2009

Dissecting the Development and Function of C. elegans Glia with Mutations of the mls-2 and vab-3 Genes

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DISSECTING THE DEVELOPMENT AND FUNCTION OF *C. ELEGANS* GLIA WITH MUTATIONS OF THE *mls-2* AND *vab-3* GENES

A Thesis Presented to the Faculty of

The Rockefeller University

in Partial Fulfillment of the Requirements for

the degree of Doctor of Philosophy

by

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June 2009

Dissecting the Development and Function of *C. elegans* Glia with Mutations of the *mls-2* and *vab-3* Genes

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The Rockefeller University 2009

The past decade has produced much evidence that glia, the major cellular component of vertebrate nervous systems, play active integral roles in a variety of processes including neuronal migration, synaptogenesis, and modulation of synaptic activity. Yet, many aspects of glia-neuron interactions remain obscure partly because manipulation of glia can result in neuronal death, hampering attempts to study glial cells *in vivo*. Moreover, a comprehensive understanding of glia differentiation is not yet achieved, although several key molecules in the process have been discovered.

C. elegans possesses glia-like cells that are morphologically reminiscent of vertebrate glia. In this thesis, I show that *C. elegans* CEPsh glia possess molecular and functional similarities to vertebrate glia. I identify transcriptional programs specifying these glia, demonstrating ventral- and dorsal-restricted roles for the *mls-2*/*Nkx*/*Hmx* and *vab-3*/*Pax6*/*Pax7* genes, respectively, in differentiation and expression of the genes *hlh-17*/*Olig* and *ptr-10*/*Patched*-related. Similar pathways regulate oligodendrocyte generation in vertebrate spinal cords. Using *mls-2* and *vab-3* mutants, as well as CEPsh glia-ablated animals, I also uncover

roles for CEPsh glia in dendrite extension and axon branching and guidance, and show that these latter functions are mediated, at least in part, by the UNC-6/Netrin protein. During the course of this study, I also confirmed that *C. elegans* CEPsh glia are not required for neuronal survival.

Overall, the conservation of molecular features between the development of *C. elegans* CEPsh glia and vertebrate oligodendrocytes, together with the lack of neurotrophic roles for these glia, suggests that *C. elegans* can serve as a unique model organism to explore, *in vivo*, basic aspects of metazoan glia development and function, as well as glia-neuron interactions.

Acknowledgements

First and foremost, I would like to thank my graduate advisor Shai Shaham for giving me the opportunity to work in his lab, and for his continued support and encouragement. Thanks also go to my thesis committee members, Cori Bargmann, Sidney Strickland, Ulrike Gaul, and Fiona Doetsch. They have given me many valuable suggestions, scientific and political.

I thank all the lab members, past and present, who'd always help me out when asked. They include Elliot Perens, Carine Waase, Mary Abraham, Max Heiman, Maya Goldmit, Taulant Bacaj, Carl Procko, Grigoris Oikonomou, Elyse Blum, Michael Chiorazzi, and Menachem Katz. I must especially thank our outstanding technician Limor Baum, as well as our lab aide Sharon White. They did a great job of keeping the lab running smoothly at all times.

Special thanks go to my collaborators John Murray and Robert Waterston. John has performed the lineage analysis, which brought important insights into the development of *C. elegans* glia. Several other people also provided help in completing the work for this thesis. I must especially thank Yun Lu for the electron microscopy and Alison North of the Rockefeller University Bio-Imaging Center for help with imaging.

Finally, I would like to thank Shohei Mitani and the Caenorhabditis Genetics Center for providing some nematode strains used in my research.

Material support was also provided by Piali Sengupta, Kang Shen, David Greenstein, and Andrew Chisholm.

Ph.D.をわざわざアメリカまで来て取るために、大変多くの方々にご迷惑をかけ、そしてご協力を頂きました。そのことにあらためて感謝の意を表明したいと思います。ありがとうございました。

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Chapter 2

Chapter 1

General Overview and Background

In general, nervous systems are described as composed of two cell types: neurons and non-neuronal cells collectively referred to as glia (Greek for “glue”). German pathologist Rudolf Virchow is often regarded as the first to describe, the non-neuronal component of nervous systems (Virchow, 1846). The exact term he used in his text to describe the component was “Nervenkitt” (nerve glue). It was somewhat unfortunate for these cells to have such a name because it brought to many people’s mind cells designed for the purpose of keeping our neurons from bumping into one another. In addition, the absence of action potential in glia, in comparison with neurons which can be studied with electrodes, has made investigation of their roles in nervous systems challenging. However, a series of studies *in vitro* and *in vivo* are now starting to reveal unexpected functions of glia and they seem to be much more actively involved in brain functions than previously thought. I would like to start this chapter by briefly stating my personal view on the meaning of studying glia.

1.1 Glia: What Are They, and Why Care?

- **Why Care About Glia?**

Glia are estimated to compose about 90% of the human brain. Functions, if

any, of the 90% of cells may have to be clarified in order to comprehensively understand our nervous system. It is possible, for example, to consider that glia have a role completely independent of neurons and are not involved in higher order brain functions such as memory. On the contrary, however, recent findings suggest interesting roles for glia and highlight the importance of viewing many activities of nervous systems as a collaboration between glia and neurons (see following sections). More importantly, glia are involved in brain diseases. For example, massive reactive gliosis is often observed in patients with epilepsy, a neurological diseases affecting 1% of the population world wide (de Lanerolle and Lee, 2005; Kim, 2001). Although the significance of this alteration is poorly understood, the generation and spread of seizure activity in epilepsy may be due to the alterations in function of astrocytes. Thus thorough understanding of basic physiology of glia offers the potential for developing treatments for such neurological disorders.

Glia can also be an interesting target for research from evolutionally point of view. Glia compose 10-20 % of cells in the *Drosophila* nervous system, and about 90 % of cells in human brain, as mentioned previously. One interpretation of this observation is that glial function is crucial for the increased complexity of neurological function that has emerged during evolution. If so, how are they crucial? Investigations of glia may provide some critical insights into this kind of question.

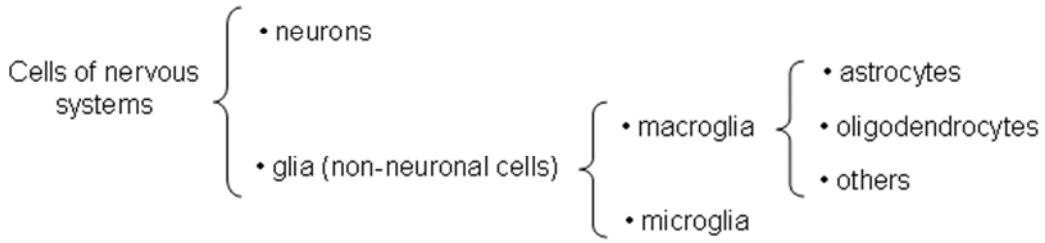
- **Defining Glia**

Traditionally, cells of the nervous systems other than neurons are often collectively referred to as glia (Figure 1.1A). However, this rough grouping is problematic, because cells categorized as glia are quite heterogeneous in structure, function, and in derivation. For example, a type of glia called microglia are haematopoietic in origin (Ling and Wong, 1993), whereas other glia, such as astrocytes and oligodendrocytes, derive from ectoderm, which also give rise to neurons (Rowitch, 2004). Radial glia, a unique type of glia typically observed during development of the central nervous systems (CNS), serve as neuronal progenitor cells (Doetsch et al., 1999; Malatesta et al., 2000; Noctor et al., 2001). In order to properly categorize this complex population of cells that comprise the "non-neuronal" part of the nervous systems, researchers may have to supplement the term "glia" with additional terms that better reflect the diversity of these cells.

For this thesis, I would like to use the term "glia" to refer to what has been recognized as "macroglia" (Figure 1.1B). Macroglia are defined by the three criteria (Shaham, 2005): first, they are always physically associated with neurons; second, they do not transmit action potentials; third, they are lineally related to neurons. Microglia are specialized macrophages that provide critical immune defense in the CNS, but they will not be discussed further in this thesis. In the following sections, all references to "glia", regardless of organismal origin, fulfill the above mentioned criteria.

Figure 1.1

A. Anatomical classification



B. Classification in this thesis

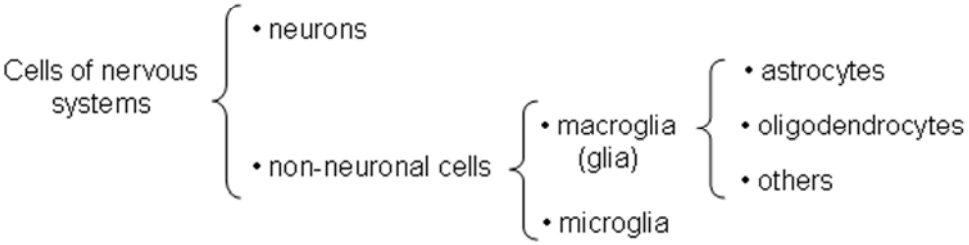


Figure 1.1. "Glia" Refers to Non-Neuronal Cells of the Nervous Systems

(**A**) The anatomical classification of cells of the nervous systems. The term "glia" refers to all non-neuronal cells.

(**B**) A slightly modified way to classify cells of the nervous systems. In this classification, "glia" refers to a part of non-neuronal cells that are lineally related to neurons. Microglia in (**A**), for example, are not lineally related to neurons.

- **Major Types of Glia in Mammals**

The principal glial subtypes in the mammalian CNS are oligodendrocytes and astrocytes. Although these cells come in diverse morphologies, it is possible to classify them according to their shapes and contacts of their processes. (Reichenbach, 1989). Oligodendrocytes produce the myelin sheaths that insulate the CNS axons. Astrocytes are characterized by endfeet that typically contact endothelial cells of blood vessels. In addition, astrocytes often possess processes to ensheath synapses and control the chemical composition of the perisynaptic space.

From the functional perspective, astrocytes are best known for their roles in providing structural support, regulating water balance and ion distribution, and maintaining the blood-brain barrier (Kettenmann and Ransom, 1995). Additionally, recent studies have shown that astrocytes also participate in synaptogenesis, neuronal metabolism, and modulation of synaptic activity (Bennett et al., 2003; Newman, 2003). Oligodendrocytes, on the other hand, engage in interactions with nerve cell bodies and axons, notably by forming myelin sheaths. Myelin is an electrically-insulating phospholipid layer which enhances conduction efficiency of neurons (Barres and Barde, 2000).

In the peripheral nervous system (PNS), the major type of glia is Schwann cells. They mainly provide myelin insulation to axons and thus are similar in function to oligodendrocytes, but they can also ensheath synapses between neurons (Jessen and Mirsky, 2005).

Some glial subtypes function primarily during development of CNS. One such example is radial glia which represent a unique class of cells that are present transiently in most brain regions during the periods when neurons are generated. Radial glia are typically located in the ventricular zone and remain mitotically active throughout neurogenesis (Misson et al., 1988). They often exhibit a bipolar morphology, extending radial processes that associate with both the ventricular surface and the pia, and these radial fibers guide migration of neurons (Noctor et al., 2001). In most regions of the mammalian brain, radial glia disappear or transform into astrocytes when neuronal generation and migration are complete (Mission et al., 1991; Schmechel and Rakic, 1979). There are exceptions, however, and some radial glia persist in the adult CNS. Those include Bergmann glia, found in the developing and adult cerebellum, Muller glia present in the retina, and radial glia that persist in the dentate gyrus of the adult hippocampus (Cameron et al., 1993; Eckenhoff and Rakic, 1984; Rickmann et al., 1987).

It is of note that glia can be categorized in a much finer manner. It is

becoming increasingly clear that the properties of astrocytes, for instance, can vary significantly depending on the brain regions. Astrocytes in different regions express different types of receptors and transporters (Matthias et al., 2003). More recently, three distinct subpopulations of astrocytes were identified in the ventral spinal cord (Hochstim et al., 2008). Thus although we refer to several types of cells with astroglial properties as “astrocytes”, the heterogeneity of these cells may have to be considered in some cases. However, we have only rudimentary understanding of these heterogeneous properties of glia and further study is necessary to unravel their biological meaning.

1.2 Functions of Glia

- **Roles of Glia in Mature Nervous Systems**

Recent studies have begun to reveal that glia are highly complex cells with a variety of intriguing aspects. Indeed, these cells seem to play active integral roles in nervous system development and possess important homeostatic and neuronal modulatory functions. Thus glia are probably not just the “glue” of the nervous system, rather they are more of a partner to neurons. Here I want to focus on four major functions of glia to illustrate their significance: a) secretion of neurotrophic

factors; b) maintenance of homeostasis; c) insulation of neurons; and d) modulation of signal transmission. Some, if not all, of these glial functions are probably essential throughout the development of nervous systems, not just in mature nervous systems as the subtitle of this section indicates. Roles of glia that are prominent during development of nervous systems will be discussed in the next section.

a) Secretion of Neurotrophic Factors

Neurons and glia control the survival of one another in order to determine the final composition of the nervous system. Indeed, neurons die when cultured separately from glia (Meyer-Franke et al., 1995). When glia are ablated in *Drosophila*, associated neurons degenerate (Booth et al., 2000) and the same is observed in *Drosophila* mutants that completely fail to specify glial fates (Hosoya et al., 1995; Jones et al., 1995).

This neurotrophic role of glia is achieved, at least in part, by producing neurotrophic factors (Althaus and Richter-Landsberg, 2000). Indeed, astrocytes can secrete, *in vivo* and *in vitro*, a number of trophic and regulatory substances, including nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (Condorelli et al., 1994; Friedman et al., 1998). It is also known that

astrocytes are equipped with a variety of receptors to modulate their production of neurotrophin synthesis and release. Among those are interleukin (IL) receptors. The secretion of NGF, for instance, is induced by activation of astrocyte IL-4 and IL-6 receptors (Brodie et al., 1998; Kossmann et al., 1996).

