAi plasmid vectors. These sequences were matched against the genomic sequence of *C. elegans* to confirm the identity and correctness of the genes that are targeted by the RNAi vectors. Out of 63 clones isolated, upon sequencing 57 clones showed homology with the corresponding genes in the *C. elegans* genome sequence database (www.wormbase.org, release WS133). Three clones contained wrong inserts, while the *nas-5* gene was represented by four RNAi clones (see Materials and Methods).

Table 2-7: Genes on chromosome I identified in feeding RNAi screen for axon guidance genes.

Gene	Description	Extra phenotype	% Axon defects (n >50)		
			Comm.	VNC	DNC
Wild-type (*hdIs17*)	-	-	19	7	6
Signaling (8 genes)					
unc-73[b]	GTPase	Gro, Unc, Egl	80	45	27
Y95B8A.10	Phosphodiesterase	-	47	47	-
F26E4.5	S/T kinase	-	42	35	-
smd-1	SAM decarboxylase	-	50	56	14
ZC581.9	S/T kinase	-	44	83	21
F10G8.4	Tyrosine phosphatase	Gro	68	52	44
ced-1	TM protein EGF domain	-	40	47	10
F47B3.1	Tyrosine phosphatase	-	58	39	27
pry-1	Axin homolog	Gro, Unc	50	45	12
Receptor (4 genes)					
unc-40[b]	Netrin receptor	Gro, Egl, Unc	80	50	25
B0041.5	Receptor?	-	52	41	30
C24G7.1	Sodium channel homolog	-	44	42	45
T23B3.4	7TM receptor	-	39	40	23
C09D4.1	Receptor	Unc?	38	33	12
Cytoskeletal (3 genes)					
nmy-2	Non-muscle myosin	Gro	47	55	13
unc-95	LIM domain	Unc, Egl	68	72	43
lem-3	Ankyrin repeats domain	-	53	50	27
Transcription/translation (10 genes)					
sur-2	Cofactor for Sp1	-	50	59	38
hmg-3[a]	HMG group homolog	Gro, Unc	81	60	25
lin-59	Trithorax homolog	Gro, Egl	73	72	42
rnp-6	RNA binding domain	Gro, Unc, Egl	66	60	33
rpl-17	Ribosomal L17 protein	Gro, Unc	58	46	20
T05E8.3	Helicase	Gro, Unc	35	50	36
Y105E8A.23	Polymease	Gro	37	55	33
C41D11.2	Initiation factor	-	47	28	7
ZC328.2	Zinc finger domain	-	35	37	-
Y95B8A.7	Zinc finger domain	-	50	44	38
Metabolism (4 genes)					
lpd-3	Lipid metabolism	-	56	45	33
lbp-5	Lipocalin	-	45	40	-
F43G9.3	Mitochondrial protein		43	56	-

Gene	Description	Extra phenotype	% Axonal defects		
W03D8.8[a]	Peroxisomal acyl-CoA thioesterase	-	29	39	11
Other (6 genes)					
unc-13[b]	C1 domain	Gro	30	12	0
Y105E8B.9[a]	Isomerase	Gro	58	69	44
unc-101	AP2 homolog	-	39	39	9
E03H4.1	Transposase	-	44	47	18
pqn-20	Prion-like	-	26	33	12
nas-5	Astacin metalloprotease	-	67	66	33
Y65B4BR.4	Ubiquitin ligase	Unc	47	38	36
Novel, conserved (9 genes)					
F25D7.1	Novel	-	42	89	0
T21G5.5	Novel	Unc, Egl	47	63	43
Y63D3A.9[a]	Novel (F-box doamin)	-	53	52	40
Y106G6H.8	Novel	Gro	78	61	27
Y47G6A.29	Novel	-	55	50	44
C55B7.9	Novel	Gro, Unc	50	45	12
F26B1.1	Novel	-	46	67	33
F46F11.9	Novel	Gro, Unc, Egl	52	42	64
D2030.9	Novel (WD-40 domain)	-	33	9	8
Novel (13 genes)					
unc-14[b]	Novel	Gro	30	7	0
Y18D10A.21	Novel	-	14	56	11
Y37H9A.1	Novel	-	41	32	9
T15D6.9	Novel	-	40	47	38
Y34D9A.3[a]	Novel	Unc	79	62	45
Y48G1C.8	Novel	Gro, Unc	31	69	50
Y106G6A.2	Novel	-	33	69	-
Y106G6A.4	Novel	-	47	47	16
Y106G6A.5	Novel	-	48	56	-
T24D1.3	Novel	-	53	46	30
F29D11.2	Novel	Unc, Dpy	58	50	46
ZK973.8	Novel	-	37	61	9
F48C1.4	Novel	Dpy, Lvr	52	38	15
C17F3.1	Novel	-	70	48	36

[a]These genes showed cell misplacement phenotypes.
[b]These genes were used to test the RNAi efficiecy of the *nre-1* mutant strain and were also isolated in the feeding RNAi screen in this work. Dpy, dumpy; Egl, egg laying defective; Lvr, larval lethal; Gro, slow growth; Unc, uncoordinated.

The RNAi phenotypes of some of the genes identified are detailed in Section 2.11. Table 2-7 summarizes types of genes identified with percent penetrant defects they cause in axon guidance in different parts of the nervous system of *C. elegans*.

2.11 Types of axon guidance RNAi phenotypes observed

This screen was planned to isolate genes that cause defects in the axons of a subset of interneurons and motor neurons as listed in Table 2-6. Based on the type of RNAi-mediated axon guidance phenotypes caused by each of the 57 genes, we classified the defects in three types; commissural defects, defects in the fasciculation of VNC axons and defects in the dorsal nerve cord (Table 2-7). The majority of RNAi targets showed defects in the dorsal-ventral guidance and outgrowth of the axons, because these defects are relatively easy to identify due to the stereotypic patterns of the labeled axons (Figure 2-4C-D). The second most frequently observed types of defects were in the fasciculation of VNC and DNC axons. Figure 2-6 shows representative examples of the types of defects caused by RNAi of the indicated genes. The most common defects were in commissural guidance and ectopic branching of axons (Figure 2-6A-C, E, G, I). The commissures in *C. elegans* have to travel from the ventral side of the animal, where somas are located, to the dorsal side running around the lateral sides of the animal. Most of the commissures are positioned during embryonic development except in the case of commissures of VA/VB and VD motor neurons that grow post-embryonically. All the commissures are unbranched and reach the dorsal side running perpendicular to the anterior-posterior axis of the animal. We frequently observed defective guidance in commissures in several RNAi targets (Table 2-5). These defects were either in the commissural growth wherein commissures did not reach the dorsal side of the worm but turned halfway in lateral directions traveling parallel to the DNC (Figure 2-6A). While in some cases commissures also traveled long distances laterally (Figure 2-6C, G) without reaching the VNC. In some cases, they were branched into two or more protrusions that travel in opposite directions (Figure 2-6B, I) or formed a tangle of branches (Figure 2-6C, E).

The defects observed in the VNC axons were largely of defasciculation of the major tract of the cord causing axons from the right tract to crossover and run through the left tract (Figure 2-6F). In the case of *unc-101* RNAi, gaps in the VNC were observed probably due to the axon outgrowth defects leading to shortening of axons (Figure 2-6D). Besides axon guidance defects described above the cell misplacement defects were also detected on

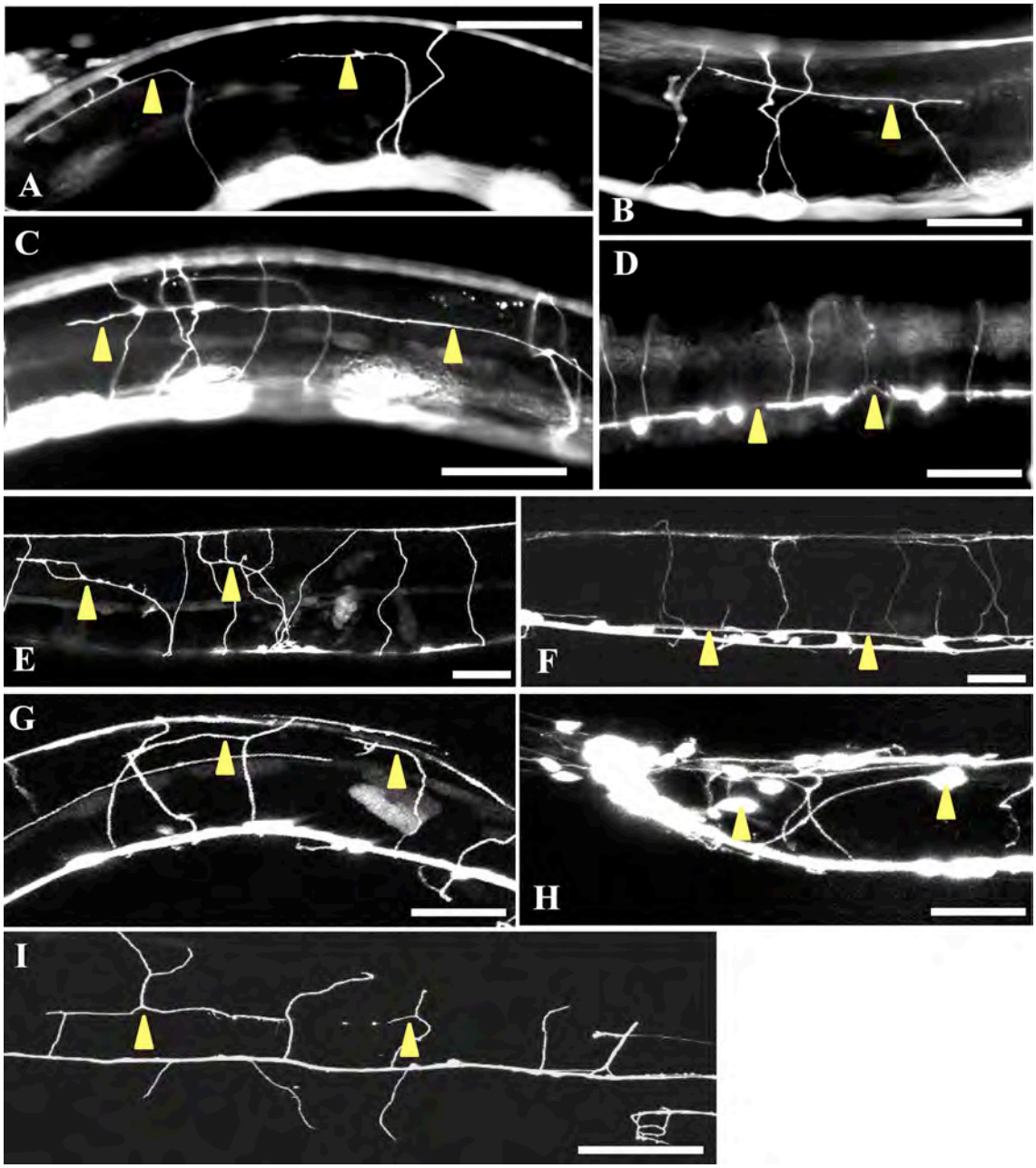

Figure 2-6: Representative RNAi-mediated axon guidance phenotypes caused by knockdown of the indicated genes. **A)** *Y105E8B.9(RNAi),* commissural dorsal-ventral outgrowth defects. **B)** *T23H4.3(RNAi),* commissural branching defects. **C)** *pry-1(RNAi),* commissural defects. **D)** *unc-101(RNAi)*, gaps in the VNC. **E)** *F29D11.2(RNAi),* excessive commissural branching and **F)** *F29D11.2(RNAi),* defasciculation in the VNC. **G)** *F10G8.4(RNAi),* commissural branching defects. **H)** *Y63D3A.9(RNAi),* cell misplacement and axon guidance defects. **I)** *Y106GH.8(RNAi),* commissural branching defects. A-C, E-G, H: side view. D, F: ventral view. I: dorsal view. In all images anterior is to the left and arrowheads indicate the defects. Scale bars, 20 μM.

RNAi of a few genes, wherein neurons located in the nerve ring or VNC were misplaced to the lateral sides of the animal (Figure 2-6H). The axons sent by these misplaced cells were randomly targeted to the nerve ring or VNC. Defects in the DNC were limited to the defasciculation; however, this phenomenon was linked to the defects observed in the commissures (Table 2-7) as defective commissures frequently caused defects in fasciculation of the DNC axons.

2.12 Bioinformatic analysis and classification of identified genes

To classify the identified genes as listed in Table 2-7 we performed bioinformatic analysis on the protein sequences of the 57 genes that gave penetrant RNAi phenotypes. At first, to confirm the identity and correctness of the RNAi clones, we sequenced the isolated feeding vectors by bidirectional DNA sequencing using primers specific to the feeding vector (see Materials and Methods). The RNAi clones representing the 57 genes listed in Table 2-7 had correct sequences corresponding to their cognate gene sequences as deposited in the *C. elegans* database. In some cases sequences of the feeding RNAi clones did not identify any open reading frame sequence representing a *C elegans* gene while a few other contained wrong inserts (see Materials and Methods), however, these clones were not considered for further analysis.