Oligodendrocytes are also the source for a number of neurotrophic factors. Northern blots and in situ hybridization combined with immunohistochemistry revealed that oligodendrocytes as well as oligodendrocyte precursors could produce NGF and brain-derived neurotrophic factor (BDNF) (Byravan et al., 1994). Schwann cells, the oligodendrocyte-equivalent cells in the PNS, are capable of producing growth factors as well. One of the factors they secrete is ciliary neurotrophic factor (CNTF), which functions as a trophic factor for motor neurons and other tissue cells (Friedman et al., 1992).

Conversely, glia depend on neurons for their survival. One of the well-characterized factors promoting glia survival *in vivo* is neuregulins (NRGs). The NRG family proteins contain an epidermal growth factor (EGF)-like motif that activates the membrane associated ErbB2, ErbB3, and ErbB4 receptor tyrosine kinases. In the PNS, Schwann cells express ErbB2/3 receptors, while axons of dorsal root ganglia (DRG) neurons express NRG1. In transgenic mice lacking either of the receptors, Schwann cells die (Riethmacher et al., 1997; Woldeyesus et al., 1999). Importantly, most PNS neurons also undergo apoptosis in these mice, illustrating the interdependence of glia and neuron survival. Similarly, the

Drosophila NRG1 homologue Vein promotes survival of longitudinal glia (Hidalgo et al., 2001). Interestingly, NRGs are also important for survival of oligodendrocytes in the CNS and myelination of axons (discussed below).

Survival of glia is also dependent on neurotrophins. Accordingly, astrocytes and oligodendrocytes express two classes of neurotrophin receptors, the p75 and Trk family of tyrosine kinase receptors, which may trigger apoptotic pathway in these cells (Althaus and Richter-Landsberg, 2000). Thus, glia are both targets and producers of neurotrophins.

b) Maintenance of Homeostasis

Another important role of glia is to regulate the internal environment of the brain, especially the fluid surrounding neurons and their synapses. Astrocytes, in particular, play a significant role in this process. They tightly control water and ionic homeostasis of perisynaptic space to accelerate smooth synaptic transmission.

The astroglial function in homeostasis is well represented in clearance of potassium ions (K^+) from synaptic cleft. Neuronal activity leads to transient increases in the extracellular K^+ concentration, which, if uncorrected, would be detrimental for neurons. Astrocytes are essential for the maintenance of proper

extracellular potassium level (Theis et al., 2005). They are connected by an extensive network of gap junctions (Cotrina et al., 1998) which are permeable to K^+, forming a syncytium for rapid redistribution of K^+ from areas with high neuronal activity. The permeability is mediated primarily by inwardly rectifying potassium channels such as Kir 4.1. A variety of other potassium channels expressed by astrocytes, including calcium-activated potassium channel *Rslo* K_{Ca}, and voltage-gated potassium channel Kv 1.5, are also involved in the spatial K^+ buffering (Higashi et al., 2001; Price et al., 2002; Roy et al., 1996). Other K^+ uptake mechanisms contribute to this process as well. For instance, high potassium concentration will stimulate Na^+, K^+-ATPase of astrocytes to actively remove K^+ from extracellular fluid (Hajek et al., 1996).

Depending on the extracellular ion concentrations and osmolarity, astrocytes can actively regulate not only potassium ions but also sodium, chloride, and hydrogen ions (H^+). Astroglial modulation of H^+ concentration, for example, results in change in the extracellular pH (Rose and Deitmer, 1994). This function is partially mediate by carbonic anhydrase in astrocytes, an enzyme that catalyzes the rapid conversion of carbon dioxide to bicarbonate and H^+ (Ghandour et al., 2000).

In addition, astrocytes are actively involved in the removal of neurotransmitters that are released by active neurons. Astroglial uptake of glutamate is well studied but this topic will be discussed below in a different section.

c) Insulation of Neurons

In vertebrates, axons are often wrapped by glial membrane extensions. A multilamellar glial ensheathment is called myelin, which is one of the most abundant membrane structures in vertebrate nervous systems. Myelin provides electrical insulations around the axons and maximizes their conduction velocity. It is produced by two types of glia, oligodendrocytes in the CNS and Schwann cells in the PNS. To form myelin, these cells first wrap their plasma membrane around axons. The spiral wrapping is followed by the extrusion of cytoplasm and the compaction of the stacked membrane bilayers. The ensheathment is punctuated by gaps, called the nodes of Ranvier, which are required for efficient and rapid propagation of action potentials.

The molecular composition of myelin is unique. In contrast to most plasma membranes, myelin is a lipid-rich membrane (lipid constitute 70% of dry myelin weight) that is highly enriched in glycosphingolipids and cholesterol. In addition, myelin contains a relatively simple array of proteins: the two major ones are myelin basic proteins (MBP) and the proteolipid proteins (PLP/DM20). Surprisingly, during the active phase of myelination, each oligodendrocyte is estimated to produce as much as 5-50x103 μm2 of myelin membrane surface area per day (Pfeiffer et al., 1993).

The physiology of myelin has provoked researchers to ask many intriguing questions. One of them is “How do organisms match the number of oligodendrocytes to the axonal surface requiring myelination?” This question brings us back to the discussion on NRGs in the previous section because they are involved in this process as well. NRGs activate ErbB on oligodendrocytes, which promote their terminal differentiation and ensheathment of axons (Park et al., 2001). Proper myelination by Schwann cells similarly requires NRGs to be expressed on the axonal surface (Taveggia et al., 2005). Interestingly, NRGs define not only whether or not an axon will be myelinated but also the thickness of the sheath. Transgenic mice with reduced NRG1 expression display hypomyelination, whereas overexpression of NRG1 induces increased myelin thickness (Michailov et al., 2004). Thus myelin forming glia apparently use NRG1 signals to know whether and to what extent axons require myelination.

Another question is related to the nodes of Ranvier and structures surrounding them (called paranodes and juxtaparanodes): what triggers the clustering of sodium channels at the nodes of Ranvier? One example of the mediators responsible for the clustering is Gliomedin, a novel glial ligand for the neuronal adhesion molecules neurofascin and NrCAM (Eshed et al., 2005). Gliomedin is particularly important in peripheral nerves, and it can induce

nodal-like clusters of sodium channels.

d) Modulation of Signal Transmission

Information processing in the nervous system is achieved primarily at chemical synapses between neurons. The modulation of synaptic activity and efficacy is critical for highly complex brain functions including learning and memory. Recent observations strongly suggest that glia may significantly affect signal transmission at synapses, and thus play an important role in regulating information processing in the nervous systems.

The Xenopus neuromuscular junction (NMJ) has been intensively studied to show glial role in the regulation of synaptic efficacy. At the NMJ, nerve terminals are ensheathed by perisynaptic Schwann cells. Robitaille and his colleague used an anatomically intact NMJ to examine whether Schwann cells modulate synaptic activity (Colomar and Robitaille, 2004; Robitaille, 1998). They have demonstrated that continued activation of G proteins in Schwann cells causes a reduction in neurotransmitter release, while blockade of G protein activation significantly increases synaptic activity. Their observations strongly suggest that glia modulate the activity of chemical synapses and regulate the information processing.

Although it is not entirely clear how glia modulate synaptic activity, it is

becoming evident that they themselves can uptake and even release neurotransmitters. Of those glial mechanisms to control the neurotransmitter concentration at the synaptic cleft, perhaps the most studied example is the clearance of glutamate.

Glutamate is the major excitatory amino acid in the brain. Glutamate uptake by astrocytes has been shown to be a crucial step to maintain a low concentration of extracellular glutamate and also to terminate its effects as a neurotransmitter (Sonnewald et al., 1997). Two key glial specific transporters involved in the uptake of glutamate are excitatory amino acid transporter 1 (EAAT1) and EAAT2 (which in rodents are known as glutamate-aspartate transporter (GLAST) and glutamate transporter 1 (GLT1), respectively). Oliet *et al.* showed that pharmacologically-induced reduction in glutamate clearance by inhibition of GLT1 decreases transmitter release through modulation of perisynaptic metabotropic glutamate receptors (Oliet et al., 2001). His study also demonstrated that when astrocytic processes are absent from the vicinity of synapses, inhibition of GLT1 does not alter neurotransmitter release, suggesting that glutamate transporters on astrocytes are responsible for the glutamate clearance.

Glia may participate in synaptic activity by secreting neurotransmitters into the synaptic cleft. There is now convincing evidence that astrocytes release glutamate as well as ATP and serine (Araque et al., 2001; Bezzi et al., 1998; Liu et al., 2004). The underlying mechanisms of this transmitter release are not clear,

but some evidence indicate that the process is dependent on an increase in Ca^{2+} level in astrocytes (Araque et al., 2001).

- **Developmental Functions of Glia**

Glia are important not only for regular functions of mature nervous systems, but also for establishing the complexity of nervous structures. The purpose of this section is to illustrate some of the glial functions in the regulation of neural development. I will highlight recent progress in understanding the roles of glia in neurogenesis, neuronal migration, process outgrowth, and synaptogenesis.

a) Neurogenesis

Glia and neurons are lineally related. A typical example is the generation of CNS neurons by radial glia. At the time of neurogenesis, radial glia divide asymmetrically, leading to both renewal of a precursor cell and the generation of a daughter neuron (Malatesta et al., 2000; Noctor et al., 2001). Interestingly, radial glia located in different brain regions differ in their gene expression pattern and the cell types they generate (Kriegstein and Gotz, 2003). This heterogeneity of

radial glia may be involved in regionalization of the developing nervous system.

It is also known that cells with glial characteristics are intimately involved in neurogenesis in the adult vertebrate brain (Doetsch, 2003). In mammals, adult neurogenesis is most extensively studied in the subventricular zone (SVZ), which is a paired brain structure that extends along the lateral walls of the lateral ventricles. Dividing astrocytes at SVZ generate neurons and act as neural stem cells (Doetsch et al., 1999). Similarly, astrocytes are the *in vivo* primary precursors of hippocampal neurons (Seri et al., 2001). These adult-formed neurons may contribute to higher-order brain functions including learning and memory formation.

Schwann cell precursors (SCPs; see below) can generate, *in vitro*, non-glial lineages, including neurons (Hagedorn et al., 1999; Morrison et al., 1999). The significance of this observation *in vivo* is not yet clear but it at least accords with a study showing that SCPs may be the source of the relatively small population of fibroblasts that is found in peripheral nerves (Joseph et al., 2004). The generation of neurons from SCPs, *in vivo*, if verified, would further demonstrate the kinship between glia and neurons.

b) Regulation of Neuronal Migration

Neurons need to be appropriately directed to their targets to establish neural networks. The first evidence indicating involvement of glia in the process of neuronal migration was provided over 30 years ago by Rakic (Rakic, 1971). Based on static observations, he proposed a hypothesis that migration of granule neurons in the developing cerebellum was guided by glia. Later in the 80s, Edmondson and Hatten directly demonstrated neuronal movement along glial fibers using purified murine cerebellar glia and granule neurons (Edmondson and Hatten, 1987). Recent study further suggests the idea of glia-guided migration. Using morphological, immunohistochemical, and electrophysiological techniques as well as time laps imaging, researchers confirmed that, in the developing cortex, neurons migrate along clonally related radial glia (Noctor et al., 2001). Glia-guided migration is not the only mechanism of neuronal migration, but it is an important aspect of nervous system development.

Similarly, glial migration is guided by neuronal processes. A good example comes from zebrafish, in which the lateral line glia follow the path of axons. In mutants where axons are misdirected around somites, glia follow the roundabout route of axons. Furthermore, laser ablation of the extending lateral line axons hampers glia migration (Gilmour et al., 2002).

c) Roles in Process Outgrowth

Another function of glia during nervous system development is to regulate axon guidance. A variety of attractive and repulsive guidance cues are necessary to achieve proper axon guidance. Five conserved families of guidance cues with prominent developmental effects have been identified: the netrins, Slits, semaphorins, ephrins, and Wnts (Dickson, 2002; Salinas and Zou, 2008; Yu and Bargmann, 2001). Although these are not the only guidance factors, glia often use these factors to guide axons.

In *Drosophila*, midline glia express Slit, a large extracellular matrix protein that act primarily as a repellent (Rothberg et al., 1988; Rothberg et al., 1990). Slit binds to a repulsive guidance receptor Roundabout (Robo) expressed in axonal growth cones and this interaction prevents neuronal processes to recross the midline (Kidd et al., 1999). In the vertebrate spinal cord, Glia-like cells of the floor plate express Netrin-1. During development, spinal commissural axons project toward floor plate cells and *netrin-1* deficient mice exhibit defects in the spinal commissural axon projections, suggesting that Netrin-1 attract these axons (Serafini et al., 1996). The floor plate cells are also the source of repellent cues, Slit and semaphorin, which prevent commissural axons from recrossing the nervous system midline (Zou et al., 2000). Some glia are known to express ephrins. Radial glia at the optic chiasm midline express ephrin-B2, and this

expression is important for guiding axons of retinal ganglion cells that express EphB1, the receptor for ephrin-B2 (Williams et al., 2003).

Recently, there has been an intensive focus on semaphorin expression in oligodendrocytes as a hurdle for successful axon regeneration. Mounting evidence indicate that axon regeneration after injury is inhibited by the glial environment of the adult CNS. The inhibitory influences are partially mediated by semaphorins 4D and 5A (Yiu and He, 2006), which are selectively expressed in oligodendrocytes and their precursors (Goldberg et al., 2004; Moreau-Fauvarque et al., 2003).

d) Roles in Synaptogenesis

In addition to neuronal migration and axon guidance, neurons need to get connected with defined strengths to establish neural circuitry. This is achieved by formation of synapses. Co-culture experiments using highly purified retinal ganglion cells (RGCs) strongly indicate that glia promote synaptogenesis. Addition of astrocytes or astrocyte conditioned medium to a culture of RGCs leads to an increase in number of synapses (Pfrieger and Barres, 1997; Ullian et al., 2001). Molecules at least partly responsible for this phenomenon are thrombospondins, extracellular matrix proteins secreted by glia. Activity of these proteins is sufficient to induce the formation of anatomically normal but silent

synapses (Christopherson et al., 2005).

For the maturation of neural circuits, it is also important to selectively eliminate inappropriate synaptic connections. Recent study suggested involvement of astrocytes and the classical complement cascade in mediating CNS synapse elimination (Stevens et al., 2007). These observations together strongly support dynamic roles of astrocytes in synapse development.

1.3 Development of Glia: Where Do They Come From?

A fundamental question in developmental neurobiology is how a relatively simple and undifferentiated neuroepithelium in the embryo can give rise to the remarkable cellular diversity of the mature nervous systems. Extrinsic signaling molecules as well as cell-intrinsic factors orchestrate to instruct multipotent progenitor cells to generate specialized neurons and glia.

Recent efforts have shed light on the complex interplays of molecular regulators that underlie the differentiation and maturation of glia. Particularly, identification of lineage-specific regulatory factors has facilitated studies on the development of oligodendrocytes. Astroglial development, on the contrary, is less understood partially due to the lack of markers that can be used to definitively identify their progenitors *in vivo*, although several interesting signaling pathways

have been shown to be involved in their development.

Reflecting the fact that development of *C. elegans* glia shares similarities with that of oligodendrocytes (shown in this study), this section first focuses on the oligodendrocyte specification. Glial development in *Drosophila* will be discussed briefly as an example of development of glia in invertebrates.

- **Glial Development in Vertebrates**

a) Production of Oligodendrocyte Precursors in the Spinal Cord

The development of oligodendrocytes is most extensively studied in the spinal cord. Pulse-chase labeling studies, coupled with differentiation markers and morphological analysis, suggested that development of neurons precedes that of glial subtypes *in vivo*, and that oligodendrocytes in particular are the last population to develop, largely at postnatal stages (Altman and Bayer, 1997). However, it is now clear that the initial specification of spinal cord oligodendrocytes takes place in the embryo and requires precise interplay of a variety of transcriptions factors.

During development, the neural plate composed of newly formed neural cells folds to form the spinal cord. The specification of neuronal subtypes in the

spinal cord is achieved by a “gradient” of signaling activity that confers positional identity along the dorsoventral axis of the neural tube (Jessell, 2000). Three cells that play critical roles in this process are cells of floor plate, notochord, and roof plate. The main signaling activities of the notochord and floor plate are mediated by a secreted protein Sonic hedgehog (Shh), whereas the roof plate cells secrete bone morphogenetic proteins (BMP). Together, these cells establish a dorsoventral gradient of Shh-BMP activity. The Shh signaling is responsible for regulating the expression of homeodomain transcription factors that define unique progenitor domains in the ventral neural tube (Briscoe et al., 2000; Jessell, 2000). Combinatorial interactions of these transcription factors within a given domain regulate neuronal progenitor identity and consequently, neuronal subtype specification.

One such domain defined by the Shh pathway is called “progenitor of motor neurons”, or pMN. As its name indicates, pMN gives rise to somatic and visceral motor neurons of the spinal cord. Interestingly, expression analysis *in situ*, using markers of oligodendrocyte precursor cells (OLPs), indicated that OLP development is initiated in the same pMN region that gives rise to motor neurons (Ono et al., 1995; Pringle and Richardson, 1993; Timsit et al., 1995). In mouse, pMN precursors give rise first to motor neuron progeny (from about embryonic day (E) 9-10.5), and then to OLPs (E12.5-) (Miller, 2002). Subsequently, OLPs migrate from the ventricular zone to lateral and dorsal regions throughout the

spinal cord and eventually, at postnatal stage, mature into myelinating oligodendrocytes.

Key factors that unify the development of motor neurons and oligodendrocytes downstream of Shh signaling are basic helix-loop-helix (bHLH) proteins Olig1 and Olig2 (Lu et al., 2000; Zhou et al., 2000). *Olig* genes can be detected in the pMN domain from very early on (E9-), and genetic studies indicate that they function downstream of Shh signaling and of the Shh-regulated homeodomain proteins Nkx6.1, Nkx6.2, and Pax6 (Liu et al., 2003; Novitch et al., 2001). Functional analysis in mice has shown that Olig1 and Olig2 are required for the establishment of the pMN domain (Zhou and Anderson, 2002), and that a null mutation of Olig2 alone results in failure of development of pMN progeny (Lu et al., 2002). These observations together suggest that Olig1 and Olig2 are essential for the specification of both neuronal and glial lineages.

Although studies of Shh-inducible factors, including Olig proteins, have provided many insights into mechanisms of cell specification in the pMN domain, it is evident that additional signaling factors need to be invoked to fully explain the glial differentiation. Especially, it is important to elucidate the machinery underlying time-dependent transition from motor neuron to OLP production. Several molecules provide further insights into the regulation of this transition mechanism. For example, proneural bHLH transcription factors Ngn1/2 are expressed in a subset of Olig2-expressing cell in the pMN at the time of motor

neuron production and are known to be downregulated as OLPs are being produced (Zhou et al., 2001). This observation led to the proposal that Ngn1/2 downregulation is a component of the neuron-glia transition. However, Ngn1/2 null mice do not accelerate the production of OLPs, indicating that there is additional complexity in the regulation (Kele et al., 2006; Rowitch, 2004). Delta-Notch signaling and Sox9 are also thought to be critical for oligodendrocyte specification. Mutations in genes that encode Delta ligands or Sox9 result in defects in the specification of oligodendrocytes (Park and Appel, 2003; Stolt et al., 2003). More recently, Deneen *et al.* demonstrated that nuclear factor I-A (NFIA), a member of a family of CCAAT box element-binding transcription factors, functions as a “pro-glial” gene to regulate the expression of Olig2 (Deneen et al., 2006). Thus NFIA and its related genes may play a key role in the oligodendrocyte specification. Above mentioned observations together suggest that the onset of OLP production in the pMN may require integration of several distinct signaling pathways.

Recent examination of *Nkx6*-/- mutant mice has provided evidence for oligodendrogenesis in dorsal regions of the spinal cord as well. Several researchers explored the positional specification of oligodendrocytes in the spinal cord and provided evidence that *Olig2* expression in cells derived from the dorsal spinal cord is regulated by an *Nkx6*-independent mechanism (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). Thus distinct genetic program seems

to regulate Olig2 activation at different positions in the spinal cord. The transcriptional code governing this dorsal expression of *Olig2* remains unknown, but may include Pax7, because Olig2 positive cells from the region are also Pax7 positive.

It is worthy of note that the Shh-Nkx/Pax signaling pathway has a role outside of the nervous system. For instance, a number of studies demonstrate that it is required for pancreatic development. The adult mammalian pancreas is a heterogeneous organ composed of three major cell types: exocrine cells, duct cells, and endocrine cells. Endocrine cells are organized into islets of Langerhans, which disperse throughout the exocrine tissue. Shh as well as Nkx6.1 and Pax6 are necessary for pancreatic islet development (diIorio et al., 2002; Sander et al., 2000; St-Onge et al., 1997). The resemblance between islet cells and neural cells of pMN domain is further highlighted by the fact that islet cell development requires transcription factors Hb9 and Isl1 (Ahlgren et al., 1997; Harrison et al., 1999), both of which are involved in motor neuron specification. The expression of these factors within endodermal endocrine cells may point to a common mechanism shared between islet cells and neurons. Although Olig1/2 are unlikely to be expressed in peripheral tissues (Lu et al., 2000), it is possible that studies on pancreatic development provide additional insights into cell specification in the pMN domain.

b) Specification of Astrocytes

During development, there are at least two distinct phases in which astrocytes are generated. Initially, astrocytes derive from the neuroepithelium through radial glia, and at later stages, their progenitors emerge from the subventricular zone (Levison and Goldman, 1993). Although genetic regulatory factors that are specific for astrocyte lineage are not yet identified, several bHLH transcription factors are known to be important for the specification of astrocytes. Ngn2, for example, promotes neurogenesis and inhibits astrocyte generation in the dorsal telencephalon (Nieto et al., 2001). Moreover, Olig2 are thought to antagonize astrocyte differentiation (Fukuda et al., 2004), and the negative HLH protein Hes1 promotes the formation of astrocytes *in vitro* presumably by down-regulating *Olig* genes (Wu et al., 2003). Olig2 may be more actively involved in astrocyte development because temporally controlled *Olig2* deletion resulted in a severe deficit in the formation of cortical astrocytes (Cai et al., 2007).

In contrast to the oligodendrocytes, majority of which derive from a specific domain of the ventral neural tube, astrocyte development in the spinal cord takes place in a broader region of the ventricular zone (VZ). Interestingly, however, different regions of the VZ produce astrocytes with different phenotypes. This positional heterogeneity among astrocyte precursors was initially hinted by a work on a bHLH transcription factor Stem Cell Leukaemia (SCL). SCL is specifically

expressed in the p2 progenitor domain, one of the domains specified by the Shh-regulated homeodomain transcription factors, and is required for the regionally restricted differentiation of astrocytes (Muroyama et al., 2005). Further evidence suggested that combinatorial activities of Pax6 and Nkx6.1 generate at least three molecularly distinct astroglial subtypes in the spinal cord (Hochstim et al., 2008). These observations indicate that Shh-Nkx/Pax signaling pathway is important not only for oligodendrocyte specification but also for differentiation of astrocytes.

c) Production of Schwann Cells in the PNS

Schwann cells in the PNS originate from the neural crest. As the neural plate folds and fuses to form the neural tube in the process of neurulation, neural crest cells segregate from the tips of the folds and start migration. Neural crest cells need to make two transitions before they become mature Schwann cells: they first generate Schwann cell precursors (SCPs), which then generate immature Schwann cells. Immature Schwann cells eventually become mature myelinating and non-myelination Schwann cells. Interestingly, this transition is partially reversible. Mature Schwann cells, for instance, can respond to nerve injury by reverting to a phenotype similar to that of immature Schwann cells

(Dupin et al., 2003; Morrison et al., 1999). This plasticity is an important feature of the Schwann cell lineage.

Each transition of neural crest cells requires different mechanism. Signaling pathways that control the transition from migrating neural crest cells to SCPs involve neureglin1 (NRG1), SRY (sex determining region Y) box 10 (Sox10), bone morphogenetic protein 2 (BMP2), and Notch, all of which function in the neural crest cells to suppress the transition, or to suppress or activate neuronal development (Morrison et al., 2000; Shah et al., 1996; Shah et al., 1994). Little is know about transcription factors that control the transition from SCPs to immature Schwann cells. However, it is known that activator protein 2 alpha (AP2 α) is strongly downregulated as SCPs become immature Schwann cells *in vitro* (Stewart et al., 2001), indicating that AP2 α negatively regulate the transition process. The final transition from immature Schwann cells to mature Schwann cells is not well characterized either. Interestingly, however, depletion of all laminin isoforms in Schwann cells results in arrest at premyelinating stage, suggesting that laminin is required for the axon-Schwann cell interactions and mediates Schwann cell differentiation (Yu et al., 2005).

- **Glial Development in Invertebrates**

In the *Drosophila* CNS, both neurons and glia are derived from neuroblasts. The choice between glial and neuronal fates is determined primarily by a Zn-finger transcription factor encoded by the *glial cells missing* (*gcm*) gene. In *gcm* loss-of-function mutant, nearly all glia differentiate into neurons, whereas *gcm* ectopic expression transformed virtually all CNS cells into glial cells (Hosoya et al., 1995; Jones et al., 1995). Thus *gcm* is considered to function as a binary switch that promotes glial fate while simultaneously inhibiting the neuronal fate. However, mammalian *gcm* homologs are mainly expressed in non-neuronal cells and appear to have no *in vivo* role in glial specification (Kim et al., 1998).

These observations support the view that invertebrate glia are developmentally distinct from their vertebrate counterparts. However, some weak similarities in the glial developmental pathway might exist. *Drosophila* possesses an *Olig*-related gene, *Oli*, that is expressed extensively in the animal's brain (Brody et al., 2002). Precise expression pattern and function of *Oli* is not yet available, but it is possible that *Oli* is expressed in glia. Furthermore, Zn-finger transcription factors may play important roles in vertebrate glial development. The vertebrate Zn-finger transcription factor *Zfp488* can cooperate with *Olig2* to control oligodendrocyte differentiation (Wang et al., 2006). Interestingly, *C. elegans* glia also express the Zn-finger transcription factor LIN-26, which is

required for terminal differentiation of these cells (Labouesse et al., 1996). Thus it may be too early to conclude that mechanisms regulating glial differentiation are not conserved among multicellular organisms.

1.4 Glia in *C. elegans*

- **Sheath and Socket Glia**

The model organism used in this study is the nematode *C. elegans*. A *C. elegans* adult hermaphrodite has fifty glia-like cells (often simply referred to as glia), all of which are part of sensory organs in the animal (Shaham, 2005). These *C. elegans* cells meet the criteria for microglia/glia described previously in section 1.1. First, they are physically associated with neurons as assessed by electron microscopy studies (Ward et al., 1975). Second, they are not neurons themselves, based on their morphological characteristics (Ward et al., 1975; White et al., 1986). Third, they are lineally related to neurons. Lineage analysis demonstrate that the sister cells of sheath and socket cells are neurons, other glia cells, or epithelial cells (Sulston et al., 1983), all of which are of ectodermal origin in vertebrates.

Of the fifty glial cells, twenty-four are sheath glia. These cells extend

processes that associate with dendrites of sensory neurons and ensheath sensory dendrites at the dendritic tip. The rest of twenty-six cells, termed socket glia, run along the terminal portion of sheath glia processes and surround the dendritic tips of a subset of sensory neurons, anterior to the sheath glia.

Although sheath and socket glia are considered *C. elegans* glia, convincing molecular evidence is lacking to prove that they are comparable to glia in other organisms.

- **Cephalic Sheath Glia: The Main Subject of This Study**

Of the twenty-four sheath glia, four cells, termed cephalic sheath (CEPsh) glia, are associated with the four cephalic (CEP) sensilla, which are symmetrically arranged (dorsal left/right, ventral left/right) in the head. These cells are the subject of this study and my research entirely focuses on the development and functions of these cells.