Next, we performed homology analysis of all the genes listed in the Table 2-7 by the protein-protein BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST) against the *C. elegans, Drosophila,* human and mouse protein sequences. The sequences for the 57 *C. elegans* genes were recovered from the WormBase database (www.wormbase.org, release WS133). The selected protein sequences were then compared with human, mouse or *Drosophila* sequences available in the GenBank database. The protein sequence comparisons that gave a p-value of $>10^{-10}$, for a sequence length of >30% between the *C. elegans* genes and that of either *Drosophila* or mammals were identified as potential homologs of the worm genes. However, genes having weak similarities may escape these comparisons. After this analysis, out of 57 genes we have identified homologs for 44 genes either in human, mouse or *Drosophila*, while 13 genes have no detectable homolog outside the nematode family. Based on their homology to the genes in other organisms and previously known genes we classified the 57 genes into 8 functional classes as list in Table 2-7. Figure 2-7 shows a pie diagram of the distribution of 57 genes into different functional classes. Of the 57 genes identified, 29 genes (51%) were from the previously

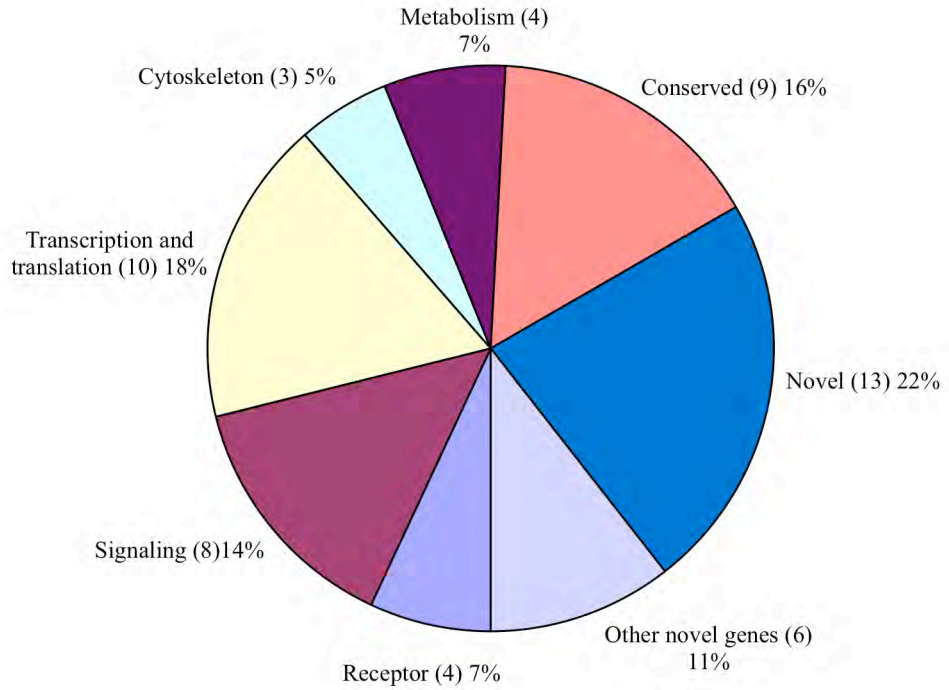

Figure 2-7: Pie chart of the 57 genes classified in functional classes based on bioinformatic analysis with well-characterized or annotated genes in the databases.

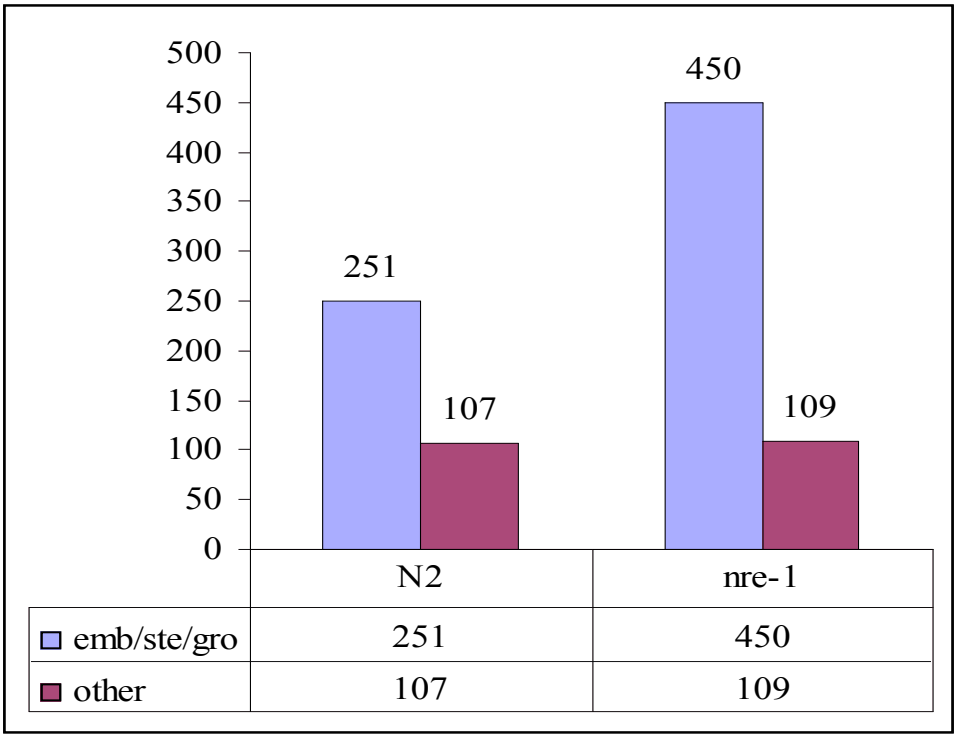

Figure 2-8: The *nre-1* strain is supersensitive to RNAi. In the *nre-1* background twice as many genes showed RNAi phenotypes compared to the wild-type background.

annotated classes and comprised 4 receptors, 8 signaling molecules, 10 transcription and translation factors, 3 cytoskeleton associated proteins and 4 metabolic genes. Out of remaining 22 (38%) genes, 9 conserved genes have homologs present either in mammals or *Drosophila*; however, they are not functionally annotated in any of the functional classes, as they are novel genes with unknown domain structures. The 13 novel genes have weak or no similarity to the genes present in databases beyond nematodes; however, they show specific and highly penetrant axon guidance defects in *C. elegans*. It will be interesting to see whether these genes have distal homologs in other animals or whether they are specific to the nematode species. The 6 genes classified as other novel genes include conserved genes that are involved in a variety of intracellular processes.

2.13 Supersensitivity of the *nre-1* strain

The *nre-1* strain is not only efficient for RNAi in the nervous system, but it is also supersensitive for non-neuronal genes. During screening for genes on chromosome I for RNAi-mediated axon guidance phenotype; we observed that in the *nre-1* background many RNAi targets caused phenotypes that were not found in the previous chromosome I RNAi screen done on wild-type animals for the same genes (Fraser *et al.,* 2000). Figure 2-8 compares the total number of RNAi phenotypes we observed in the *nre-1* background to that of the same found in the screen performed by Fraser *et al.* In wild-type background the previous screen has identified 251 genes that cause embryonic lethality (Emb), sterility (Ste) or slow growth (Gro) phenotypes. In the *nre-1* background we have identified 450 genes that give rise to Emb, Ste, or Gro phenotypes. The 251 genes identified by Fraser *et al.* are mostly the genes that are required for basic cell maintenance and metabolism processes. On the other hand, from about 200 genes most that gave an extra phenotype in our screen have not been previously reported to have any other phenotypes. Therefore, it will be interesting to study these genes for their possible roles in the embryonic and postembryonic developmental events in *C. elegans.* The other visible phenotypes like Dpy, Unc, etc., observed in the *nre-1* background are similar to that of the same phenotypes observed in wild-type animals, i.e. 107 in WT vs. 109 in the *nre-1* mutant (Figure 2-8,). As listed in Table 2-7 several genes that cause axon guidance phenotypes also give other extra phenotypes like Dpy, Egl, Gro, and/or Unc. Therefore, further studies on these genes should shed more light on the development of the nervous system in *C. elegans*.

2.14 Proof of principle

To validate the ability of our RNAi screening approach to correctly identify genes that are involved in axon guidance mechanisms in *C. elegans*, we took advantage of genetic mutations available in some of the genes identified in this screen (Table 2-7). Out of the 57 genes identified that showed axon guidance defects upon RNAi, we selected 6 genes (*ced-1, lin-59, pry-1, nmy-2, sur-2* and *unc-101*) that have genetic mutations available and have been implicated in a variety of signaling pathways. Of these 6 genes, we were able to cross GFP/YFP transgenes into the mutants of 4 genes, namely *ced-1, lin-59, pry-1* and *unc-101*. Two genes cause severe defects in the worm; *nmy-2* is embryonic lethal (Guo and Kemphues, 1996) and *sur-2* gives 100% vulvaless phenotype limiting mating experiments in the mutant animals (Howard and Sundaram, 2002). Of the four genes in which axons were labeled with the fluorescent proteins, *lin-59(sa489)* did not show any detectable axon guidance phenotype in the marked neurons. In the case of *ced-1(e1735), pry-1(mu38)* and *unc-101(m1)* mutants distinct axon guidance phenotypes were observed (Figure 2-9) that were comparable to the RNAi phenotypes observed for these genes (Table 2-7).

The *pry-1* gene encodes the axin homolog of *C. elegans*, which is involved in a Wnt pathway as a component of the β-catenin complex (Korswagen *et al.*, 2002). It is a negative regulator of Wnt signaling and inhibits the stabilization of β-catenin preventing its translocation into the nucleus to act as a transcriptional regulator. In *C. elegans*, the RNAi knockdown and genetic mutants of the *pry-1* gene gave consist phenotypes of severe defects in the commissural axon branching and the defasciculation of the VNC axons. These defects were strong and about 90% of *pry-1(mu38)* animals showed the defects. In *C. elegans* the roles of Wnt signaling molecules are not yet established with regard to nervous system development. Hence, it will be interesting to study the role of *pry-1* in axon guidance in *C. elegans* to further investigate the Wnt signaling pathways.

The second gene whose genetic mutations gave phenotypes similar to that observed in the RNAi experiments is *ced-1.* This gene was previously identified in a screen for cell death genes and it is a receptor tyrosine kinase (Zhou *et al.,* 2001). In *ced-1* mutants engulfment of dead cells after apoptosis is inhibited by unknown mechanisms as signaling molecules upstream and downstream of the gene are not identified. However, *ced-1* is strongly expressed in the nervous system of *C. elegans*, especially the VNC and the motor neurons. Its significance is not yet known in any of the axon guidance mechanism and our identification of this gene as an axon guidance molecule should provide more

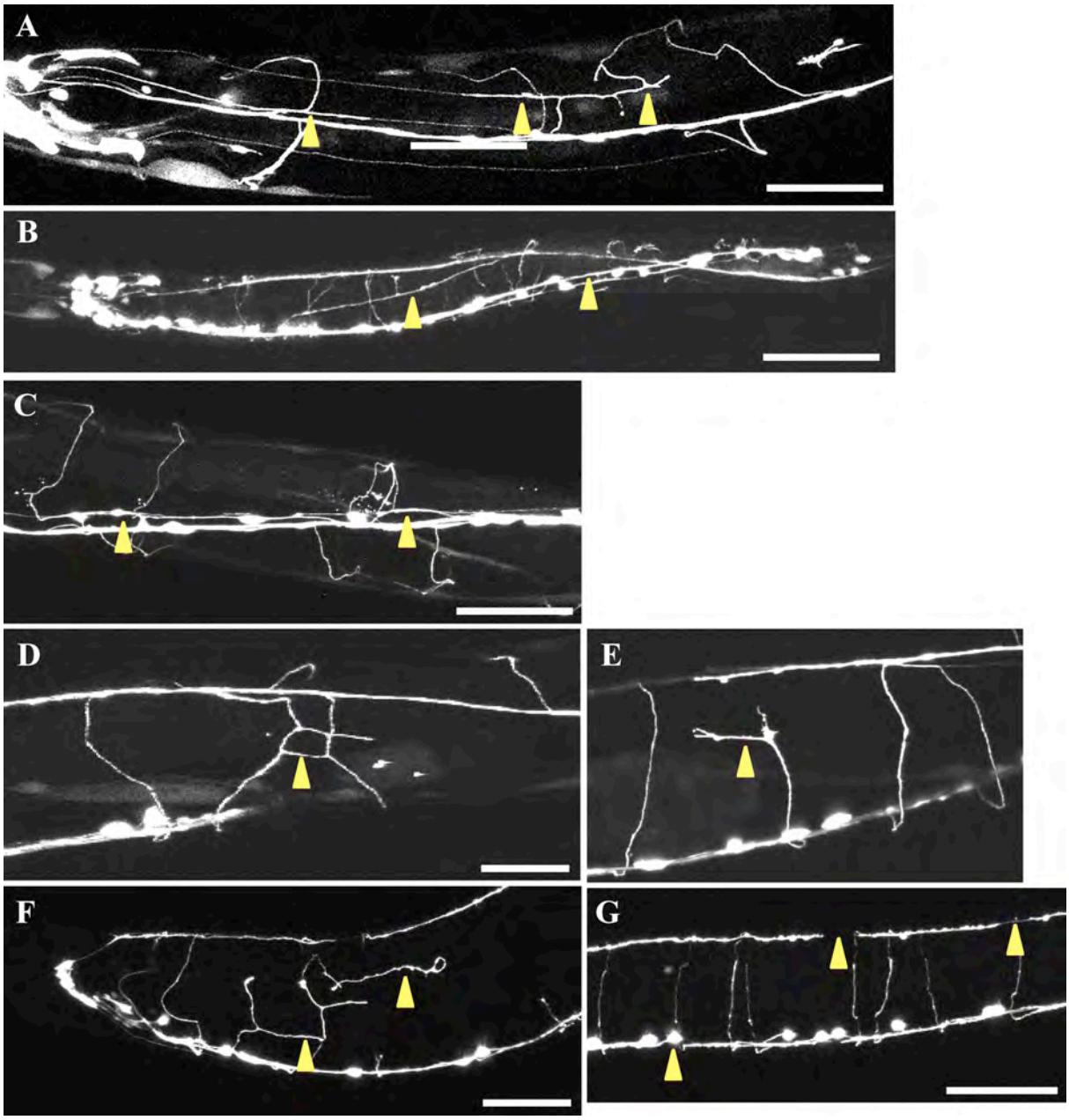

Figure 2-9: Axon guidance phenotypes observed in genetic mutants. **A-C)** *pry-1(mu38),* defects were observed in commissural axon branching (A) and VNC defasciculation (B-C); axons were labeled with the *hdIs17* marker. **D-E)** *ced-1(e1735)*, commissural axon defects; axons were labeled with the *hdEx191* marker. **F-G)** *unc-101(m1)*, commissural axon defects (F) and gaps in the ventral and dorsal nerve cord tracts (G); axons were labeled with the *oxIs12* marker. In all images anterior is to the left and arrowheads indicate the defects. Scale bars, 20 μM.

information on its biological function in the nervous system. The third gene, *unc-101,* is a component of clathrin-associated protein complex and is required for many aspects of *C. elegans* development and behavior (Lee *et al.,* 1994). The involvement of this gene in axon guidance could be due to its role in the intracellular vesicle trafficking that is obligatory for the transport of proteins to the growing growth cones of axons.

Here, our identification of abovementioned genes has validated the strategy developed in this work to rapidly screen for axon guidance genes in *C. elegans*. This study demonstrates that the feeding RNAi can be used for the identification of genes involved in axon guidance pathways in *C. elegans*, provided that the refractive neurons are made susceptible to feeding RNAi by taking advantage of the mutants that are efficient for RNAi in the nervous system. The approach used here is rapid and gives information on a large number of genes that are potentially involved in axon guidance in the worm. Moreover, future screens covering other chromosomes of the worm should give us more information and candidate genes to understand the complex mechanisms of axon guidance.

3 DISCUSSION

3.1 Background to the RNA interference phenomenon in *C. elegans*

RNA interference (RNAi) is the process wherein double-stranded RNA (dsRNA) induces the homology dependent degradation of cognate mRNA in the cell. RNAi was first discovered in *C. elegans* (Fire *et al.*, 1998), however, similar phenomena called post-transcriptional gene silencing in plants and quelling in fungi have been known for several years (reviewed by Hannon, 2002 and Montgomery, 2004). Recently, the molecular mechanism of RNAi has been elucidated and a few genes involved in this phenomenon have been discovered and their homologs identified across species. In *C. elegans* exogenously introduced or endogenously produced dsRNA is acted upon by different components of RNAi machinery (Figure 1-6), at first dsRNA is cleaved into small-interfering RNA molecules (siRNAs) of ~21 nucleotides by an RNaseIII-like endonuclease *dcr-1* (*Drosophila* Dicer homolog in *C. elegans*) in the cell. Then the siRNAs, along with a helicase and other factors, are assembled in a protein–siRNA complex called RNA-induced silencing complex (RISC). The RISC complex provides the specificity to siRNAs to target their cognate mRNA molecules. Upon priming by siRNAs cognate mRNA is either degraded by cellular endonucleases or is converted into new dsRNAs by the action of RNA-dependent RNA polymerases (RdRPs) and fed into a new RNAi cycle thereby amplifying the effect, and in the process silencing the expression of cognate mRNA (Figure 1-6). The polymerases and endonucleases that are components of the RISC complex are of special interest, because of their ability to control the efficiency of RNAi in different tissues of the animal. The RNAi phenomenon has a variety of functions in *C. elegans* as it controls the expression of many genes at post-translational level (Lippman and Martienssen, 2004). It is active in all the tissues of the animal and can uniquely spread from one cell to another by systemic mechanisms (Winston *et al.,* 2002). However, the nervous system of *C. elegans* is largely refractory to the systemic spread of RNAi upon feeding of dsRNAs to the animals (Tavernarakis *et al.* 2000; Kennedy *et al.,* 2004). The mechanisms behind this refractory nature the *C. elegans* neurons to systemic RNAi are poorly understood. This has also hampered the use of RNAi as a technique to study the genes involved in the development of the nervous system of *C. elegans*.

3.2 RNAi-mediated genetic screens in *C. elegans*

On the discovery and recognition of importance of RNAi in *C. elegans* several RNAi experiments were performed by injecting dsRNA molecules into the gonads or intestine of worms to study the effect of down regulation of cognate gene expression on the phenotype of treated animals (Fire *et al.*, 1998). Subsequently, a chromosome-wide injection RNAi screen was performed to identify genes required for early embryonic development in *C. elegans* (Gonczy *et al.* 2000). Though rapid this approach was laborious for large-scale screens and it required injecting several animals to get a sufficient number of progeny. However, after the discovery that the ingestion of bacterially expressed dsRNA can produce specific and potent genetic interference, feeding RNAi became a standard RNAi method in *C. elegans* genetics (Timmons and Fire 1998; Kamath *et al.*, 2000; Timmons *et al.*, 2001).

Recently, a genome-wide library of bacteria producing dsRNA for ~86% of the genes of *C. elegans* has been created as a permanent reagent (Kamath *et al.*, 2003). Using this library several genome-wide screens have been performed to define genes involved in a variety of biological processes (Fraser *et al.*, 2000; Kamath *et al.*, 2003; Ashrafi *et al.,* 2003). The advantage of this strategy is that the whole genome of *C. elegans* can be screened for genes required in a particular biological process in a relatively short time. The first paradigm large-scale feeding RNAi screen was performed to define genes involved in early embryonic development in *C. elegans* (Fraser *et al.*, 2000). This screen identified a phenotype for ~14% of the genes on chromosome I showing the high-throughput nature of the screening method. This first feeding RNAi screen has also set a paradigm for the future feeding RNAi screens in which novel molecular biology and genetics approaches may be used to identify genes involved in specific biological processes. In this study, we took advantage of the availability of the chromosome I feeding-RNAi library to identify genes involved in axon guidance in *C. elegans*.

3.3 Efficiency of RNAi in the nervous system of *C. elegans*

It has been reported that "feeding RNAi" approach in *C. elegans* is greatly influenced by the extrinsic factors like the growth temperature at the time of RNAi, the extent of induction of feeding bacteria to produce dsRNAs, etc (Kamath *et al.*, 2000; Timmons *et al.*, 2001). To check if "feeding RNAi" works in our conditions in the nervous system of the worm, we performed feeding RNAi experiments on a transgenic *C. elegans* strain. The

worm strain VH41 contains a chromosomally integrated GFP marker transgene (*unc-119::gfp*) that is expressed in all neurons and some head muscle cells of *C. elegans* (Figure 2-1). We treated the VH41 animals with an optimized feeding RNAi protocol (see Materials and Methods). Then the loss of GFP expression in the nervous system and muscle cells was examined 24 and 48 h after the RNAi treatment of the animals. In the case of muscle cells of *C. elegans* the expression of GFP was completely suppressed with in 24 h, however, the neuronal GFP levels were not affected by this treatment (Figure 2-1; Table 2-1, see data for VH41). Longer than 48 h treatment did not make any difference in the expression level of GFP in the neurons. This data of ours is in line with the similar results obtained by others on the ineffectiveness of RNAi in the nervous system of the worm (Tavernarakis *et al.*, 2000; Timmons *et al.*, 2001; Kamath *et al.*, 2003). In addition, manipulation of conditions like the growth temperature or the amount of dsRNA producing bacteria food on the feeding plates did not change the efficiency of RNAi in the nervous system.

The muscle cells of *C. elegans* have been shown to be sensitive to RNAi (Kamath *et al.*, 2001), our experiments with transgenic strains VH41 and VH288 (in which GFP is only expressed in the muscle cells) validated the previous studies on the high efficiency of RNAi in this tissue. Moreover, RNAi experiments on muscle cells demonstrated that our experimental conditions used for feeding RNAi are sufficient to induce RNAi in the cells of *C. elegans*, except the nervous system. Based on this data we reasoned that the refractory nature of the nervous system of *C. elegans* to systemic RNAi could be attributed to genetic factors and a mutational approach to isolate mutants of *C. elegans* that are efficient for RNAi in the nervous system should be explored. And further these mutants can be used in large-scale feeding RNAi screens to isolated genes involved in axon guidance in *C. elegans*. The main reasoning behind such an approach was to identify the genes by RNAi that function cell-autonomously in the neurons affecting the mechanisms of axon guidance.

3.4 Isolation of neuronal RNAi efficient (*nre*) mutants

C. elegans is a powerful model system for genetics and molecular biology studies due to its many advantages over other model systems. One of the major advantages of *C. elegans* is the ease of isolation of mutants with unique phenotypes in complex experimental conditions. In order to isolate mutants of *C. elegans* that are efficient for RNAi in the

nervous system, we performed a chemical mutagenesis screen on a transgenic strain that expresses GFP in the nervous system of the worm (Figure 2-2). The worm strain VH41 was used for the screen as it expresses GFP in all the neurons of the animal at a moderate level and any change in the GFP expression pattern should be readily identified in the mutants with effective RNAi in the neurons. After ethyl-methane-sulfonate (EMS) mutagenesis and screening of about 20000 genomes of F2 progeny, we identified two independent mutants (*hd20, hd21*) that showed feeding RNAi-mediated suppression of GFP expression in the nervous system. The mutant *hd20* showed a robust and consistent RNAi effect in the neurons (Figure 2-3; Table 2-1, see data for VH624). It was named *nre-1* (for neuronal RNAi efficient) mutation and further characterized with regard to its chromosomal location and biological characters. Recently, with a similar approach an RNAi efficient mutant of *C. elegans* was isolated that showed enhanced RNAi in a subset of neurons (Simmer *et al.,* 2002; Kennedy *et al.,* 2004). Here our identification of *nre* mutants is important for further studies on understanding the mechanisms of RNAi in the nervous system. Secondly, molecular characterization of these mutants may provide more information on the types of molecules that take part in the RNAi phenomenon in different organs of the worm.

3.5 Characteristics of the *nre-1* mutant

The *nre-1* mutant we identified shows robust down regulation of GFP expression in the nervous system of *C. elegans* upon the delivery of dsRNA molecules by the feeding RNAi method (Figure 2-3; Table 2-1). This phenotype of the *nre-1* mutant was specific and reversible as shifting the treated animals to control bacteria food from the *GFP* RNAi bacteria food caused reappearance of the GFP fluorescence in the nervous system within a few hours after the shift. We further characterized the *nre-1* mutant with regard to its ability to phenocopy the mutant phenotypes of well-known neuronal genes that have been associated in different biological processes in the worm. To this end, we selected 5 neuronal genes: *unc-13, unc-14, unc-40, unc-73* and *lin-11,* which are required cell-autonomously for the development of the nervous system of *C. elegans* (see Section 2.6). RNAi of these genes in the *nre-1* mutants significantly affected the function of the genes leading to 30-80% defects in the progeny of treated animals (Table 2-5). The ability of the *nre-1* mutant to phenocopy the phenotypes of genetic null mutantions of neuronal genes showed that the *nre-1* mutant animals are significantly more efficient in RNAi-mediated

gene knockdown in the nervous system. Besides the RNAi-mediated phenotypes observed in the *nre-1* mutant background are comparable to that of the same phenotypes found in the genetic mutants of the 5 genes (Table 2-5) proving the efficiency of the *nre-1* mutation to RNAi in the neurons.