In hermaphrodites, each of the CEP sensillum contains one dopaminergic neuron (CEP neuron) as well as one sheath and one socket glia (Figure 1.2A). In males, one extra neuron termed CEM can be found in each sensillum. CEP neurons do not penetrate the cuticle and thus are suggested to be mechanosensory, whereas CEM neurons are exposed to the external

environment and may function as chemosensory neurons (Perkins et al., 1986). Although roles of these sensilla are not entirely clear, some experiments suggest that CEP neurons, together with other dopaminergic neurons, are involved in modulation of locomotory rate and habituation to tap stimulation (Kindt et al., 2007; Sanyal et al., 2004; Sawin et al., 2000).

CEPsh glia are unique in morphology since they are bipolar: they extend both dendrite-associated processes and sheet-like processes that ensheath the nerve ring, the largest neuropil in *C. elegans*. The nervering is composed of many neuronal processes and is generally considered as the animal's brain (Figure 1.2). Although very little is known about function of CEPsh glia, there are two interesting observations. First, CEPsh ventral left and right glia express *C. elegans* netrin *unc-6*, suggesting that they may have roles in axon guidance or cell migration (Wadsworth et al., 1996). Secondly, CEPsh glia send fine processes into the nerve ring, where they can be found closely associated with some synapses, indicating that they may have roles in the modulation of synaptic activity (White et al., 1986). A role for CEPsh glia in synaptogenesis has recently been suggested (Colon-Ramos et al., 2007). These predicted functions have not been rigorously verified, making these cells attractive targets to work on.

Figure 1.2

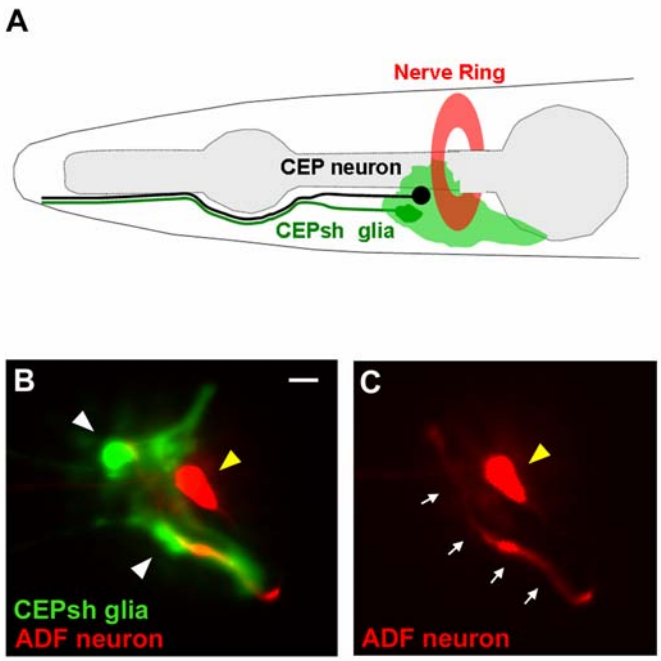


Figure 1.2. CEP Sheath Cells are Associated with the Nerve Ring

(**A**) A schematic diagram of the head region of *C. elegans* depicting a CEPsh glial cell (green), an associated CEP neuron (black), and the nerve ring (pink). The axon of CEP neuron is not depicted in order to simplify the diagram. In all larval and adult schematics and images in this document dorsal is up and anterior is to the left. The posterior region of the ventral CEPsh glial cells (green), ensheaths the ventral ganglion leading in to the nerve ring.

(**B**, **C**) Fluorescence images of a wild-type adult expressing the *hlh-17*::GFP reporter transgene in CEPsh glia (white arrowheads) and the T08G3.3::RFP reporter transgene in ADF neurons (yellow arrowheads). (**B**) Overlay. (**C**) The ADF neuron alone. The ADF axon (white arrows) is a component of the nerve ring. Scale bar, 5um.

- **Advantages of Working with *C. elegans* Glia**

Despite the growing realization of the importance of glia in nervous systems, we are still in the initial stage of understanding the physiology of glia. This is partially due to the fact that neurons and glia control the survival of one another. The reciprocal control of survival significantly hampers study of glia because when neurons die in the absence of glia, it becomes difficult to determine whether phenotypes you observe are caused by the glial loss or merely represent a secondary effect of neuronal death. Researchers have overcome this problem in a number of ways. For example, a method was introduced to culture neurons without the physical presence of glia (Meyer-Franke et al., 1995; Ullian et al., 2001). Approaches to perturb glia in a more subtle way have also been informative (Robitaille, 1998).

Another solution to circumvent the hurdle is to use a natural setting in which glia are not required for neuronal survival. Such glia may be significantly different from vertebrate glia in terms of neurotrophic roles, but can be very useful to study other basic physiology of glia. Previous studies in our lab suggested that *C. elegans* provide such a setting (Perens and Shaham, 2005) and this study also confirmed that ablations of *C. elegans* glia are not accompanied with neuronal death. These observations, combined with the facility of genetic studies in *C. elegans*, suggest that this organism may provide a unique setting in which to

explore basic *in vivo* aspects of metazoan glial cell development and function, as well as glia-neuron interactions.

1.5 Aims of This Study

I have two aims for this research. The first is to define molecular similarities between *C. elegans* glia and metazoan glia. This is especially important because the similarities could indicate usefulness of the model organism for understanding general physiology of glia. To achieve this, mutagenesis screens have been performed to isolate new mutants affecting development of CEPsh glia. The second is to show that the absence of CEPsh glia results in developmental or behavioral defects. It was critical to prove that these cells have functions *in vivo* in order to motivate further work on these cells. The second aim is achieved primarily through cell ablation technique.

The results of the mutagenesis screens and basic phenotypes of the CEPsh mutants are discussed in Chapter 2. Chapter 3 describes the axonal defects observed in CEPsh ablated animals and these observations are further supported by defects identified in CEPsh mutants. I was constantly supported by Shai with a myriad of helpful suggestions to carry out these studies, and thus the word “we” used in the following chapters refers to Shai and myself.

Chapter 2

Developmental Analysis of CEPsh Glia

Isolation and Basic Characterization of *mls-2*, *vab-3*, and *hlh-17* Mutants

Summary

When we started working on cephalic sheath (CEPsh) glia in September of 2004, there were no markers available for these cells. In this chapter, we will first describe how we identified two genes, *hlh-17*/*Olig*-related and *ptr-10*/*Patched*-related, that are expressed in CEPsh glia. Subsequently, the process of isolation and characterization of mutants of *Nkx*/*Hmx*-related gene *mls-2* and *Pax6*/*7*-related gene *vab-3* will be discussed. We have also isolated *hlh-17* deletion mutants and observations in these mutants will be presented as well. These results together demonstrate that the ventral and dorsal CEPsh glia develop through molecularly distinguishable pathways regulated by *mls-2* and *vab-3*, respectively. The molecular similarities between the development of *C. elegans* CEPsh glia and vertebrate oligodendrocytes suggest that *C. elegans* glia may provide a unique setting to explore basic *in vivo* aspects of metazoan glia development and function.

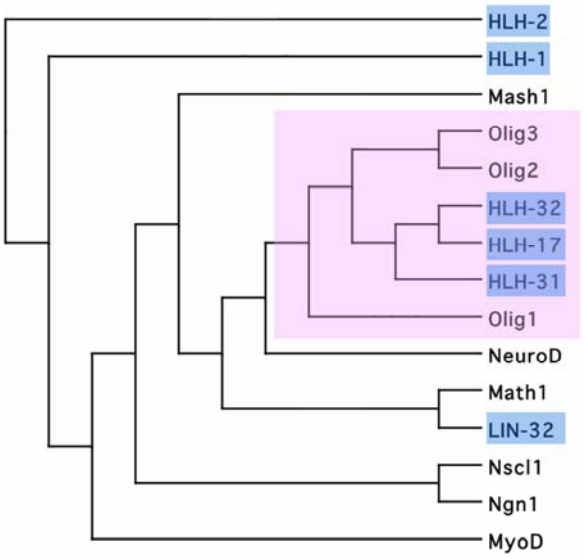
2.1 Visualization of CEPsh Glia

- ***hlh-17*/*Olig*-Related and *ptr-10*/*Patched*-Related are Expressed in CEPsh Glia and Other *C. elegans* Glia**

To begin to investigate the roles of CEPsh glia in the development of the *C. elegans* nervous system, reporter transgenes were identified in order to visualize these cells. We used two approaches. First, we explored the possibility that *C. elegans* genes similar in sequence to known vertebrate regulators of glial cell fate might be expressed in *C. elegans* glia. In vertebrates, the *Olig2* gene encodes a bHLH transcription factor involved in the development of both oligodendrocytes and motor neurons (Lu et al., 2002; Lu et al., 2000; Takebayashi et al., 2002; Zhou and Anderson, 2002; Zhou et al., 2000). Comparison of the *Olig2* protein sequence to the predicted *C. elegans* proteome using the BLAST program (Altschul et al., 1990) revealed three genes, *hlh-17*, *hlh-31*, and *hlh-32*, situated within a 140 kb region of chromosome IV, and encoding proteins with extensive similarity to the bHLH domain of *Olig2* (71%, 59%, 71% identity, respectively; see also Figure 2.1). To determine whether any of these genes is expressed in glia, we generated animals carrying reporter transgenes in which putative promoter regions of each gene were placed directly upstream of either the green fluorescent protein (GFP) or DsRed reporter genes. Neither the *hlh-31*

Figure 2.1

A



B

```
human Olig2 : RLKINSRERKRMHDLNIAMDGLREVMPYA
mouse Olig2 : RLKINSRERKRMHDLNIAMDGLREVMPYA
    HLH-17 : RLSINLRERCRMHDLNEALDDLRAVIPYA

human Olig2 : HGPSVRKLSKIATLLLARNYILMLTNSLE
mouse Olig2 : HGPSVRKLSKIATLLLARNYILMLTNSLE
    HLH-17 : HGGSVRKLSKIATLLLAKNHIIMQAKAIE
```

C

```
HLH-17 : RLSINLRERCRMHDLNEALDDLRAVIPYAHGGSVRKLSKIATLLLAKNHIIMQAKAIE
HLH-32 : RLSINLRERCRMHDLNEALDDLRAVIPYAHGGSVRKLSKIATLLLAKNHIIMQAKAIE
HLH-31 : MKSSKLLKRCRMHDLNEALDDLRAVIPYAHGGSVRKLSKIATLLLAKNHIIMKAKAIE
```

Figure 2.1. Three *C. elegans* Genes are Homologous to Vertebrate *Olig2*

(**A**) Dendogram depicting similarity of HLH-17 to Olig and other human and *C. elegans* (blue) bHLH protein subfamilies.

(**B**) Alignment of the bHLH domains of human Olig2, mouse Olig2, and *C. elegans* HLH-17 proteins. Pink, identical residues in all three species. Blue, identical residues in two species.

(**C**) Alignment of the bHLH domains of the HLH-17, HLH-32, and HLH-31 proteins. Pink indicates identity among all three proteins. Blue indicates identity in two of three proteins.

nor the *hlh-32* reporter transgenes we generated were expressed in glia. The *hlh-32* transgenes were expressed in a pair of unidentified neurons in the head of the animal, whereas no expression was detected for the *hlh-31* reporters (data not shown). However, it is possible that we did not include sufficient regulatory sequences to obtain the full expression pattern of these genes.

A transgene containing a 2.7 kb region, including 1.9 kb of sequences directly upstream of the *hlh-17* translation start site (see below) and 0.8 kb downstream of the start site fused to GFP, was expressed strongly in CEPsh glia at all developmental stages. Reporter expression was first detected in several anteriorly situated cells in embryos at around 400 minutes post-fertilization. In first-stage (L1) larvae, expression was evident in glial cells of inner and outer labial sensilla, as well as in CEPsh glia, and in several unidentified neurons in the head and tail (Figure 2.2A-D). In L2 and L3 larvae, expression in CEPsh glia persisted, but expression in other glia and in head neurons weakened. Furthermore, in animals of these stages, some motor neurons of the ventral nerve cord also expressed the transgene (Figure 2.2E and 2.2F). Finally, in adults, our *hlh-17* reporter transgene was expressed exclusively in the four CEPsh glia, consistent with results of another study (McMiller and Johnson, 2005; Figures 2.3B and 2.3D).

The expression pattern we observed for *hlh-17* is reminiscent of that of *Olig2* in vertebrates, which is expressed in oligodendrocyte and motor neuron

Figure 2.2

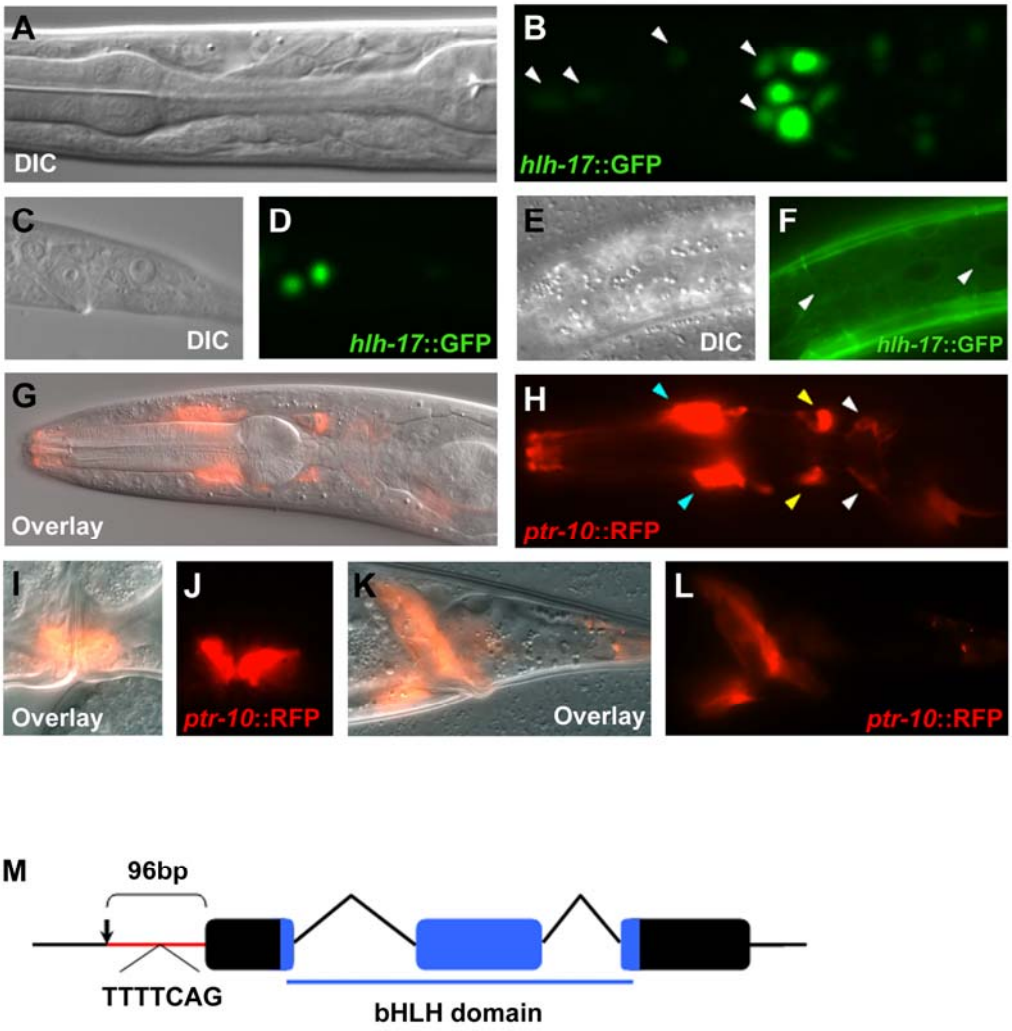

A
DIC
B
hlh-17::GFP
C
DIC
D
hlh-17::GFP
E
DIC
F
hlh-17::GFP
G
Overlay
H
ptr-10::RFP
I
Overlay
J
ptr-10::RFP
K
Overlay
L
ptr-10::RFP
M
96bp
TTTTCAG
bHLH domain

Figure 2.2. Expression Patterns of *hlh-17*::GFP and *ptr-10*::myrRFP Reporter Transgenes

(**A**, **C**) DIC images and (**B**, **D**) fluorescence images of an L1-stage animal expressing *hlh-17*::GFP. Expression in the head (**A**, **B**) and tail (**C**, **D**) is shown. Arrowheads in (**B**) indicate expression of GFP in some IL, OL, and OLQ sheath and/or socket glia. (**E**) DIC image and (**F**) fluorescence image of an L3-stage animal expressing *hlh-17*::myrGFP. Arrowheads indicate the expression of myrGFP in motor neuron commissures.

(**G**, **I**, **K**) Merged DIC and fluorescence images and (**H**, **J**, **L**) fluorescence images of an adult animal expressing *ptr-10*::myrRFP. Expression in the head (**G**, **H**), vulva (**I**, **J**), and rectum (**K**, **L**) is shown. Arrowheads in (**H**) indicate the expression in sheath and socket glia of IL, OL (blue), and OLQ (yellow) sensilla, as well as in CEPsh glia (white).

(**M**) *hlh-17* gene structure. Boxes, exons; thick Λ-shaped lines, introns. The arrow indicates the position of a previously predicted translation start site differing from the one described here (see Experimental Procedures). TTTCAG indicates the position where trans-splicing to the SL1 spliced-leader was found. The 96 bp segment (red) was not present in the reporter transgene previously described (McMiller and Johnson, 2005). Blue regions encode the bHLH domain.

Figure 2.3

A

Allele	Phenotypes
ns147, *ns148*, *ns149*	misexpress *hlh-17*::GFP in the body
ns150, *ns151*, *ns153*	L1 lethal, CEPsh glia are anteriorly displaced
ns152	L1 lethal, fails to express *hlh-17*::GFP in all four CEPsh glia
ns156, *ns158*, *ns159*	fails to express *hlh-17*::GFP in the CEPshVL/R glia
ns157	fails to express *hlh-17*::GFP in the CEPshDL/R glia
ns160, *ns161*	fails to express *hlh-17*::GFP in all four CEPsh glia

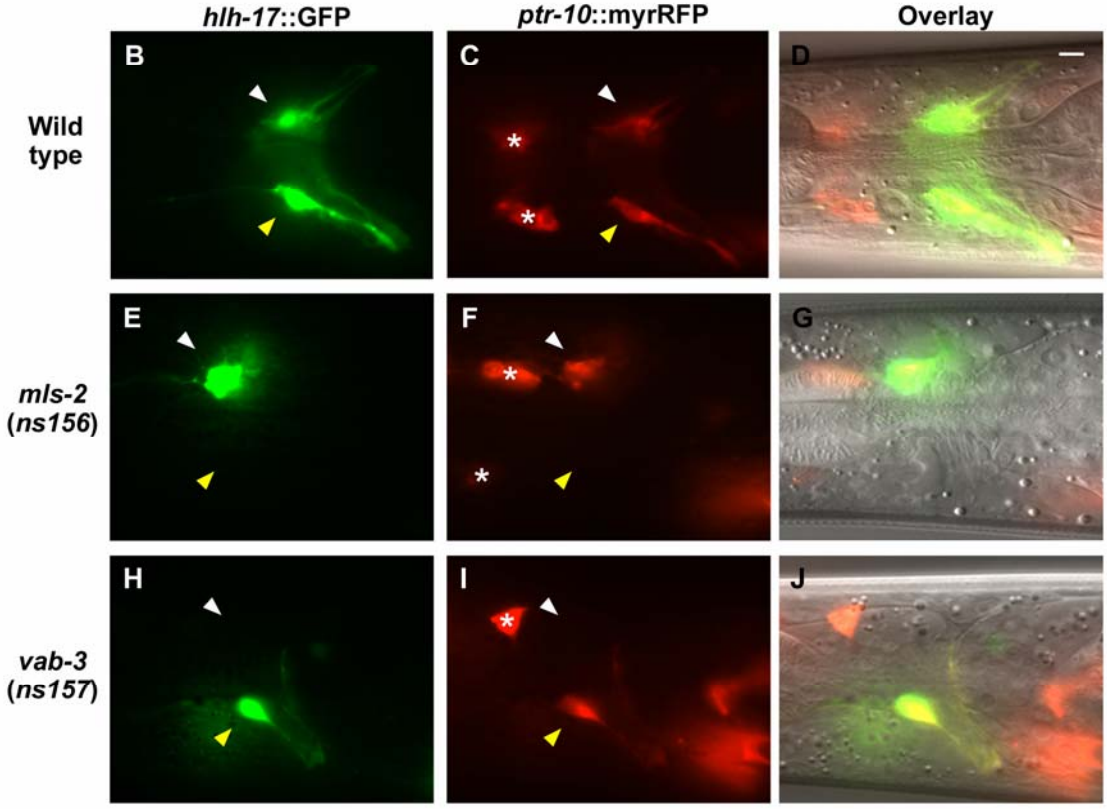


Figure 2.3. *mls-2* and *vab-3* Preferentially Regulate the Development of Ventral and Dorsal CEPsh Glia, Respectively

(**A**) Table describing the phenotypes of the thirteen alleles isolated in the screen.

(**B-D**) Fluorescence images (**B**, **C**) and merged DIC and fluorescence image (**D**) of a wild-type adult expressing *hlh-17*::GFP and *ptr-10*::myrRFP reporter transgenes. The expression in dorsal and ventral CEPsh glia is indicated by white and yellow arrowheads, respectively. Asterisks in (**C**) indicate expression in non-CEPsh glial cells. Scale bar, 5um.

(**E-G**) Same as (**B-D**), respectively, except that an *mls-2*(*ns156*) adult is shown. Note lack of reporter expression in ventral CEPsh glia.

(**H-J**) Same as (**B-D**), respectively, except that a *vab-3*(*ns157*) adult is shown. Note that reporter expression is missing in dorsal CEPsh glia.

progenitor cells (Lu et al., 2000; Zhou et al., 2000), and is consistent with the notion that *hlh-17* may be the *C. elegans* ortholog of *Olig2*.

It is of note that a previous study failed to reveal expression of an *hlh-17* reporter transgene in cells other than CEPsh cells (McMiller and Johnson, 2005). To define an appropriate promoter element for *hlh-17*, we sequenced cDNAs for the gene and identified some that contained the SL1 trans-spliced leader sequence (Krause and Hirsh, 1987) at their 5' ends. The 1.9 kb promoter fragment we used to generate our *hlh-17* reporter transgene included a 96 bp segment immediately upstream of the first in-frame ATG following the SL1 leader. This segment was not present in the reporter transgene previously described (McMiller and Johnson, 2005), presumably accounting for the differences in expression we observed (Figure 2.2M).

As a second approach to identify reporter transgenes for the CEPsh glia, we turned to previous studies from our laboratory, demonstrating that the *daf-6* gene, encoding a protein similar to the Hedgehog receptor Patched, is expressed in sheath and socket glia of the amphid and phasmid sensory organs (Perens and Shaham, 2005). *C. elegans* possesses 24 *Patched*-related genes (Kuwabara and Labouesse, 2002; Kuwabara et al., 2000), and we surmised that, by analogy to *daf-6*, some of these might be expressed in CEPsh glia. Indeed, we showed that the *ptr-10* gene was expressed in these cells. Specifically, a *ptr-10* promoter::myrRFP reporter transgene, expressing myristoylated RFP under the

control of a 300 bp *ptr-10* promoter fragment, was expressed in all four CEPsh glia (Figures 2.3C and 2.3D), as well as in sheath and socket glia of inner and outer labial sensilla, in the anterior deirid sheath glia, and in cells of the excretory system, vulva, and rectum (Figure 2.2G-L).

2.2 Identification of CEPsh-Defective Mutants

- **Genetic Screen and Isolation of Four Mutants**

The availability of reporter transgenes labeling CEPsh glia allowed us to begin to explore the roles played by these cells during nervous system development. We reasoned that mutants defective in CEPsh glia development would inform us not only about the process of glial cell differentiation, but might also possess neuronal defects, uncovering hitherto unexplored roles for *C. elegans* glia in neuronal development.

To identify such mutants, we mutagenized animals carrying a genomically-integrated *hlh-17* promoter::GFP reporter transgene with ethyl methanesulfonate (EMS; see Experimental Procedures), and scanned resulting F2 progeny for alterations in CEPsh glia using a dissecting microscope equipped with epifluorescence, to visualize GFP. From 3,200 mutagenized haploid

genomes examined, we recovered thirteen mutants exhibiting either abnormal CEPsh glia cell-body positioning or abnormal reporter transgene expression (Figure 2.3A). For further analysis, we chose four mutants of the latter class, which, based on linkage and complementation studies (see Experimental Procedures), form two complementation groups: one containing the alleles *ns156*, *ns158*, and *ns159*, and another containing the *ns157* allele.

- **The Development of Dorsal and Ventral CEPsh Glia is Molecularly Distinguishable**

The two complementation groups we selected were especially intriguing for us because of their reciprocal effects on CEPsh glia development (Figures 2.3B-J). Specifically, *ns156*, *ns158*, and *ns159* mutants lacked expression of both *hlh-17*::GFP and *ptr-10*::myrRFP preferentially in ventral CEPsh glia, whereas *ns157* animals lacked expression of these markers preferentially in dorsal CEPsh glia. For example, as shown in Figure 2.4, 93% and 94% of *ns156* animals completely lacked expression of *hlh-17*::GFP and *ptr-10*::myrRFP in ventral CEPsh glia, respectively; whereas 85% and 83% of these mutants, respectively, maintained a wild-type expression pattern of these reporters in dorsal CEPsh glia. Reciprocally, only 3% and 4% of *ns157* animals exhibited wild-type expression of