The *nre-1* mutation we isolated not only affects RNAi in the nervous system, but is also supersensitive to RNAi in the non-neuronal tissues of *C. elegans*. This fact is demonstrated by our finding of significantly more non-neuronal RNAi phenotypes in the *nre-1* background compared to wild-type animals when the large-scale RNAi screen was performed for the chromosome I genes. When RNAi of 2416 genes located on chromosome I was performed to identify genes involved in axon guidance (Figure 2-8; Section 2.13), we isolated about 450 genes that give rise to different embryonic phenotypes, for example, Emb, Ste or Gro phenotypes. The number of extra phenotypes we identified is about two times more than that of the same phenotypes observed in the wild-type background (Fraser *et al.,* 2000). This recovery of extra phenotypes that were not previously reported in wild-type animals demonstrated the usefulness of the *nre-1* mutant to find a function for previously unknown genes. Besides the *nre-1* mutation also shows temperature sensitive sterile phenotype at an elevated growth temperature of 25°C, similar phenomenon has also been reported in previously isolated RNAi supersensitive genes *rrf-3* and *eri-1* (Simmer *et al.*, 2002; Kennedy *et al.,* 2004), pointing out the possible roles for these genes in the germline development in *C. elegans*. The *rrf-3* mutation was isolated during studies on the role of RNA-amplification in dsRNA-triggered gene silencing processes (Sijen *et al.*, 2001). Later the *rrf-3* mutant animals were shown to be hypersensitive to RNAi (Simmer *et al.*, 2002). The *rrf-3* gene encodes an RNA-directed RNA polymerase, but the exact mechanism of its function in the RNAi phenomenon and enhancement of RNAi in some tissue is not clear. It is assumed that RRF-3 competes with other components of the RISC complex during the amplification step of RNAi (Simmer *et al.*, 2002). On the other hand, the *eri-1* gene encodes an siRNA-degrading RNase that negatively regulates RNAi in specific tissues like the nervous system (Kennedy *et al.,* 2004). These two genes and *nre-1* have some common phenotypes as described above and this could be due to their involvement in the regulation of RNAi in different tissues of the animal. Further molecular characterization of *nre-1* should provide more information on these mechanisms. However, in this study we have used the *nre-1*

mutant as a tool for large-scale feeding RNAi-mediated genetic screens to isolate genes involved in axon guidance.

3.6 Feeding RNAi-mediated genetic screen for axon guidance genes

In *C. elegans* many genetic screens have been performed to identify genes required for axon guidance of different types of neurons, however, these forward genetic screens are laborious and in many screens the same genes were repetitively identified. We decided to take advantage of the RNAi technology to identify genes that cause axon guidance defects upon down regulation of their expression in the nervous system of *C. elegans*. The *nre-1* mutant we isolated is useful tool for such RNAi-mediated genetic screens as it provides a way to knockdown genes in the nervous system. Using the *nre-1* mutation we created a screening strain VH715 for large-scale feeding RNAi-mediated screens in the worm to identify genes involved in the development of the nervous system. The VH715 strain contains a transgenic array (*hdIs17*) of three YFP fusion genes under the control of promoters of *unc-47, unc-129* and *glr-1* genes. These genes are specifically expressed in the nervous system of *C. elegans* and they label a subset of interneurons and motor neurons that send their axons along the anterior-posterior and dorsal-ventral axes of the animal (Figure 2-5; Table 2-6). The pattern of neurons labeled by the *hdIs17* marker is simple and stereotypic in different animals providing an advantage while looking for defects in the axons of labeled neurons.

Next, using an RNAi-mediated screening strategy we screened the feeding RNAi library available for 2416 genes located on chromosome I of *C. elegans* (Figure 2-4). During the screening procedure we selected the clones that consistently gave >30-50% defects in the labeled axons of the treated animals. These genes were retested several times by RNAi to identify the genes that caused specific axon guidance phenotypes. Of the 2416 genes tested, 57 genes gave highly penetrant RNAi phenotypes in the nervous system leading to axon guidance defects in the neurons that were labeled by the YFP transgenes. Most of these are novel genes and their identification, as being involved in the mechanisms of axon guidance, should provide more information on the development of the nervous system. Furthermore this screen also assigns a function for about 200 previously unknown genes, significantly increasing the number of genes on chromosome I of *C. elegans* with a function in the development of the animal.

3.7 Limitations of RNAi-mediated genetic screens

Although RNAi is used as a very quick method for determining loss-of-function phenotypes of genes for which no genetic mutations are available, it has a few limitations with respect to its efficacy in knocking down genes in different conditions and in different tissues in *C. elegans* (Sugimoto, 2004). RNAi phenomenon is affected by several factors like, the timing and method of dsRNA treatment, sensitivity of the gene under investigation for reduced expression levels, and the temperature at which the RNAi experiments were performed (Sugimoto, 2004). Therefore, it is necessary to confirm the observed effect of RNAi for a gene with alternative methods. In addition, there is significant experimental variability in different RNAi results, which may be due to the differences in experimental setups, such as the developmental stage at which the animals are treated or the concentration of dsRNA supplied to the worms during RNAi experiments (Simmer *et al.,* 2003). Here it is important to take careful note of the penetrance and variability of the RNAi effects during interpretation of the RNAi results.

In this screen we have identified genes that give RNAi-mediated axon guidance phenotypes in the nervous system of *C. elegans*. The nervous system of the worm is refractory to RNAi by feeding method (Travernarakis *et al.,* 2000). However, we took advantage the *nre-1* mutant strain for identification of the genes involved in the nervous system of the worm. It is possible that our screen has missed several genes that cause subtle defects in axon guidance or are sensitive to the factors that cause a variability in RNAi process itself. To identify axon guidance genes that give consistent and penetrant defects, we subjected about 150 candidates genes identified in the first round of the screening to two more rounds of feeding RNAi experiments to isolate genes that showed axon guidance phenotypes in all experiments. After final round of RNAi experiments, we identified 63 clones representing 57 genes as axon guidance genes. This data showed that experimental variability existed in our RNAi results and we may have missed several genes in this screen that may show axon guidance phenotypes. Indeed, we were unable to identify the clone for the *lin-11* gene, which is also involved in the process of axon guidance and the development of the nervous system (Hutter, 2003). In addition, upon sequencing we observed that a few RNAi clones showing axon guidance phenotypes did not contain or represent the reported genes. The limitations listed above are difficult to control in large-scale screens and hence the absence of a phenotype for a gene in RNAi experiments does not rule out a role for that gene in the said phenomoneon.

3.8 Types of axon guidance genes identified on chromosome I

The *C. elegans* chromosome I has a total of 2856 genes as identified by the genome sequencing project (*C. elegans* Sequencing Consortium, 1998). The feeding RNAi library of chromosome I created by Fraser *et al.* contains 2425 RNAi clones representing about 2416 genes of the worm. This library has been previously used for feeding RNAi-mediated genetic screens to identify genes involved in variously biological processes (Fraser *et al.*, 2000; Kamath *et al.*, 2003; Ashrafi *et al.,* 2003). By using this library in the *nre-1* supersensitive background, we were able to identify 57 genes (Table 2-7) that gave significant defects in axon guidance of a subset of interneurons and motor neurons of *C. elegans*. The genes identified are of different types ranging from metabolic genes to transcription factor and signaling molecules (Figure 2-7). Based on the BLAST protein sequence analyses with genes in the databases, we found conserved homologs for 29 of the 57 identified genes. The 35 conserved genes have well-characterized or annotated homologs in other organisms, while the remaining 22 genes are novel with some having homologs to genes in other model organisms like *Drosophila* and mammals (Table 2-5), but their functions are unknown. Of the 35 conserved, ten genes are transcription or translation factors and remaining genes encode signaling molecules (eight genes) and transmembrane receptor (four genes), respectively. Another three conserved genes represent components of the cytoskeleton and are likely to be regulated by the signaling molecules during the process of axon guidance. While six genes of other genes group are conserved genes involved in various intracellular processes (see Table 2-7). Of the remaining genes, we have identified 22 genes that are novel with no previously described annotated homologs, 9 of them are conserved in higher organisms while 13 genes are apparently nematode specific. Table 2-7 also lists the four genes, i.e. *unc-13, unc-14, unc-40* and *unc-73,* which are previously known to be involved in the nervous system of *C. elegans*. These were used to test the neuronal RNAi efficiency of the *nre-1* mutant strain (Table 2-5) and they serve as positive controls in the screening approach used.

3.8.1 Transcription factors

Out of the 57 genes identified, four genes (Table 2-7) represent transcription factors. In different model organisms, a number of transcription factors have been implicated from the selection of neuronal precursors to the specification of neuronal subtypes during the development of the nervous system (reviewed by Shirasaki and Pfaff, 2002). Of the

different families of transcription factors, the LIM-homeodomain transcription factors have been extensively studied in the development of motor neuron subtypes, where they work in a combinatorial fashion to control the gene expression networks (Jacob *et al.,* 2001; Shirasaki and Pfaff, 2002). However, these transcription factors are expressed only in a few subsets of neurons and are unlikely to define the neuronal complexity of the nervous system. This points out the need of identification of other transcriptions factors that are involved in the development of the nervous system.

In the case of axon guidance mechanisms in *C. elegans,* only a few transcription factors are studied to demonstrate the link between the activity of the transcription factors and the types of axon guidance molecules they may control. In the nematode, a nuclear hormone receptor gene, *fax-1*, has been shown to be required for guidance of axons in the nerve ring, the VNC, and for normal expression of a few neurotransmitters in the AVK interneurons (Much *et al.,* 2000). Here *fax-1* coordinately regulates the transcription of genes that function in the selection of axon pathways, neurotransmitter expression and, perhaps, other aspects of the specification of neuron identity. The second example of transcription factor regulating axon guidance in *C. elegans* is the gene *unc-3,* a member of the O/E (Olf-1/EBF) family of transcription factors. *unc-3* is expressed in certain chemosensory neurons throughout their development and is also expressed transiently in developing motor neurons when these cells undergo axonal outgrowth controlling guidance of the axons of the motor neurons (Prasad *et al.,* 1998). In addition, recently a zinc finger transcription factor, *zag-1*, has been shown to be required for control of neuronal differentiation and axon outgrowth in the worm (Wacker *et al.,* 2003). In this study we have identified two new zinc-finger domain transcription factors (ZC328.2 and Y95B8A.7) along with two known transcription factors (*hmg-3* and *lin-59*) as regulators of axon guidance mechanisms. It will be important to study the expression pattern and the types of genes these zinc-finger domain genes regulate in the nervous system to get insight into their roles in the developing nervous system of the worm. In the case of *hmg-3* (HMG group homolog, www.wormbase.org) and *lin-59* (trithorax homolog, Chamberlin and Thomas, 2000), they have been implicated in various biological processes in *C. elegans*, however, their roles in axon guidance are not known.

3.8.2 Signaling molecules and receptors

Several of the genes identified in this study are receptors and intracellular signaling molecules. Among intracellular signaling genes our identification of a Wnt signaling molecule *pry-1* (axin homolog in *C. elegans*) as involved in axon guidance is important as the Wnt signaling pathway has been implicated in various developmental events in model organisms. In *C. elegans* it plays important roles in the development of embryo, cell fate specification and the determination of cellular polarity and migration (Korswagen, 2002). Concerning the roles of Wnt signaling in axon guidance, some Wnt ligands have been implicated in the mechanisms of axon guidance in *Drosophila* and mouse (reviewed by Zou, 2004). However, the roles of intracellular molecules that specifically control these mechanisms in not clearly understood. In *C. elegans,* several Wnt signaling molecule mutants are available and our identification of *pry-1* as an axon guidance gene should provide a starting point to future studies on how the Wnt signaling factors interact to perform a complex process like axon guidance in a simple animal like the nematode worm *C. elegans*. Secondly, mutants of several Wnt pathway genes that grow to adulthood can be tested for their roles in the development of the nervous system in the worm in general as currently no information is available on these genes with respect to their potential roles in axon guidance mechanisms in *C. elegans*.