Figure 2.4

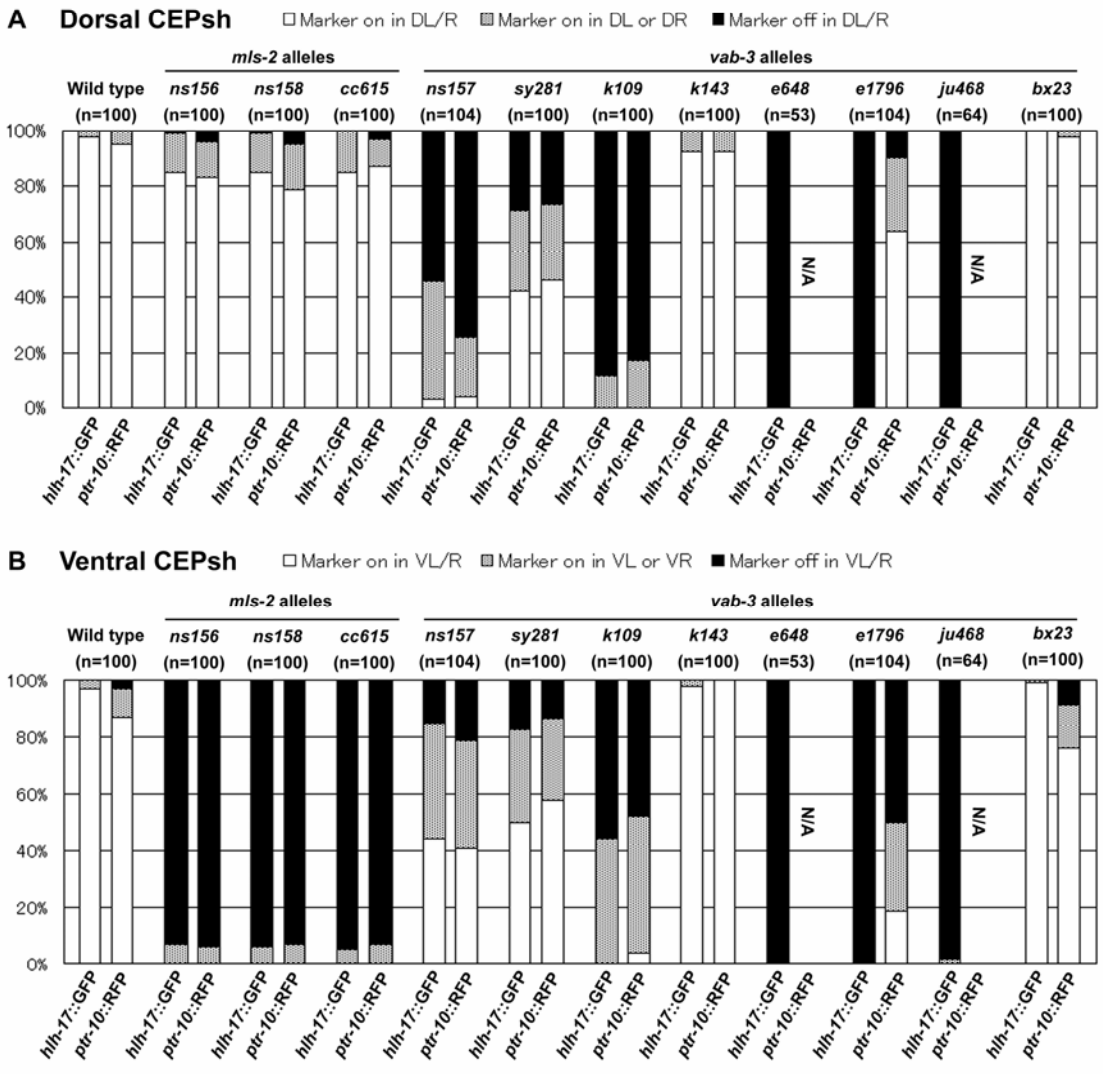

A Dorsal CEPsh
Marker on in DL/R
Marker on in DL or DR
Marker off in DL/R
mls-2 alleles
vab-3 alleles
Wild type (n=100)
ns156 (n=100)
ns158 (n=100)
cc615 (n=100)
ns157 (n=104)
sy281 (n=100)
k109 (n=100)
k143 (n=100)
e648 (n=53)
e1796 (n=104)
ju468 (n=64)
bx23 (n=100)
100%
80%
60%
40%
20%
0%
N/A
N/A
hlh-17::GFP
ptr-10::RFP
B Ventral CEPsh
Marker on in VL/R
Marker on in VL or VR
Marker off in VL/R
mls-2 alleles
vab-3 alleles
Wild type (n=100)
ns156 (n=100)
ns158 (n=100)
cc615 (n=100)
ns157 (n=104)
sy281 (n=100)
k109 (n=100)
k143 (n=100)
e648 (n=53)
e1796 (n=104)
ju468 (n=64)
bx23 (n=100)
100%
80%
60%
40%
20%
0%
N/A
N/A
hlh-17::GFP
ptr-10::RFP

Figure 2.4. Multiple *mls-2* and *vab-3* Alleles Affect CEPsh Glia Differentiation

(**A**) Histogram assessing *hlh-17*::GFP and *ptr-10*::myrRFP reporter transgene expression in dorsal CEPsh glia of animals of the indicated genotype. Vertical axis, % animals. White bars represent animals expressing reporters in both dorsal left and dorsal right (DL/R) CEPsh glia. Gray bars represent animals lacking expression of reporters in either dorsal left (DL) or dorsal right (DR) CEPsh glia. Black bars represent animals lacking expression in both DL/R CEPsh glia. n, number of animals examined. N/A, not applicable: in these animals many head cells are mispositioned, making it difficult to verify the identities of CEPsh glia and score transgene expression.

(**B**) Same as (**A**), except that expression in ventral CEPsh glia is depicted. VL/R, ventral left and right CEPsh glia. VL, ventral left. VR, ventral right.

hlh-17::GFP and *ptr-10*::myrRFP in dorsal CEPsh glia, respectively; whereas 44% and 41% of animals, respectively, maintained wild-type expression patterns in ventral CEPsh glia. Expression of the reporter genes in at least some cells other than the CEPsh glia was essentially unaffected in these mutants (data not shown).

Although defects in reporter transgene expression were evident in both dorsal and ventral CEPsh glia in each of the mutants described above (e.g., *hlh-17*::GFP and *ptr-10*::RFP expression was missing in one or more dorsal CEPsh glia in 15% and 17% of *ns156* animals, respectively), the dorsal/ventral bias in these defects suggests that despite morphological similarities (Figure 2.3B; White et al., 1986), dorsal and ventral CEPsh glia may be molecularly distinguishable. This observation is consistent with a previous study demonstrating that the *C. elegans* netrin-encoding gene, *unc-6*, is expressed in ventral but not dorsal CEPsh glia (Wadsworth et al., 1996)

Interestingly, *Olig2* expression in the developing vertebrate spinal cord also exhibits dorsal/ventral segregation of expression, controlled by independent transcriptional programs (Cai et al., 2005; Fogarty et al., 2005; Liu et al., 2003; Lu et al., 2000; Vallstedt et al., 2005; Warf et al., 1991; Yu et al., 1994; Zhou et al., 2000), in line with the possibility that *hlh-17* may be the *C. elegans* ortholog of *Olig2*.

- **CEPsh Glia are Generated but are Abnormal in *ns156* and *ns157* Mutants**

The absence of *hlh-17*::GFP and *ptr-10*::myrRFP expression in *ns156* and *ns157* mutants could reflect either defects in the generation of ventral and dorsal CEPsh glia, respectively, or defects in CEPsh glia terminal differentiation. To distinguish between these possibilities, we collaborated with John Murray working in Bob Waterston's lab. John used an automated 4-D lineage tracing setup (Bao et al., 2006) to follow the pattern of cell divisions leading to the generation of CEPsh glia in *ns156* and *ns157* mutants. In four of four *ns156* animals he examined, all CEPsh glia were generated. Similarly, in four of four *ns157* animals examined, all CEPsh glia were formed (Figure 2.5). Furthermore, CEPsh glia were properly positioned within the embryo in these animals, with the exception of a single anteriorly displaced dorsal CEPsh glia in one *ns156* embryo examined. Taken together, these results suggest that the *ns156* and *ns157* mutations do not affect CEPsh glia generation, and must, therefore disrupt CEPsh glia terminal differentiation.

- **Ensheathment of the Nerve Ring is Defective in *ns156***

Since CEPsh glia of *ns156* and *ns157* mutants are defective for expression

Figure 2.5

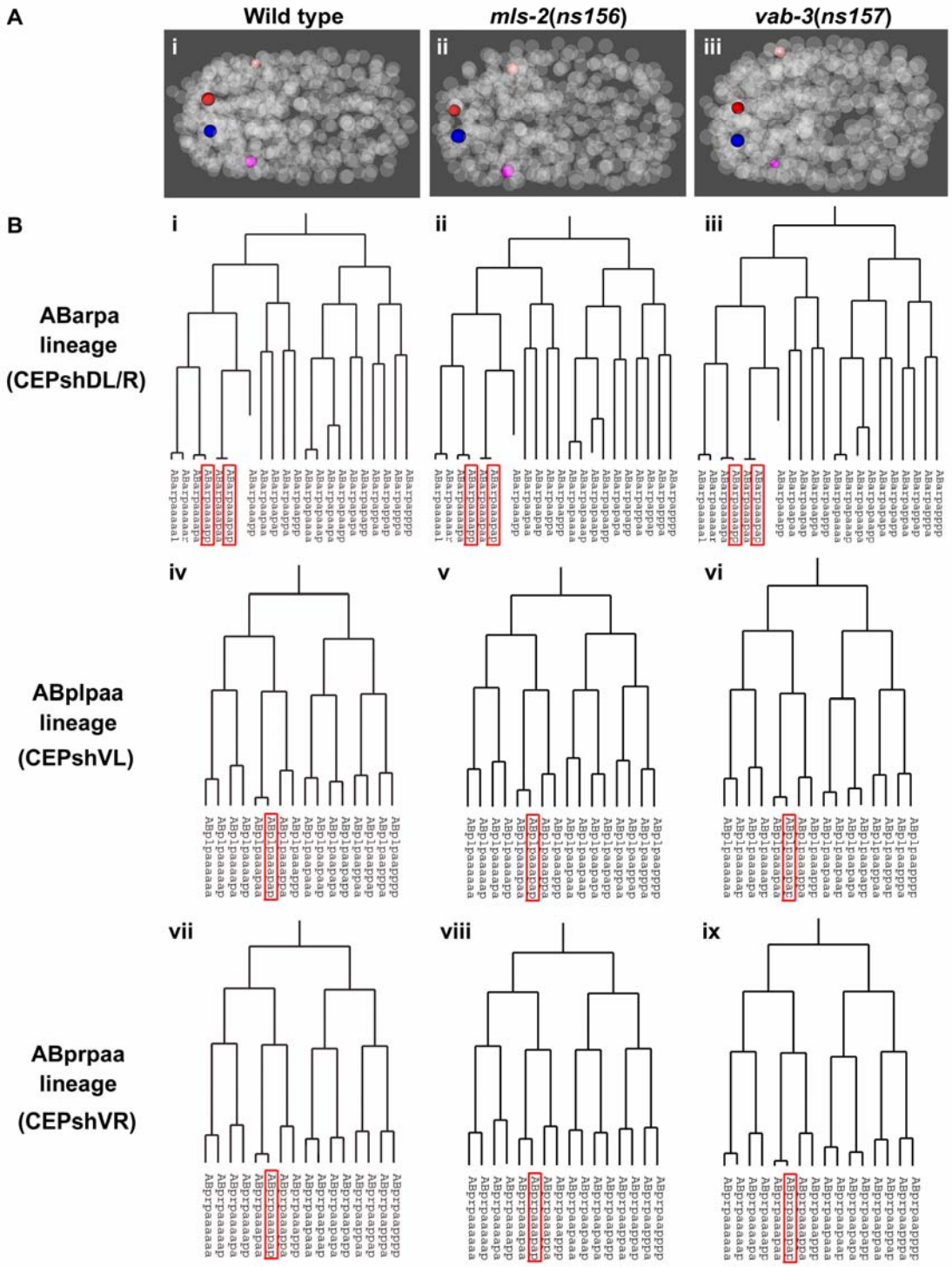

A
Wild type
mls-2(ns156)
vab-3(ns157)
i
ii
iii
B
ABarpa lineage (CEPshDL/R)
ABplpaa lineage (CEPshVL)
ABprpaa lineage (CEPshVR)
iv
v
vi
vii
viii
ix

Figure 2.5. Cell Lineage is Not Altered in *mls-2*(*ns156*) and *vab-3*(*ns157*) Mutant Embryos

(**A**) Fluorescence images of embryos of the indicated genotype containing *pie-1*::H2B::GFP and *his-72*::H3.3-GFP, labeling all nuclei. The red, blue, magenta, and pink dots indicate the nuclei of CEPshDR, CEPshDL, CEPshVL, and CEPshVR glia, respectively, immediately after their births.

(**B**) Representative lineage trees constructed from tracing the divisions executed by the ABarpa, ABplpaa, and ABprpaa precursor cells and their progeny in wild-type (i, iv, and vii, respectively), *mls-2*(*ns156*) (ii, v, and viii, respectively), and *vab-3*(*ns157*) (iii, vi, and ix, respectively) animals. Note that all three genotypes give rise to essentially identical lineage trees.

of *hlh-17* and *ptr-10*, we wondered whether other differentiated features of CEPsh glia were also perturbed in these mutants. Specifically, we examined whether CEPsh glia still extended processes that ensheathed the nerve ring. Two *ns156* and two *ns157* adults were serially sectioned and examined by electron microscopy for glial ensheathment of the nerve ring and ventral ganglion. In both *ns157* mutants, we were able to see glial ensheathment of the nerve ring and ventral ganglion leading into the nerve ring.

Unlike in *ns157* mutants, however, ventral ensheathment was absent in *ns156* animals (Figure 2.6). Furthermore, the ventral ganglion leading into the nerve ring of *ns156* mutants was highly disorganized, lacking its characteristic bilateral symmetry (Figure 2.6). Taken together, these results suggest that the *ns156* mutation may disrupt CEPsh terminal differentiation to a larger extent than the *ns157* lesion. This is a result consistent with our studies of CEP neuron dendrite extension in these mutants (see Chapter 3).

2.3 Basic Characterization of *mls-2*

- ***ns156* Carries a Mutation in *Nkx*/*HMX*-Related Gene *mls-2***

To identify the gene affected by the *ns156* mutation, we used single

Figure 2.6

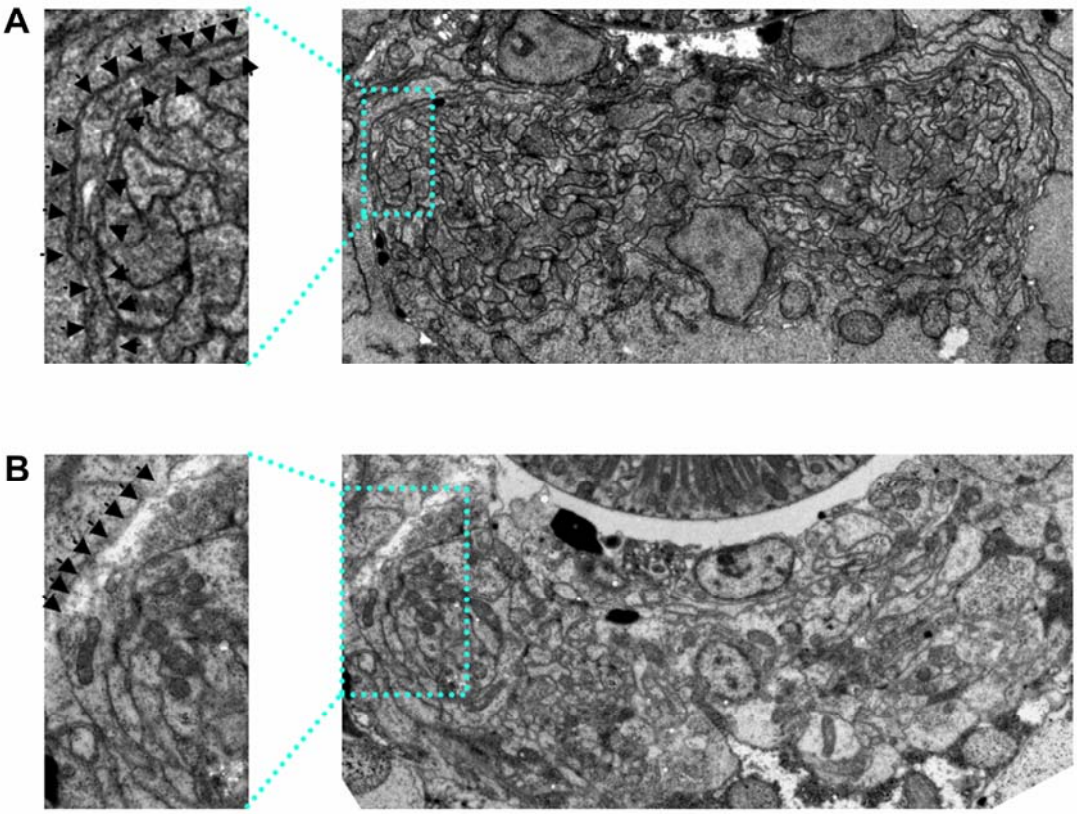


Figure 2.6. *mls-2*(*ns156*) Mutants Lack Ventral CEPsh Glia Processes

(**A**) Right, electron micrograph of a section of a wild-type adult showing the ventral ganglion leading into the nerve ring. Left, magnification of the boxed region indicated in the right panel. Arrows demarcate the CEPsh glial process.