Further genes that have been studied previously for different biological processes and isolated here as causing RNAi-mediated axon guidance defects include *ced-1* (a receptor protein kinase; Zhou *et al.,* 2001), *nmy-2* (non-muscle myosin homolog; Guo and Kemphues, 1996), *sur-2* (a novel gene; Howard and Sundaram, 2002) and *unc-101* (a clathrin coat component; Lee *et al.,* 1994). These genes have many mutants available in *C. elegans*. However, they have not yet been implicated in any of the mechanisms of axon guidance. The *ced-1* gene was first identified as a gene that when mutated prevents the engulfment of corpses of the cells that undergo apoptosis (Zhou *et al.,* 2001). It is a novel receptor protein kinase that is expressed strongly in the nervous system of the worm, however, the molecules upstream and downstream of it are unknown (Zhou *et al.,* 2001). Our identification of *ced-1* as an axon guidance gene provides new information on the potential extra roles of this gene in *C. elegans*, besides being involved in the scavenging of dead cells. The second gene, *unc-101*, is a component of intracellular membrane trafficking machinery, and axon guidance phenotype caused by this gene could be a secondary effect of its role in the transport of molecules among different compartments of

the cell. The other two genes, *nmy-2* and *sur-2*, are not studied with respect to their roles in the nervous system. However, *nmy-2* is a non-muscle myosin and mutations in this genes are embryonic lethal precluding its use to study its roles in the nervous system of *C. elegans* (Guo and Kemphues**,** 1996). *sur-2* is a novel gene with no appreciable homology outside the nematode species, however, mutations in this gene cause very penetrant vulvaless phenotype and it is associated with the Wnt signaling pathway in *C. elegans* (Howard and Sundaram, 2002).

We have also identified several genes that include protein kinases, phosphotases and membrane receptors (Table 2-7). The soluble or membrane associated protein kinases and phosphotases are the molecular effectors of the signaling pathways (Pires-daSilva and Sommer, 2003). They play important roles in the transduction and regulation of signaling events in the cell. Defects in these genes cause many diseases, cancers and developmental defects in organisms. In *C. elegans* several protein kinases and phosphotases have been identified in various genetic screens and have been implicated in a variety of biological processes from the embryonic development to axon guidance mechanisms. Our identification of five genes with a kinase or phosphotase domain (F26E4.5, ZC581.9, F10G8.4, *ced-1* and F47B3.1) as new axon guidance genes further advances the number of genes known to be involved in axon guidance mechanisms. Further characterization of these genes should provide more insight into the developing nervous system of *C. elegans*. It will be also interesting to test the possible roles of these protein kinases and phosphatases in the pathways of well-characterized signaling molecules like UNC-5, UNC-40 and SAX-3 (see Table 1-1). These genes were identified in different forward genetic screens for axon guidance molecules, however, the signaling cascades of these molecules are unknown in *C. elegans*. Besides protein kinases and phosphotases we have also identified four genes (B0041.5, C24G7.1, T23B3.4 and C09D4.1) that are potential receptor molecules, however, their biological functions are yet unknown. Characterizing these genes will be important for future studies as they may act as important regulators of signaling processes.

3.8.3 Other conserved genes

The other genes that are listed in the Table 2-7 have a variety of biological functions, like several of them are involved in basic metabolic processes and cytoskeleton organization in the cell. The RNAi phenotypes observed in these genes is likely to be due to the

disruption of metabolic processes or cytoskeleton in the cell leading to developmental defects including axon guidance defects. However, notable among these genes is the gene *nas-5*, which is a member of astacin family of metalloproteases (Mohrlen *et al.,* 2003). Astacins are extracellular enzymes postulated to be involved in modulation of extracellular matrix components and/or processing of extracellular proteins that acts as ligands for signaling pathways in a variety of biological processes and in different organisms (Basbaum and Werb, 1996). In *C. elegans* a few genes having metalloprotase domains have been identified as mediators of developmental and signaling pathways, for example, *hch-1*, a gene required for normal hatching and normal migration of a neuroblast in *C. elegans*, encodes a protein related to TOLLOID and BMP-1 family of metalloproteases (Hishida *et al.,* 1996).

Other genes like Y65B4BR.4 (a ubiquitin ligase), Y95B8A.10 (a phosphodiesterase) and novel genes like Y63D3A.9 and D2030.9 are interesting candidate genes to characterize in axon guidance mechanisms as several orthologs of these genes have been involved in important developmental and signaling processes. However, they have not been implicated in any of the mechanisms involved in the development of nervous system and axon guidance.

3.9 Validation of the screening approach

The screening strategy used here is novel and rapid, however, to validate that it is able to identify genes that are specifically involved in axon guidance in the nervous system of *C. elegans*, it was necessary to show that the genes identified in this screen are indeed required for axon guidance by alternative methods. To this end, we took advantage of genetic mutations available in three previously characterized genes *ced-1, pry-1* and *unc-101* and that we identified in this study as axon guidance genes. The mutants of these genes were crossed to GFP/YFP marker strains that labeled the axons of a subset of motor neurons and interneurons. Then the resultant transgenic mutants were examined for axon guidance defects in their nervous systems (Figure 2-9). The null mutants of these genes indeed gave axon guidance defects in axons of the fluorescently labeled neurons, which were comparable to the defects observed in the RNAi treated animals of the same genes (Figure 2-6). These results demonstrated the effectiveness of the RNAi screening strategy used for identification of axon guidance genes.

In addition, we were also able to isolate the four genes, namely *unc-13, unc-14, unc-40* and *unc-73* of the five genes located on chromosome I (Table 2-5) that were used to test the neuronal RNAi effectiveness of the *nre-1* strain. These genes have been previously shown to be required for different developmental and axon guidance mechanisms in the nervous system of *C. elegans* . They have been extensively studied with respect to their roles in axon guidance mechanisms and the nervous system development. They provide positive controls to the screening strategy of this study establishing its workability in the neurons of *C. elegans*. However, we could not identify the fifth gene *lin-11*, which is also involved in axon guidance in the ventral cord of *C. elegans* (Hutter, 2003), probably due to lack of RNAi clone corresponding to this gene in the feeding RNAi library or variability observed in feeding RNAi protocols (see Section 3.7).

3.10 Conclusions and perspective

A goal of this study was to identify genes that caused axon guidance phenotypes in nervous system of *C. elegans* by a large-scale RNAi-based approach. RNAi is a very effective technology for rapid and specific down regulation of genes and has been used in several genetic screens to identify genes required for a variety of biological functions in *C. elegans.* However, the feeding RNAi strategy did not work in the nervous system of *C. elegans*, and to overcome this problem we started with a genetic screen to isolate mutants of the worm that are efficient for RNAi in the nervous system. We isolated and characterized one such mutant *nre-1*, which shows efficient RNAi of neuronally expressed genes. Using this supersensitive mutant we screened the entire chromosome I feeding RNAi library representing 2416 genes to isolate 57 new genes that showed significance defects in the guidance of axons of a subset of interneurons and motor neurons in *C. elegans*. Further studies on these genes should provide us with more information on the biological reactions behind the phenomenon of axon guidance. They may also provide information on the missing components of currently known signaling pathways as in the case of several axon guidance genes in *C. elegans* the upstream and/or downstream molecules are not known. However, many of the genes we identified in this screen have no genetic mutants available and isolation of mutants by genetic screens or deletion by PCR-based strategy will be required.

The screening strategy developed here is also applicable to the study of genes involved in different biological processes in the nervous system, which are otherwise not accessible to

RNAi-mediated knockdown procedure in the wild-type animals. For example, our screening strategy can be used for identification of genes required for processes like chemotaxis and thermotaxis in the worm. The *nre* mutants are valuable tools for these types of screens and molecular characterization of these mutants may also provide new insight into the RNAi mechanisms involved in the nervous system. Especially, the molecular identity of the *nre-1* mutant should be helpful to better understand the mechanism of RNAi in the nervous system of *C. elegans*. On the other hand, this study has exclusively dealt with the genes present on chromosome I of *C. elegans*. However, extension of the screening strategy to the complete genome of the worm is desirable as this will lead to identification and assignment of several genes that are potentially involved in the development of the nervous system. Secondly, it should also facilitate rapid characterization of interesting genes that have important structural features, for example, transmembrane segments, kinase or phosphatase domains.

4 MATERIALS AND METHODS

4.1 *C. elegans* strains and culture conditions

The wild-type strain was *C. elegans* variety Bristol strain N2. The Hawaii polymorphic wild-type strain of the worm was CB4856. All strains were maintained at 20°C unless otherwise mentioned using standard methods (Brenner, 1974). They were maintained on the nematode growth media (NMG) plates containing antibiotics as indicated. Strains were kept free of contaminates like bacteria and fungi by periodic clean-up procedures that included use of antifungal and/or antibiotic agents. The synchronous cultures of worms were obtained from starving populations of daure larvae. Several of the strains listed below were obtained from *Caenorhabditis* Genetics Center.

The mutations and integrated or extrachromosomal array transgenic strains used in this study were: CB61, *dpy-5(e61) I;* CB128, *dpy-10(e128) II;* CB224, *dpy-11(e224) V;* CB184, *dpy-13(e184) IV;* CB164, *dpy-17(e164) IV;* CB450, unc-*13(e450) I;* CB57, *unc-14(e57) I;* CB271, *unc-40(e271) I;* CB936, *unc-73(e936) I;* EG1306, *oxIs12[unc-47::gfpntx, lin-15(+)] X;* MT1196, *lin-11(n566) I;* VH15, *rhIs4[glr-1::gfp, dpy-20(+)] III;* VH41, *rhIs13[unc-119::gfp, dpy-20(+)] V;* VH288, *hdIs8 [him-4::gfp, rol-6(su1006)];* VH414, *hdIs14[odr-2::cfp, unc-129::yfp, glr-1::dsred, hsp-16::rol-6(su1006)] IV;* VH455 (in CB4856 background), *hdEx194[glr-1::yfp, unc-129::yfp, unc-47::yfp, rol-6(su1006)].* VH477 (in CB4856 background), *hdIs17[glr-1::yfp, unc-47::yfp, unc-129::yfp, rol-6(su1006)] I;* VH525, *nre-1(hd20) X, hdIs10[glr-1::yfp, unc-47::dsred, unc-129::cfp, hsp-16::rol-6(su1006)] V;* VH545 (VH477 three times outcrossed to N2), *hdIs17[glr-1::yfp, unc-47::yfp, unc-129::yfp, rol-6(su1006)] I*; VH624, *nre-1(hd20) X, rhIs13[unc-119::gfp; dpy-20(+)] V;* VH715, *nre-1(hd20) X, hdIs17[glr-1::yfp, unc-47::yfp, unc-129::yfp, rol-6(su1006)] I, hdIs10[glr-1::yfp, unc-47::dsred, unc-129::cfp, hsp-16::rol-6(su1006)] V;* VH972, *pry-1(mn38) I, hdIs17[glr-1::yfp, unc-47::yfp, unc-129::yfp, rol-6(su1006)] I;* VH982, *unc-101(m1) I, oxIs12[unc-47::gfpntx, lin-15(+)] X* and VH1101, *ced-1(e1735) I, hdEx191[glr-1::yfp, unc-129::yfp, unc-47::yfp, rol-6(su1006)].* The strain VH715 for the feeding RNAi experiments was created from crossings of the strains VH545 and VH525 and selecting for the presence of both the *nre-1* mutation and the *hdIs17* array in the F2 progeny. However, VH715 also contains the *hdIs10* array of VH525 origin. All the strains that contained the *hdIs17* array are partially Hawaii for chromosome I due to the original transgenic strain VH477, which was created in CB4856 background. This strain also

contains the dominant *rol-6(su1006)* allele that causes a rolling (Rol) phenotype in the transgenic worms.

4.2 Plasmid construction and germline transformation

To make the *glr-1::yfp* construct, the Pst1-BamHI fragment upstream of the *glr-1* gene of *C. elegans* from the plasmid pVH14.01 was cloned into the multicloning site of plasmid pVH20.01 (this plasmid is a derivative of Andy Fire's pPD95.75 that contains the promoterless yellow fluorescent protein (YFP) coding region for creating transcriptional gene fusions) in front of the YFP coding sequence to created the plasmid pVH10.13. To make the *unc-129::yfp* construct, the XbaI-BamHI fragment upstream of the *unc-129* gene from the plasmid pVH14.03 was cloned into pVH20.01 to created the plasmid pVH10.14. Similarly, to create the *unc-47::yfp* fusion, the Kpn1-SpeI fragment upstream of the *unc-47* gene from the plasmid pVH10.18 was cloned into pVH20.01 in front of the YFP coding sequence to created plasmid pVH10.34. The plasmids pVH10.13, pVH10.14, pVH10.18 and pVH10.34 have been made by Suse Zobeley.

The triple YFP transgenic worm strains were produced by co-injecting plasmids pVH10.13, pVH10.14, pVH10.34 and pRF4*[rol-6(su1006)]* at a concentration of 25-35 ng/µl each into the gonads of young adult worms as described (Mello *et al.,* 1991). For example, the polymorphic Hawaii strain CB4856 was used to create the triple YFP transgenic worm strain VH455 containing the stably propagating extrachromosomal array *hdEx194* with the three YFP fusions genes. Then the *hdEx194* array was integrated into the genome by UV mutagenesis to create the integrated transgene *hdIs17* line in the Hawaii background. In brief, ~300 young adult animals with the *hdEx194* array were places on a 10-cm NMG plate and exposed to 200x100 µJoule of UV radiation in a UV-wave oven. The treated animals were allowed to recover and then healthy transgenic animals were cloned on several 5-cm NMG plates. F1 progeny of the treated animals were cloned further and animals showing 100% propagation of the transgene were isolated. One stable isolate was outcrossed several times to CB4856 animals to remove background mutations and named VH477. The dominant transformation marker pRF4 encodes an abnormal cuticle collagen ROL-6 that when incorporated into the cuticle creates rolling movement in the transformed worms (Mello *et al.,* 1991).

4.3 Isolation of *nre* mutants

The *nre* mutants were isolated after EMS mutagenesis of the worm strain VH41 *(rhIs13 V [unc-119::gfp, dpy-20(+)]).* VH41 contains the transgene (*rhIs13*) that expresses the GFP in the entire nervous system of the worm under the control of promoter of the *unc-119* gene (Maduro and Pilgrim, 1995). We decided to use this transgenic strain with the reasoning that the expression level of GFP in the nervous system is moderate and any subtle change should be readily detectable after treatment with RNAi for the GFP in the nervous system of the animals. As depicted in Figure 2-2, ~1000 healthy L4 hermaphrodites of VH41 strain grown on the OP50 bacteria food were treated with 50 mM EMS for 4 h in M9 buffer (6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4 \cdot 7H_2O$ per liter) and allowed to recover on an NGM plate for further 4 h. Then 5-7 healthy looking and normally crawling animals were allowed to lay eggs onto 10 NGM plates with *GFP* RNAi bacteria as food. The *GFP* RNAi bacteria food was induced with IPTG on the NGM plates for about 48 h at room temperature. The treated animals were allowed to grow for two generations leading to ~20000 mutagenized genomes. Care was taken during the growth of worms so that the progeny worms did not starve and had sufficient amount of *GFP* RNAi bacteria food on the feeding plates. Occasionally, animals were transferred to new plates so as to prevent the starvation of growing population. All worms were grown at 20°C, however, they were occasionally stored at 15°C for no more than a day in order to reduce their growth rate for the convenience during screening protocol. After 5 days the F2 progeny were screened for the mutants with loss of GFP in the nervous system in the presence of sufficient amount *GFP* RNAi bacteria. Mutants that showed reversible loss of GFP in the nervous system were isolated and retested more that three times. Then were backcrossed to the wild-type or parental (i.e. VH41) strain for three times. In this screen we isolated two *nre* mutants, namely *hd20* and *hd21*. The *hd20* mutation was named *nre-1(hd20)* and as it showed robust neuronal RNAi efficiency it was used for further experimentations.

4.4 Mapping of the *nre-1* mutation

To facilitate the use of *nre-1(hd20)* mutation in genetic experiments we mapped the genomic position of the *nre-1(hd20)* by two-factor analysis. We used mapping strains with mutations in *dpy* genes and chromosomally integrated GFP marker transgenes linked to different chromosomes. The *nre-1(hd20)* mutant animals carrying the pan-neuronal GFP

transgene *rhIs13* were three times outcrossed with wild-type or parental (i.e. *rhIs13* containing strain VH41) animals and maintained in the *rhIs13* background for easy detection of Nre phenotype. The outcrossed strain VH624 *(nre-1(hd20), rhIs13 V [unc-119::gfp; dpy-20(+)])* was used for all subsequent mapping experiments. In the first round of two factor mapping experiments, VH624 hermaphrodites were crossed to wild-type males to obtain *nre-1(hd20)*/+; *rhIs13*/+ males. These males were then crossed to hermaphrodites of various Dpy mutants representing linkage groups (LG) I-V (see Table 2-3) on normal bacteria food. From the F1 progeny non-Dpy, GFP positive hermaphrodites were selected and further grown on GFP RNAi plates. Next, the Dpy, GFP positive F2 animals were scored for loss of GFP in the nervous system due to the presence of *nre-1(hd20)* mutation. Here the advantage of a few neurons that did not show RNAi effect was taken to score for animals with *rhIs13* transgene. From this analysis the location of *nre-1(hd20)* mutation was not expected to be present on LGI-V (Table 4.1). *nre-1(hd20)* also did not show any relation to LGV due to the fact that the transgene *rhIs13* is located on chromosome V and they were easily separable form each other. These experiments pointed to the presence of *nre-1(hd20)* mutation on chromosome X. Secondly, during crossing experiments we observed that the number of males expected from crosses between wild-type males and *nre-1(hd20)* hermaphrodites was unexpectedly low and in some cases no cross progeny males were observed. These experiments led us to reason that *nre-1(hd20)* must be located on chromosome X. To test the presence of *nre-1(hd20)* on chromosome X, a X-chromosomally integrated GFP transgene (*oxIs12 X*) was used in mapping experiments. *nre-1(hd20)* animals are temperature sensitive sterile and produce almost no progeny at 25°C and hemizygous *nre-1(hd20)* males are also not viable at 25°C. However, the hemizygous males grow normally at 20°C. Therefore, males heterozygous for *oxIs12* were crossed to *nre-1(hd20)* hermaphrodites and GFP positive F1 animals were allowed to grow at 25°C. In the case of F2 progeny if *nre-1(hd20)* is linked to chromosome X and is recessive then all the progeny should be GFP positive as the *nre-1(hd20)* homozygous animals do not develop and heterozygous animals are all GFP positive. As depicted in Table 2-4, of 9 F1 GFP/+; *nre-1(hd20)* /+ animals all gave rise to GFP positive F2 progeny. This confirmed that the GFP positive chromosome X complements the temperature sensitive sterility of the *nre-1(hd20)* mutation and therefore they are on the same chromosome. Similar tests were carried out for chromosome III and IV carrying GFP positive integrated transgenes. In the case of chromosome III, out of 11

F1 animals (*rhIs4*/+, *hd20*/+), 3 gave all GFP positive and 8 gave mix GFP/no-GFP F2 progeny. While in the case of chromosome IV, out of 10 F1 animals (*hdIs14*/+, *hd20*/+) 3 gave all GFP positive and 7 gave mix GFP/no-GFP F2 progeny indicating that the *nre-1(hd20)* is not associated with either of the chromosomes. These data confirmed the location of *nre-1(hd20)* on chromosome X of *C. elegans*.

4.5 Feeding RNAi experiments

The bacteria used for feeding RNAi experiments contained an inducible plasmid vector (also called feeding vector, L4440) carrying a fragment corresponding to a gene for production of dsRNAs between two T7 promoters in inverted orientation (Timmons and Fire, 1998). This feeding vector was then transformed into *E. coli* strain (HT115(DE3)) lacking the RNasesIII gene and carrying a transgenic element integrated in the genome for IPTC (iso-propyl-β-D-thio-galactoside) inducible expression of a T7 RNA polymerase fusion gene. The T7 RNA polymerase is highly processive T7-bacteriophage enzyme that initiates transcription at T7 promoter sites. Upon induction by IPTG the T7 RNA polymerase produced in bacterial cells rapidly synthesizes complementary ssRNAs corresponding to the fragment between two T7 promoters. These ssRNAs anneal to form dsRNAs in vivo in bacteria. When these bacteria are fed to worms, the dsRNAs systemically spread to different cells of the worm through gut cells, initiating the RNAi-mediated destruction of the cognate mRNAs.

The chromosome I feeding-RNAi clone library was created by Fraser *et al.* (2000) and was obtained from MRC gene service in 384-well plate format. We transferred the RNAi clones from the 384-well plate format to the 96-well plate format by a 96-pin replicator. Bacterial cultures were grown from the 96-well plate clones incubated overnight in 96-deep well bacteria culture plates containing LB medium with 100 µg/ml amphicilin. The feeding RNAi experiments in *C. elegans* were performed with these overnight grown bacteria cultures as described previously (Timmons and Fire, 1998; Fraser *et al.,* 2000). About 300 µl of each culture was seeded onto 3.5-cm NGM plates containing 1 mM IPTG and 25 µg/ml carbenicilin and incubated for about 24 h at room temperature. Care was taken that the bacterial cultures on the plates are completely dry before placing animals for feeding experiments. To screen for the genes on chromosome I by feeding RNAi method, each clone from the chromosome I RNAi library was fed to the transgenic strain VH715 by placing about 5 L3-L4 stage hermaphrodite worms onto the

incubated plates that had expressed dsRNA in bacteria for each gene. The worms were allowed to feed on the RNAi bacteria for 5-7 days to at least one generation. The phenotypes of adults and progeny of about 50 worms were scored under a fluorescence microscope by washing the worms in M9 buffer and amounting it on glass slides with agar pads. The feeding RNAi clones that showed axon guidance defects in about 50% of the fed animals were isolated and retested for their ability to cause axon guidance defects in the *nre-1(hd20)* strain.

4.6 Microscopy and imaging techniques

Animals were washed from growing populations and were incubated with 10 mM levamisol or 100 mM NaN_3 in M9 buffer for 1 h to immobilize them and mounted onto 3% agar pads on object slides. For large-scale screening immobilized animals were placed onto agar pads and observed under a conventional fluorescence microscope (Axioscope or Axiovert135, Zeiss) with 20X, 40X and/or 100X objective lenses using suitable fluorescence color filters for fluorescence experiments or Normaski filter for differential interference phase contrast examinations. Stacks of confocal images were taken on a Leica TCS SP2 microscope at about 0.2 mm vertical pitch distance. Images were stored and maximum intensity projections of all images from a given animal were generated by ImageJ software available at http://rsb.info.nih.gov/ij. The images were edited using routine imaging softwares, for example, Adobe PhotoshopCS 8.0. Under the conditions of microscopic examinations described above the mounted specimens remained intact for more than two hours with no detectable change in their morphology or fluorescence properties.

4.7 DNA Sequencing

The RNAi clones that showed axon guidance phenotypes were isolated from the host bacteria for DNA sequencing using standard plasmid preparation kits (Qiagen) to confirm the correctness of the inserted RNAi gene fragments and also to remove false positive clones that did correspond to the reported genes in the feeding RNAi library, but had wrong or no inserts in the plasmids. The RNAi clones were sequenced bidirectionally on an automated DNA sequencer (ABI 3100 DNA Sequencer) by using the feeding vector (plasmid L4440) specific primers HH_sepr_13 (3`-accgtattaccgcctttgagtgag-5`) and HH_sepr_14 (3`-ccagctggcgaaaggggatgtgctgc-5`), which are located upstream and

downstream of the polycloning site of the feeding vector L4440, respectively. For amplifying PCR experiments, the BigDye cycle sequencing kit (Perkin Elmer) was used as per manufacturer's protocol. The sequences were matched to the corresponding genes in the worm genome database to confirm the identities of the positive clones. Out of the 63 clones, 57 clones contained the correct sequences corresponding to the cognate genes they represent. However, the three clones representing the genes M01G2.4, Y106G6E.6 and ZK973.6 contained the worm sequences that did not correspond to any part of the curated genes. The remaining three clones contained sequences corresponding to the *nas-5* (T23H4.3) gene and not for the reported genes showing possible contamination of stocks of the bacteria clones.

4.8 Bioinformatic analysis and classification of genes

The bioinformatic analysis of the protein sequences of the 57 RNAi clones that represented correctly the cognate genes was performed to identify their homologs in other organisms. The protein sequences of the 57 genes were collected from the *C. elegans* database (www.wormbase.org, release WS133) and used to perform protein-protein BLAST comparisons with human, mouse and *Drosophila* protein sequences available at the GenBank database (www.ncbi.nlm.nih.gov/Genbank). Each individual gene sequence was tested against human, mouse or *Drosophila* sequences with a p-value of $>10^{-10}$ for a sequence length of >30% between the *C. elegans* gene sequence and that of the other organisms. The *C. elegans* genes were categorized based on their homology with the characterized or annotated genes from GenBank for the purpose of their putative functions. In the case of genes that did not show any homology with the GenBank protein sequences were labeled novel genes. However, these genes may have some similarity to the genes of other organisms, as the parameters used above to perform sequence comparisons do not consider low similarities between the sequences. Genes that have homologs in the organisms, but their functions are unknown were labeled as conserved novel genes. Annotated genes were classified based on their categorization reported in the databases or literature.

REFERENCES

Ackley B.D., *et al.* (2001) The NC1/endostatin domain of *C. elegans* type XVIII collagen affects cell migration and axon guidance. *Journal of Cell Biology* **152,** 1219-1232.