(**B**) Same as (**A**) except that an *mls-2*(*ns156*) mutant is shown. Arrows point to the border of the ventral ganglion showing the absence of a CEPsh glial process. Note the disorganization of the ventral ganglion (right) as compared with the bilaterally symmetric structure of the wild type (**A**).

nucleotide polymorphism differences between the *ns156* and CB4856 *C. elegans* strains (Wicks et al., 2001) to map the mutation to a 0.98 map unit interval on the X chromosome between cosmids T13H2 and T07F12 (see Experimental Procedures). Introduction of cosmid C39E6, contained within this interval, into *ns156* animals, restored *hlh-17*::GFP and *ptr-10*::myrRFP expression in CEPsh glia of these animals (Figure 2.7A). An 8.3 kb subclone of this cosmid, composed of the homeodomain transcription factor gene *mls-2*, 5.5 kb of sequences upstream of the putative *mls-2* translation start site, and 300 bp downstream of the *mls-2* stop codon, rescued *ns156* animals as efficiently as the cosmid (Figure 2.7A), suggesting that the *ns156* allele, as well as the *ns158* and *ns159* alleles, disrupt *mls-2* gene function. Supporting this notion, we identified point mutations in *mls-2* in all three mutants we isolated (Figure 2.7B). Specifically, *ns156* mutants contain a G-to-A point mutation at nucleotide 189 of *mls-2*, disrupting the splice-donor site of the first intron of the gene; and *ns158* and *ns159* mutants harbor identical G-to-A point mutations at position 481, disrupting the splice-donor site of the second intron of *mls-2*.

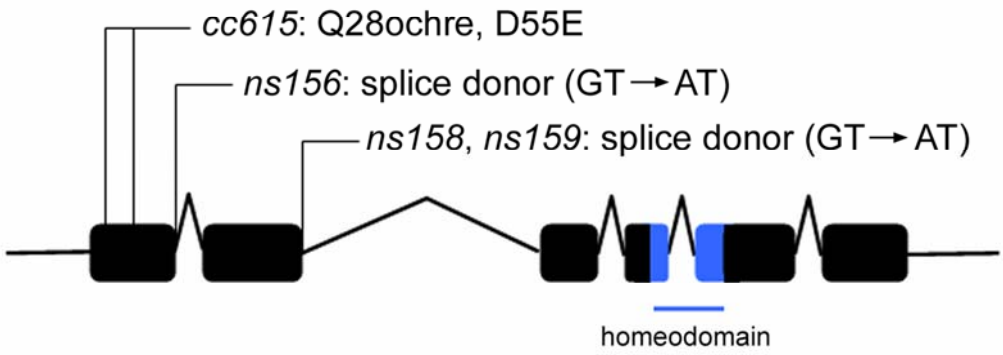
To further confirm that *mls-2* is the gene responsible for the defects seen in *ns156*, *ns158*, and *ns159* mutants, we introduced the *hlh-17*::GFP and *ptr-10*::myrRFP CEPsh reporter transgenes into *mls-2*(*cc615*) animals, previously characterized as defective in the differentiation of the muscle blast cell, M (Jiang et al., 2005). As in the mutants we identified, *mls-2*(*cc615*) animals preferentially

Figure 2.7

A

Strain (n)	*hlh-17*::GFP expression pattern		
	on in VL/R	on in VL or VR	off in VL/R
Wild type (100)	97%	3%	0%
mls-2(*ns156*) (100)	0%	5%	95%
mls-2(*ns156*) + C39E6 (57)	47%	34%	19%
mls-2(*ns156*) + *mls-2* genomic locus (51)	39%	24%	37%

B



C

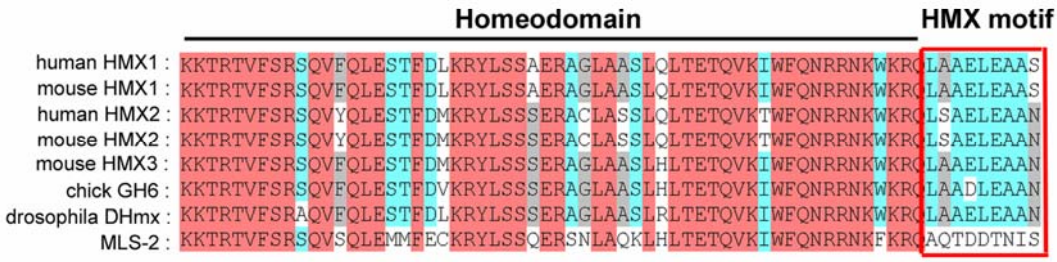


Figure 2.7. *mls-2* Encodes an Nkx/HMX-Related Homeodomain Protein

(**A**) Table summarizing the results of *mls-2*(*ns156*) rescue studies. Strains of the indicated genotype were scored as in Figure 2.4. VL/R, ventral left and right CEPsh glia. VL, ventral left. VR, ventral right. n, number of animals observed. C39E6, the cosmid clone containing *mls-2*. A single representative transgenic line is shown for each rescue construct used. The rescuing *mls-2* genomic locus construct is described in the text.

(**B**) *mls-2* gene structure. Boxes, exons. Λ-shaped lines, introns. Mutation sites and nucleotide and protein alterations are indicated. Blue areas encode the homeodomain.

(**C**) An alignment of homeodomains and HMX motifs of *C. elegans* MLS-2 protein and the indicated related proteins. Pink, identical in all proteins. Blue, identical in all but one or two proteins. Gray, present in five of the proteins. Note that MLS-2 lacks a recognizable HMX motif.

lost reporter transgene expression in ventral CEPsh glia (Figure 2.4). Taken together, these results suggest that *mls-2* regulates at least some aspects of CEPsh glia differentiation.

mls-2 encodes a protein with a predicted homeodomain. Within its homeodomain, *mls-2* is most similar to the HMX family of transcriptional regulators (Jiang et al., 2005). However, *mls-2* lacks the HMX motif, A/SAE/DLEAAN/S, located immediately downstream of the homeodomain of HMX-related proteins (Figure 2.7C; Wang et al., 2000). Nkx homeodomain proteins are closely related to HMX proteins, but lack the HMX motif, suggesting that *mls-2* may be more appropriately classified as belonging to the Nkx/HMX superfamily of homeodomain-containing transcriptional regulators. Intriguingly, murine embryos lacking Nkx6.1 function, display a reduction in Olig2 expression in the ventral aspect of the developing spinal cord (Liu et al., 2003). Thus, it may be that a module in which an *Nkx*-related gene regulates ventral expression of an *Olig2*-related gene in the developing nervous system, is conserved from *C. elegans* to mammals.

- ***mls-2* is Expressed in Both Dorsal and Ventral CEPsh Precursor Cells**

To determine where *mls-2* functions to regulate CEPsh glia differentiation,

we generated animals carrying a transgene consisting of the *mls-2* promoter driving expression of a gfp::*mls-2* transgene, fused to a 2.1 kb genomic fragment located immediately downstream of the endogenous *mls-2* stop codon. When introduced into *mls-2*(*ns156*) animals, this transgene restored wild-type expression of *hlh-17*::GFP and *ptr-10*::GFP (data no shown), suggesting that the pattern of GFP expression we observed in transgenic animals reflects, at least to some degree, the expression pattern of endogenous *mls-2*.

We failed to detect *mls-2* expression in CEPsh glia in wild-type larvae or adults, suggesting that the gene may function to regulate early stages of CEPsh glia development, consistent with its effects on *hlh-17* expression. To test this, we examined embryos at around 300 minutes post-fertilization, near the time at which CEPsh glia are born. At this stage, MLS-2::GFP is detectable in a restricted set of cells in the anterior region of the animal. Although we could not reliably confirm GFP expression within CEPsh cells at this stage, due to the high density of cells in this region of the embryo at this point in development, we could unambiguously show that gfp::*mls-2* was expressed in the nuclei of the direct precursor cells of the left and right ventral CEPsh glia, ABplpaaapap and ABprpaaapap, respectively (Figures 2.8A-G). We confirmed these assignments by demonstrating that in animals transgenic for the *hlh-17*::GFP reporter, ablation of a putative CEPsh precursor cell, resulted in absence of GFP expression at the appropriate position in the adult (see Chapter 3). GFP expression was also detected in the direct

Figure 2.8

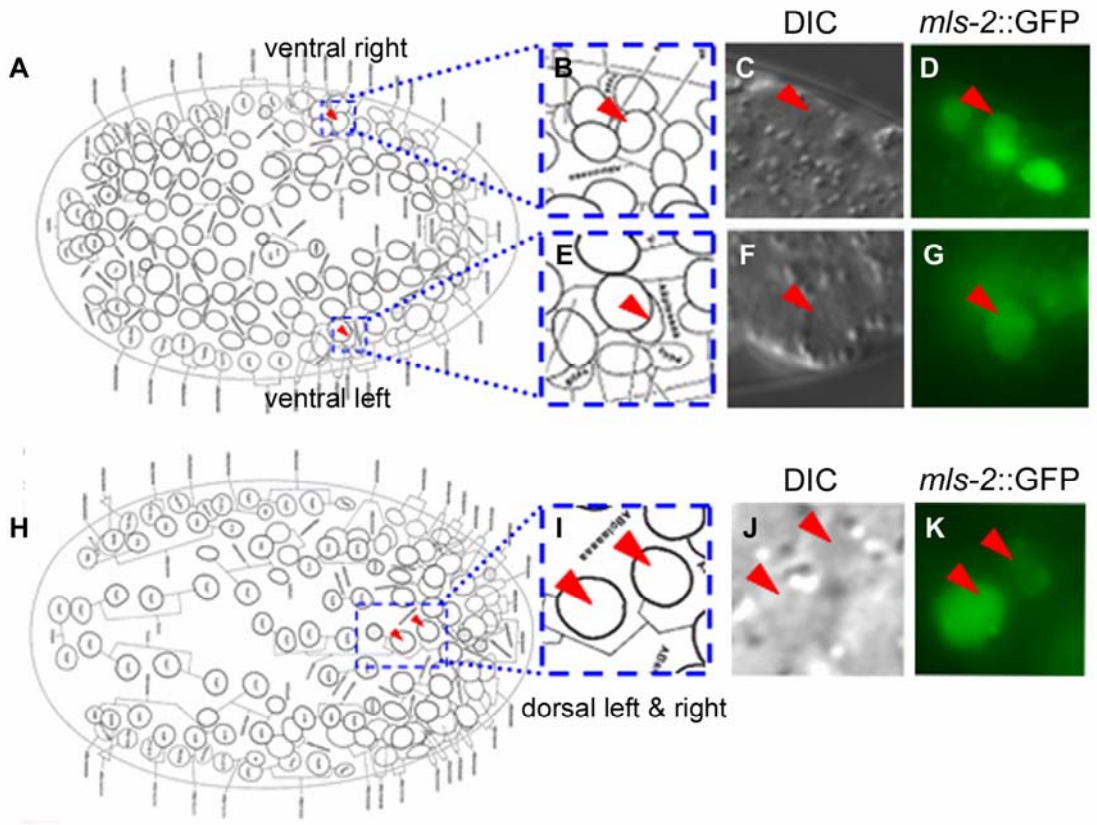


Figure 2.8. *mls-2* is Expressed in CEPsh Precursor Cells

(**A**) A diagram showing the positions of ventral nuclei in an embryo at about 270 min after the first cleavage (adapted from Sulston et al., 1983). Dashed boxed areas are expanded in (**B**) and (**E**).

(**B**) Enlarged region of (**A**), (**C**) DIC image, and (**D**) fluorescence image of the precursor cell of a ventral right CEPsh glial cell (red arrowhead) in a 270 min stage embryo expressing an *mls-2*::GFP reporter transgene.

(**E-G**) Same as (**B-D**), respectively, except that the precursor cell of a ventral left CEPsh glial cell (red arrowhead) is shown.

(**H-K**) Same as (**A-D**) showing dorsal left and right CEPsh glia precursors.

precursor of dorsal CEPsh glia (Figure 2.8H-K), consistent with our observations that *mls-2* weakly affects expression of *hlh-17*::GFP and *ptr-10*::myrRFP in these glia (Figure 2.4).

Taken together, our results suggest that *mls-2* functions in the CEPsh cell lineage, probably within the CEPsh glia themselves, to regulate their differentiation (see also mosaic analysis studies in Chapter 3).