Albertson D.G., Rose A.M. and Villeneuve A.M. (1997) *C. elegans* II (Eds. Riddle D.L., Blumenthal B., Meyer J.R. and Priess J.R.). *Cold Spring Harbor Press,* 47-78.

Antebi A., Norris C.R. and Hedgecock E.M. (1997) Cell and growth cone migrations in *C. elegans* II. *Cold Spring Harbor Laboratory Press,* 583–609.

Araujo S.J. and Tear G. (2003) Axon guidance mechanisms and molecules: lessons from invertebrates. *Nature Review Neuroscience* **4,** 910-922.

Ashrafi K., *et al.* (2003) RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. *Nature* **421,** 268-272.

Augsburger A., *et al.* (1999) BMPs as mediators of roof plate repulsion of commissural neurons. *Neuron* **24,** 127–141.

Avery L. and Horvitz H.R. (1989) Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of *C. elegans*. *Neuron* **3,** 473-485.

Bagasra O. and K. R. Prilliman (2004) RNA interference: The molecular immune system. *Journal of Molecular Histology* **35,** 545-553.

Bagri A., *et al.* (2003) Stereotyped pruning of long hippocampal axon branches triggered by retraction inducers of the semaphorin family. *Cell* **113,** 285-299.

Bargmann C.I. and Horvitz H.R. (1991) Control of larval development by chemosensory neurons in Caenorhabditis elegans. *Science* **251,** 1243-1246.

Bargmann C.I. and Avery L. (1995) *C. elegans:* Modern biological analysis of an organism (Eds. Epstein F.E. and Shakes D.C.). *Academic Press,* 225-250.

Bargmann C.I. (1998) Neurobiology of the *C. elegans* genome. *Science* **282,** 2028-2033.

Baum P.D. and Garriga G. (1997) Neuronal migrations and axon fasciculation are disrupted in *ina-1* integrin mutants. *Neuron* **19,** 51-62.

Bessereau J.L., *et al.* (2001) Mobilization of a *Drosophila* transposon in the *Caenorhabditis elegans* germ line. N*ature* **413,** 70-74.

Brenner S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77,** 71–94.

Broadbent I.D. and Pettitt J. (2002) The *C. elegans hmr-1* gene can encode a neuronal classic cadherin involved in the regulation of axon fasciculation. *Current Biology* **12,** 59-63.

Brose K., *et al.* (1999) Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* **96,** 795–806.

Bruckner K., Pasquale E.B. and Klein R. (1997) Tyrosine phosphorylation of transmembrane ligands for Eph receptors. *Science* **275,** 1640–1643.

Bulow H.E., *et al.* (2002) Heparan sulfate proteoglycan-dependent induction of axon branching and axon misrouting by the Kallmann syndrome gene *kal-1*. *PNAS* **99,** 6346-6351.

Bulow H.E., Boulin T. and Hobert O. (2004) Differential functions of the *C. elegans* FGF receptor in axon outgrowth and maintenance of axon position. *Neuron* **42,** 367-374.

***C. elegans* Sequencing Consortium, The.** (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282,** 2012-2018.

Chan S.S., *et al.* (1996) UNC-40, a *C. elegans* homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues. *Cell* **87,** 187-195.

Chang C., *et al.* (2004) Inhibition of netrin-mediated axon attraction by a receptor protein tyrosine phosphatase. *Science* **305,** 103-106.
Cheng H.J. and Flanagan J.G. (1994) Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. *Cell* **79,** 157–168.
Chalfie M. and White J. (1988) The nematode *C. elegans* (Ed. Wood W.B.). *Cold Spring Harbor Press,* 337-391.
Chalfie M., *et al.* (1994) Green fluorescent protein as a marker for gene expression. *Science* **263,** 802-805.
Chalfie M. and Jorgensen E.M. (1998) *C. elegans* neuroscience: genetics to genome. *Trends Genetics* **14,** 506-512.
Chin-Sang I.D., *et al.* (2002) The divergent C. elegans ephrin EFN-4 functions in embryonic morphogenesis in a pathway independent of the VAB-1 Eph receptor. *Development* **129,** 5499-5510.
Clarke D.L., *et al.* (2000) Generalized potential of adult neural stem cells. *Science* **288,** 1660-1663.
Colavita A. and Culotti J.G. (1998) Suppressors of ectopic UNC-5 growth cone steering identify eight genes involved in axon guidance in *C. elegans*. *Developmental Biology* **194,** 72-85.
Colavita A., *et al.* (1998) Pioneer axon guidance by UNC-129, a *C. elegans* TGF-beta. *Science* **281,** 706-709.
Cox E.A., Tuskey C. and Hardin J. (2004) Cell adhesion receptors in *C. elegans*. *Journal of Cell Science* **117,** 1867-1870.
Culotti J.G. and Merz D.C. (1998) DCC and netrins. *Current Opinion in Cell Biology* **10,** 609-613.
Dalpe G., *et al.* (2004) Conversion of cell movement responses to Semaphorin-1 and Plexin-1 from attraction to repulsion by lowered levels of specific RAC GTPases in *C. elegans*. *Development* **131,** 2073-2088.
Dent E.W., Tang F. and Kalil K. (2003) Axon guidance by growth cones and branches: common cytoskeletal and signaling mechanisms. *Neuroscientist* **9,** 343-353.
Dickson B.J. (2002) Molecular mechanisms of axon guidance. *Science* **298,** 1959-1964.
Drescher U., *et al.* (1995) *In vitro* guidance of retinal ganglion cellaxons by RAGS, a 25kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell* **82,** 359–370
Driessens M.H., *et al.* (2001) Plexin-B semaphorin receptors interact directly with active Rac and regulate the actin cytoskeleton by activating Rho. *Current Biology* **11,** 339–344.
Durbin R.M. (1987) Studies on the development and organization of the nervous system of *Caenorhabditis elegans* (Ph.D. thesis) *University of Cambridge*.
Evgrafov O.V., *et al.* (2004) Mutant small heat-shock protein 27 causes axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. *Nature Genetics* **36,** 602-606.
Estevez M., *et al.* (1993) The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. *Nature* **365,** 644-649.
Ferguson E. and Horvitz, H.R. (1985) Identification and characterization of 22 genes that affect the vulval cell lineages of *Caenorhabditis elegans*. *Genetics* **110,** 17–72.
Fire A., *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in C*aenorhabditis elegans*. *Nature* **391,** 806–811.
Forrester W.C. and Garriga G. (1997) Genes necessary for *C. elegans* cell and growth cone migrations. *Development* **124,** 1831-1843.

Fraser A.G., *et al.* (2000) Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408,** 325-330.
Fricke C., *et al.* (2001) astray, a zebrafish roundabout homolog required for retinal axon guidance. *Science* **292,** 507-510.
Gitai Z., *et al.* (2003) The netrin receptor UNC-40/DCC stimulates axon attraction and outgrowth through enabled and, in parallel, Rac and UNC-115/AbLIM. *Neuron* **37,** 53-65.
Gonczy P., *et al.* (2000) Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* **16,** 331-336.
Guan K.L. and Rao Y. (2003) Signaling mechanisms mediating neuronal responses to guidance cues. *Nature Review Neuroscience* **4,** 941-956.
Guo S. and Kemphues K.J. (1996) A non-muscle myosin required for embryonic polarity in *Caenorhabditis elegans. Nature* **382,** 455-458.
Hao J.C., *et al.* (2001) *C. elegans* slit acts in midline, dorsal-ventral, and anterior-posterior guidance via the SAX-3/Robo receptor. *Neuron* **32,** 25-38.
Hannon G.J. (2002) RNA interference. *Nature* **418,** 244-251.
Hart A.C., Sims S. and Kaplan J.M. (1995) Synaptic code for sensory modalities revealed by *C. elegans* GLR-1 glutamate receptor. *Nature* **378,** 82-85.
Hedgecock E.M., *et al.* (1987) Genetics of cell and axon migrations in *Caenorhabditis elegans. Development* **100,** 365–382.
Hedgecock E.M., Culotti J.G. and Hall D.H. (1990) The *unc-5, unc-6,* and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. *Neuron* **4,** 61-85.
Herman R.K. (1988) The nematode *C. elegans* (Ed. Wood W.B.). *Cold Spring Harbor Press,* 17-45.
Hobert O., Johnston R.J. and Chang S. (2002) Left-right asymmetry in the nervous system: the *Caenorhabditis elegans* model. *Nature Review Neuroscience* **3,** 629-640.
Hogan B.L. (1996) Bone morphogenetic proteins in development. *Current Opinion in Genettics and Development* **6,** 432–438.
Holland S.J. *et al.* (1996) Bidirectional signaling through the EPH family receptor Nuk and its transmembrane ligands. *Nature* **383,** 722–725.
Howard R.M. and Sundaram M.V. (2002) *C. elegans* EOR-1/PLZF and EOR-2 positively regulate Ras and Wnt signaling and function redundantly with LIN-25 and the SUR-2 Mediator component. *Genes Dev.* **16,** 1815-1827.
Howe A., *et al.* (1998) Integrin signaling and cell growth control. *Current Opinion in Cell Biology* **10,** 220-231.
Huber A.B., *et al.* (2003) Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annual Review of Neuroscience* **26,** 509-563.
Huang X., *et al.* (2002) MAX-1, a novel PH/MyTH4/FERM domain cytoplasmic protein implicated in netrin-mediated axon repulsion. *Neuron* **34,** 563-576.
Huang X., *et al.* (2003) UNC-71, a disintegrin and metalloprotease (ADAM) protein, regulates motor axon guidance and sex myoblast migration in *C. elegans*. *Development* **130,** 3147-3161.
Hutter H. (2000) Conservation and novelty in the evolution of cell adhesion and extracellular matrix genes. *Science* **287,** 989-994.
Hutter H. (2003) Extracellular cues and pioneers act together to guide axons in the ventral cord of *C. elegans. Development* **130,** 5307-5318.

Hutter H. (2004) Five-colour in vivo imaging of neurons in *Caenorhabditis elegans*. *Journal of Microscopy* **215,** 213-218.
Ikegami R., *et al.* (2004) Integration of semaphorin-2A/MAB-20, ephrin-4, and UNC-129 TGF-beta signaling pathways regulates sorting of distinct sensory rays in *C. elegans. Developmental Cell* **6,** 383-395.
Ishii N., *et al.* (1992) UNC-6, a laminin-related protein, guides cell and pioneer axon migrations in *C. elegans*. *Neuron* **9,** 873-881.
Jansen G., *et al.* (1997) Reverse genetics by chemical mutagenesis in *Caenorhabditis elegans*. *Nature Genetics* **17,** 119-121.
Jen J.C., *et al.* (2004) Mutations in a human ROBO gene disrupt hindbrain axon pathway crossing and morphogenesis. *Science* **304,** 1509-1513.
Jorgensen E.M. and Mango S.E. (2002) The art and design of genetic screens: *Caenorhabditis elegans*. *Nature Review Genetics* **3,** 356-369.
Kapfhammer J.P. and Raper J.A. (1987) Collapse of growth cone structure on contact with specific neurites in culture. *Journal of Neuroscience* **7,** 201–212.
Kaprielian Z., *et al.* (2000) Axon guidance at the midline of the developing CNS. *Anatomical Record* **26,** 176-197.
Kandel E.R., Schwartz J.H. and Jessell T.M. (2000) Principles of Neural Science (4 edition). *McGraw-Hill/Appleton & Lange,* 5-36.
Kamath R.S., *et al.* (2000) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans. Genome Biology* **2,** 1-10.
Kamath R.S., *et al.* (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421,** 231-237.
Keleman K., *et al.* (2002) Comm sorts robo to control axon guidance at the *Drosophila* midline. *Cell* **110,** 415-427.
Kennedy T.E., *et al.* (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78,** 425-435.
Kennedy S., Wang D. and Ruvkun G. (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* **427,** 645-649.
Kenwrick S. and Doherty P. (1998) Neural cell adhesion molecule L1: relating disease to function. *Bioessays* **20,** 668-675.
Kidd T., Bland K.S. and Goodman C.S. (1999) Slit is the midline repellent for the robo receptor in *Drosophila*. *Cell* **96,** 785–794.
Kim S. and Wadsworth W.G. (2000) Positioning of longitudinal nerves in *C. elegans* by nidogen. *Science* **288,** 150-154.
Kiryushko D., Berezin V. and Bock E. (2004) Regulators of neurite outgrowth: role of cell adhesion molecules. *Ann N Y Acad Sci* **1014,** 140-154.
Knobel K.M., Jorgensen E.M and Bastiani M.J. (1999) Growth cones stall and collapse during axon outgrowth in *Caenorhabditis elegans. Development* **126,** 4489-4498
Kokel M., *et al.* (1998) *clr-1* encodes a receptor tyrosine phosphatase that negatively regulates an FGF receptor signaling pathway in *C. elegans*. *Genes and Development* **12,** 1425-1437.
Kolodkin A.L., *et al.* (1992) Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. *Neuron* **9,** 831–845.
Korswagen H.C. *et al.* (2002) The Axin-like protein PRY-1 is a negative regulator of a canonical Wnt pathway in *C. elegans. Genes Dev.* **16,** 1291-1302.

Korswagen H.C. (2002a) Canonical and non-canonical Wnt signaling pathways in *C. elegans*: variations on a common signaling theme. *Bioessays* **24,** 801-810.
Kramer J.M. (1997) *C. elegans* II (Eds. Riddle D.L., Blumenthal B., Meyer J.R. and Priess J.R.). *Cold Spring Harbor Press,* 471-500.
Kullander K. and Klein R. (2002) Mechanisms and functions of Eph and ephrin signaling. *Nature Review Molecular Cell Biology* **3,** 475–486.
Lee J., Jongeward G.D. and Sternberg P.W. (1994) *unc-101,* a gene required for many aspects of *Caenorhabditis elegans* development and behavior, encodes a clathrin-associated protein. *Genes Dev.* **8,** 60-73.
Lee S.S., *et al.* (2003) A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity. *Nature Genetics* **33,** 40-48.
Leung-Hagesteijn C., *et al.* (1992) UNC-5, a transmembrane protein with immunoglobulin and thrombospondin type 1 domains, guides cell and pioneer axon migrations in *C. elegans*. *Cell* **71,** 289-299.
Lewis J.A., *et al.* (1980) The genetics of levamisole resistance in the nematode *Caenorhabditis elegans*. *Genetics* **95,** 905-928.
Lundquist E.A., *et al.* (2001) Three *C. elegans* Rac proteins and several alternative Rac regulators control axon guidance, cell migration and apoptotic cell phagocytosis. *Development* **128,** 4475-4488.
Luo Y., Raible D. and Raper J.A. (1993) Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* **75,** 217–227.
Lyuksyutova A.I., *et al.* (2003) Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. *Science* **302,** 1984-1988.
Maduro M. and Pilgrim D. (1995) Identification and cloning of *unc-119*, a gene expressed in the *Caenorhabditis elegans* nervous system. *Genetics* **141,** 977-988.
Maeda I., *et al.* (2002) Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. *Current Biology* **11,** 171-176.
Mazelin L., *et al.* (2004) Netrin-1 controls colorectal tumorigenesis by regulating apoptosis. *Nature* **431,** 80-84.
McIntire S.L., *et al.* (1992) Genes necessary for directed axonal elongation or fasciculation in *C. elegans*. *Neuron* **8,** 307–322.
McIntire S.L., *et al.* (1997) Identification and characterization of the vesicular GABA transporter. *Nature* **389,** 870-876.
Mello C.C., *et al.* (1991) Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO Journal* **10,** 3959–3970.
Miller M.A., *et al.* (2003) An Eph receptor sperm-sensing control mechanism for oocyte meiotic maturation in *C. elegans*. *Genes and Development* **15,** 187-200.
Mohrlen F., Hutter H. and Zwilling R. (2003) The astacin protein family in Caenorhabditis elegans. *Europian Journal of Biochememisrty* **270,** 4909-4920.
Moon R.T., *et al.* (2002) The promise and perils of Wnt signaling through β-catenin. *Science* **296,** 1644–1646.
Montgomery M.K. (2004) RNA interference: historical overview and significance. *Methods inMolecular Biology* **265,** 3-21.
Much J.W., *et al.* (2000) The *fax-1* nuclear hormone receptor regulates axon pathfinding and neurotransmitter expression. *Development* **127,** 703-712.
Murai K.K. and Pasquale E.B. (2004) Eph receptors, ephrins, and synaptic function. *Neuroscientist* **10,** 304-314.

Nishikura K. (2001) A short primer on RNAi: RNA-directed RNA polymerase acts as a key catalyst. *Cell* **107,** 415-418.
Ogura K., *et al.* (1997) The UNC-14 protein required for axonal elongation and guidance in *Caenorhabditis elegans* interacts with the serine/threonine kinase UNC-51. *Genes and Development* **11,** 1801-1811.
Otsuka A.J., *et al.* (1995) An ankyrin-related gene (*unc-44*) is necessary for proper axonal guidance in *C. elegans*. *Journal Cell Biology* **129,** 1081-1092.
Pasterkamp R.J. and Kolodkin A.L. (2003) Semaphorin junction: making tracks toward neural connectivity. *Current Opinion Neurobiology* **13,** 79–89.
Pires-daSilva A. and Sommer R.J. (2003) The evolution of signaling pathways in animal development. *Nature Review Genetics* **4,** 39-49.
Poinat P., *et al.* (2002) A conserved interaction between beta1 integrin/PAT-3 and Nck-interacting kinase/MIG-15 that mediates commissural axon navigation in *C. elegans. Current Biology* **12,** 622-631.
Prasad B.C., *et al.* (1998) *unc-3*, a gene required for axonal guidance in Caenorhabditis elegans, encodes a member of the O/E family of transcription factors. *Development* **125,** 1561-1568.
Richmond J.E., Davis W.S. and Jorgensen E.M. (1999) UNC-13 is required for synaptic vesicle fusion in *C. elegans*. *Nature Neuroscience* **2,** 959-964.
Riddle D.L., *et al.* (1997) *C. elegans* II (Eds. Riddle D.L., Blumenthal B., Meyer J.R. and Priess J.R.). *Cold Spring Harbor Press,* 1-22.
Rothberg J.M., *et al.* (1988) Slit: an EGF-homologous locus of *D. melanogaster* involved in the development of the embryonic central nervous system. *Cell* **55,** 1047-1059.
Rugarli E.I., *et al.* (2002) The Kallmann syndrome gene homolog in *C. elegans* is involved in epidermal morphogenesis and neurite branching. *Development* **129,** 1283-1294.
Ruvkun G. and Hobert O. (1998) The taxonomy of developmental control in *Caenorhabditis elegans*. *Science* **282,** 2033-2041.
Sarafi-Reinach T.R., *et al.* (2001) The lin-11 LIM homeobox gene specifies olfactory and chemosensory neuron fates in *C. elegans. Development* **128,** 3269-3281.
Schnabel R. and Priess J.R. (1997) *C. elegans* II (Eds. Riddle D.L., Blumenthal B., Meyer J.R. and Priess J.R.). *Cold Spring Harbor Press,* 361-382.
Serafini T., *et al.* (1994) The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell* **78,** 409-424.
Shen K. and Bargmann C.I. (2003) The immunoglobulin superfamily protein SYG-1 determines the location of specific synapses in *C. elegans*. *Cell* **112,** 619-630.
Shen K., Fetter R.D. and Bargmann C.I. (2004) Synaptic specificity is generated by the synaptic guidepost protein SYG-2 and its receptor, SYG-1. *Cell* **116,** 869-881.
Simmer F., *et al.* (2002) Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. elegans* hypersensitive to RNAi. *Current Biology* **12,** 1317-1319.
Simmer F., *et al.* (2003) Genome-wide RNAi of *C. elegans* using the hypersensitive *rrf-3* strain reveals novel gene functions. *PLoS Biology* **1,** 77-84.
Sijen T., *et al.* (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107,** 465-476.
Sonnichsen B., *et al.* (2005) Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. *Nature* **434,** 462-469.

Steven R., *et al.* (1998) UNC-73 activates the Rac GTPase and is required for cell and growth cone migrations in *C. elegans*. *Cell* **92,** 785-795.
Strange K. (2003) From genes to integrative physiology: ion channel and transporter biology in *Caenorhabditis elegans*. *Physiological Review* **83,** 377-415.
Struckhoff E.C. and Lundquist E.A. (2003) The actin-binding protein UNC-115 is an effector of Rac signaling during axon pathfinding in *C. elegans*. *Development* **130,** 693-704.
Su M., *et al.* (2000) Regulation of the UNC-5 netrin receptor initiates the first reorientation of migrating distal tip cells in *C. elegans*. *Development* **127,** 585-594.
Sugimoto A. (2004) High-throughput RNAi in *Caenorhabditis elegans*: genome-wide screens and functional genomics. *Differentiation* **72,** 81-91.
Sulston, J. and Horvitz, H.R. (1977) Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Developmental Biol*ogy 5**6,** 110–156.
Sulston, J. and Horvitz, H.R. (1981) Abnormal cell lineages in mutants of the nematode *Caenorhabditis elegans*. *Developmental Biol*ogy **82,** 41–55.
Sulston J.E., *et al.* (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Developmental Biology* **100,** 64–119.
Tabara H., Grishok A. and Mello C.C. (1998) RNAi in *C. elegans*: soaking in the genome sequence. *Science* **282,** 430-431.
Tavernarakis N., *et al.* (2000) Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nature Genetics* **24,** 180–183.
Teichmann S.A. and Chothia C. (2000) Immunoglobulin superfamily proteins in *Caenorhabditis elegans*. *Journal of Molecular Biology* **296,** 1367-1383.
Tessier-Lavigne M. and Goodman C.S. (1996) The molecular biology of axon guidance. *Science* **274,** 1123-1133.
Timmons L. and Fire A. (1998) Specific interference by ingested dsRNA. *Nature* **395,** 854.
Timmons L., Court D.L. and Fire A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* **263,** 103–112.
Vogel B.E. and Hedgecock E.M. (2001) Hemicentin, a conserved extracellular member of the immunoglobulin superfamily, organizes epithelial and other cell attachments into oriented line-shaped junctions. *Development* **128,** 883-894.
Wacker I., *et al.* (2003) *zag-1*, a Zn-finger homeodomain transcription factor controlling neuronal differentiation and axon outgrowth in *C. elegans*. *Development* **130,** 3795-3805.
Walsh F.S. and Doherty P. (1997) Neural cell adhesion molecules of the immunoglobulin superfamily: role in axon growth and guidance. *Annual Review of Cell and Developmental Biology* **13,** 425-456.
Wang K.H., *et al.* (1999) Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. *Cell* **96,** 771–784.
White J.G., *et al.* (1976) The structure of the ventral nerve cord of *Caenorhabditis elegans*. *Philos Trans R Soc Lond B Biol Sci.* **275,** 327-348.
White J.G., *et al.* (1983) Factors that determine connectivity in the nervous system of *Caenorhabditis elegans*. *Cold Spring Harb. Symp. Quant. Biol.* **48,** 633-640.
White J.G., *et al.* (1986) The structure of the nervous system of the nematode *Caenorhabditis elegans. Phil. Trans. Royal Soc. London. Series B, Biol. Scien.* **314,** 1-340.

Wieschaus E., Nusslein-Volhard C. and Kluding H. (1984) Kruppel, a gene whose activity is required early in the zygotic genome for normal embryonic segmentation. *Developmental Biology* **104,** 172-186.
Winston W.M., Molodowitch C. and Hunter C.P. (2002) Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. *Science* **295** 2456-2459.
Wood W.B. (1988) The nematode *C. elegans* (Ed. Wood W.B.). *Cold Spring Harbor Press,* 1-16.
Yoshikawa S., *et al.* (2003) Wnt-mediated axon guidance via the *Drosophila* Derailed receptor. *Nature* **422,** 583-588.
Yu H.H., *et al.* (2001) Multiple signaling interactions of Abl and Arg kinases with the EphB2 receptor. *Oncogene* **20,** 3995–4006.
Yu T.W. and Bargmann C.I. (2001) Dynamic regulation of axon guidance. *Nature Neuroscience* **4,** 1169-1176.
Yu T.W., *et al.* (2002) Shared receptors in axon guidance: SAX-3/Robo signals via UNC-34/Enabled and a Netrin-independent UNC-40/DCC function. *Nature Neuroscience* **5,** 1147-1154.
Zallen J.A., Kirch S.A. and Bargmann C.I. (1999) Genes required for axon pathfinding and extension in the *C. elegans* nerve ring. *Development* **126,** 3679-3692.
Zallen J.A., Yi B.A. and Bargmann C.I. (1998) The conserved immunoglobulin superfamily member SAX-3/Robo directs multiple aspects of axon guidance in *C. elegans*. *Cell* **92,** 217-227.
Zhou Z., Hartwieg E. and Horvitz H.R. (2001) CED-1 is a transmembrane receptor that mediates cell corpse engulfment in *C. elegans. Cell* **104,** 43-56.
Zipperlen P., *et al.* (2001) Roles for 147 embryonic lethal genes on C.elegans chromosome I identified by RNA interference and video microscopy. *EMBO Journal* **20,** 3984-3992.
Zhu Y., *et al.* (1999) Cellular and molecular guidance of GABAergic neuronal migration from an extracortical origin to the neocortex. *Neuron* **23,** 473–485.