2.4 Basic Characterization of *vab-3*

- ***ns157* Carries a Mutation in the *Pax6/7*-Related Gene *vab-3***

We used single nucleotide polymorphism differences between the *ns157* and CB4856 *C. elegans* strains (Wicks et al., 2001) to map *ns157* to a 0.51 map unit interval on chromosome X between clones Y49A10A and T01C1. In addition to defects in the expression of dorsal CEPsh markers, we noticed that *ns157* animals also displayed highly penetrant defects in the migration of the gonadal distal tip cell (data not shown). Similar defects had been described in animals containing mutations in the *vab-3* gene (Chisholm and Horvitz, 1995; Cinar and Chisholm, 2004), which is located within the interval to which we mapped *ns157*. To test whether *ns157* was an allele of *vab-3*, we generated transgenic *ns157*

animals carrying either cosmid F14F3, containing *vab-3*, or a 24 kb fragment derived from this cosmid, containing only *vab-3* coding and regulatory sequences. As shown in Figure 2.9A, both transgenes effectively restored *hlh-17*::GFP (and *ptr-10*::myrRFP expression; not shown) in CEPsh glia, suggesting that *ns157* was indeed an allele of *vab-3*.

The *vab-3* genetic locus encodes two DNA binding domains, an N-terminal paired domain (PD) and a C-terminal homeodomain (HD), and generates at least three different transcripts, A, B and C, of which isoform A includes both the PD and HD (Chisholm and Horvitz, 1995). We identified a G-to-A missense mutation, at position 5261 of the gene, in *ns157* mutants. This mutation is situated within the PD, and is predicted to change amino-acid 120 of the VAB-3 isoform A protein from a highly conserved Serine to Asparagine (Figure 2.9B, see description below).

Taken together, our results strongly suggest that *vab-3* is the gene altered in *ns157* mutants.

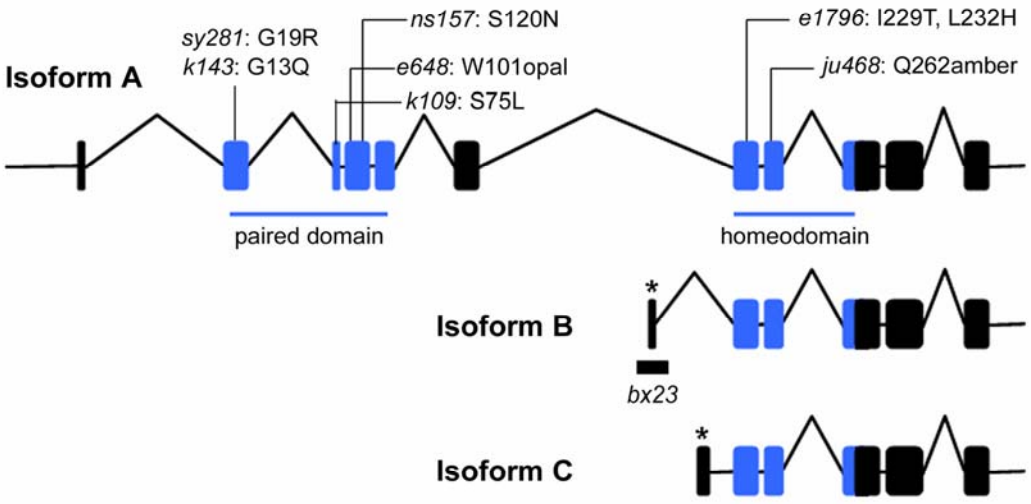
Pax-related proteins, containing both a PD and a HD, play key roles in the developing vertebrate spinal cord and in the expression of Olig2. A reduction in Pax6 function can lead to a concomitant loss in Olig2 expression in rostral regions of the ventral spinal cord (Mizuguchi et al., 2001; Novitch et al., 2001). Furthermore, oligodendrogenesis is delayed in *Pax6* mutant spinal cords (Sun et al., 1998). The Pax7 protein is expressed in the dorsal portion of the developing

Figure 2.9

A

Strain (n)	*hlh-17*::GFP expression pattern					
	on in DL/R	on in DL or DR	off in DL/R	on in VL/R	on in VL or VR	off in VL/R
Wild type (100)	99%	1%	0%	97%	3%	0%
vab-3(*ns157*) (104)	3%	43%	54%	44%	41%	15%
vab-3(*ns157*) + F14F3 (51)	73%	11%	16%	86%	9%	5%
vab-3(*ns157*) + *vab-3* genomic locus (50)	60%	24%	16%	73%	20%	7%

B



C

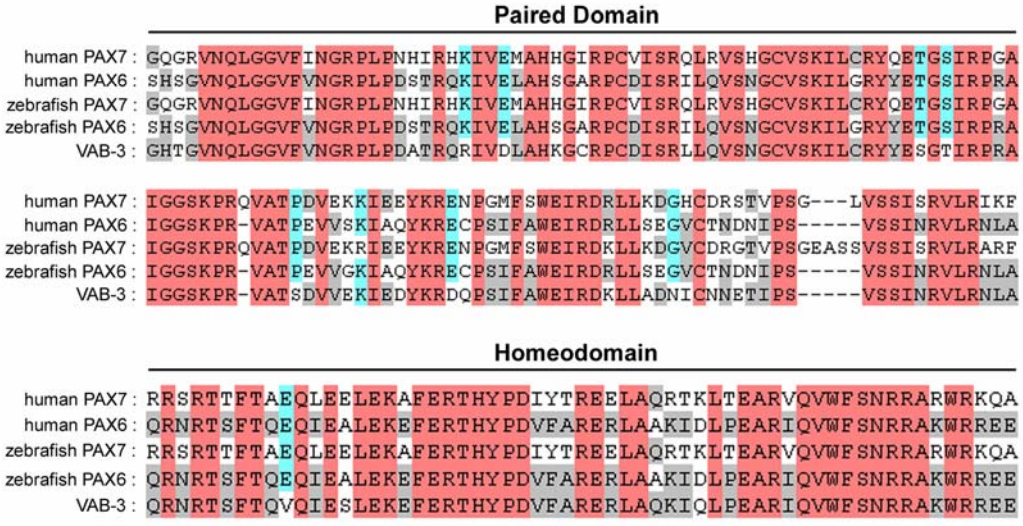


Figure 2.9. *vab-3* Encodes a Pax6/7-Related Protein

(**A**) Table summarizing the results of *vab-3*(*ns157*) rescue studies. Strains of the indicated genotype were scored as in Figure 2.4. DL/R, dorsal left and right CEPsh glia. DL, dorsal left. DR, dorsal right. VL/R, ventral left and right. VL, ventral left. VR, ventral right. n, number of animals observed. F14F3, a cosmid clone containing the *vab-3* locus. A single representative transgenic line is shown for each rescue construct used. The rescuing *vab-3* genomic locus construct is described in the text.

(**B**) *vab-3* gene structure. Three isoforms are depicted. Boxes, exons. Λ- shaped lines, introns. Mutation sites and nucleotide and protein alterations in alleles used in this study are indicated. Exons with asterisks are those specific for isoforms B and C. Blue areas encode the Paired domain and homeodomain. *bx23* is a deletion of the first exon of isoform B.

(**C**) An alignment of Paired domains and homeodomains of *C. elegans* VAB-3 protein and the indicated related proteins. Pink, identical in all proteins. Blue, identical in all but one protein. Gray, present in three of the proteins.

vertebrate spinal cord, and although the control of Olig2 expression in the dorsal spinal cord has not been extensively studied, dorsal Olig2 expressing cells are derived from Pax7 positive cells (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). It is possible, therefore, that Pax7 plays a role in the control of dorsal Olig2 expression.

It is of note that the closest related *C. elegans* protein to Pax6 and to Pax7 is VAB-3, exhibiting 79% and 93% identity to the human Pax6 PD and HD, respectively (Chisholm and Horvitz, 1995), and 71% and 65% identity to the PD and HD of human Pax7, respectively (Figure 2.9C), although VAB-3 seems to lack an octapeptide sequence present in Pax7 (Jostes et al., 1990). These results suggest that, similarly to our studies of *mls-2*, a Pax/Olig2 module may also regulate glia formation in both *C. elegans* and vertebrates.

- ***vab-3* May Function within CEPsh Glia to Regulate Their Development**

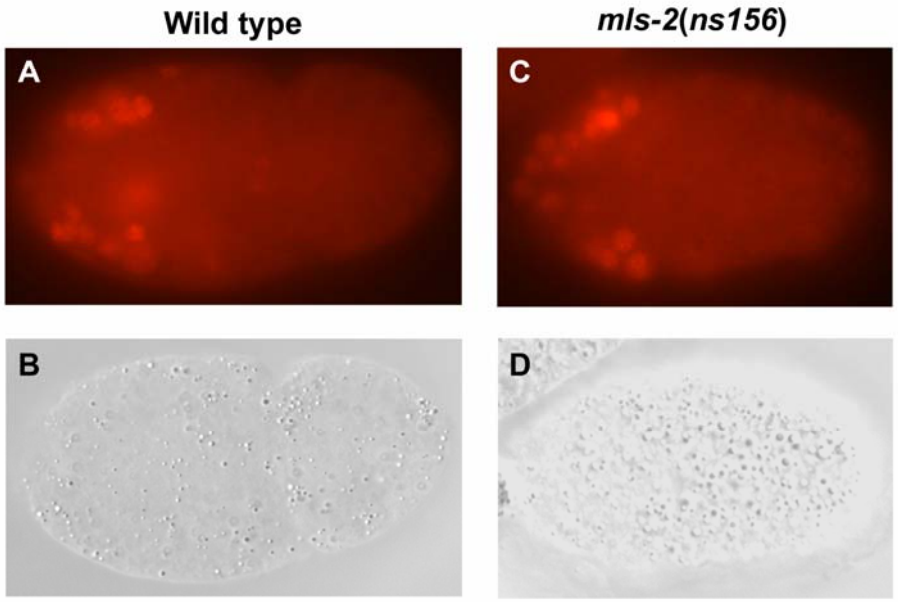
To determine whether *vab-3* functions within CEPsh glia to regulate *hlh-17* and *ptr-10* expression, we initially sought to determine whether *vab-3* was expressed in these cells. Specifically, we examined GFP expression in animals expressing this reporter under the control of either 3 kb of sequences upstream of the predicted *vab-3* translation start site, or an 18 kb DNA fragment including most

of the *vab-3* coding sequences (Chisholm and Horvitz, 1995). Neither transgene was expressed within CEPsh glia in larvae or adults, and embryonic expression, although present, was restricted to late embryogenesis in animals carrying the 3 kb promoter transgene, or was present in earlier embryos in animals containing the 18 kb transgene, but was very faint, thus precluding an analysis of earlier stages similar to the one we performed for *mls-2*.

vab-3 expression was previously examined using anti-VAB-3 antibodies generated by David Greenstein (Zhang et al., 1998). Using one of those antibodies, we tried antibody staining to identify VAB-3 protein in embryos. Although we managed to obtain an image of a stained embryo (Figure 2.10A and 2.10B), we were unable to identify the expression of the protein in CEPsh glia or in their precursors by lineage or by position, because VAB-3 expression is broad in early embryos, and the conditions for antibody staining destroy cells.

As a different approach to determine *vab-3* site of action, we introduced a transgene consisting of the 5.5 kb *mls-2* promoter driving expression of a *vab-3* isoform A cDNA, and fused to a 2.1 kb genomic fragment located immediately downstream of the endogenous *mls-2* stop codon, into *vab-3*(*ns157*) mutants. As described in the previous section, a similar *mls-2* fusion protein was expressed in the dorsal CEPsh lineage (Figure 2.8H-K). The transgene exhibited weak but significant rescue of *hlh-17* and *ptr-10* reporter expression. Specifically, 18 of 100 (18%) animals carrying the transgene expressed *hlh-17*::GFP in both dorsal left

Figure 2.10



E

	CEPsh VL	CEPsh VR	CEPsh DL/R	n
Transgene	+	+	+	20
hlh-17::GFP expression	+	+	+	
Transgene	–	+	+	1
hlh-17::GFP expression	–	+	+	
Transgene	+	–	+	2
hlh-17::GFP expression	+	–	+	
Transgene	–	–	+	3
hlh-17::GFP expression	–	–	+	
Transgene	+	+	–	6
hlh-17::GFP expression	+	+	–	

Figure 2.10. *vab-3* May Function within CEPsh Glia

(**A**, **B**) Fluorescence image (**A**), and DIC image (**B**) of a wild-type embryo stained with anti-VAB-3 antibody.

(**C**, **D**) Same as (**A**, **B**), respectively, except that the staining in an *mls-2*(*ns156*) embryo is shown.

(**E**) Table summarizing the results of mosaic studies of *vab-3* control of *hlh-17*::expression (see text and Experimental Procedures for details). VL, ventral left. VR, ventral right. DL/R, dorsal left and right. Transgene +, transgene present in indicated cell. Transgene -, transgene inferred not to be present in indicated cell. *hlh-17*::GFP expression +, *hlh-17*::GFP reporter expressed in indicated cell. *hlh-17*::GFP expression -, *hlh-17*::GFP reporter not expressed in indicated cell. n, number of animals examined.

and right CEPsh glia, whereas only 3 out of 104 (2.9%) of animals without the transgene expressed *hlh-17*::GFP in these cells, consistent with the idea that *vab-3* functions in the CEPsh lineage to control CEPsh glia differentiation.

To further narrow down the possible site of action of *vab-3*, we performed a mosaic analysis of *vab-3* with respect to defects in *hlh-17*::GFP expression. We introduced an extrachromosomal array containing the *vab-3* cosmid and *ptr-10*::myrRFP into *vab-3*(*ns157*) mutants carrying an integrated *hlh-17*::GFP reporter. We found a perfect correlation between expression of *hlh-17*::GFP and expression of *ptr-10*::myrRFP (Figure 2.10E). Importantly, we never observed animals in which *hlh-17*::GFP was expressed in a given cell, but *ptr-10*::myrRFP was not, consistent with *vab-3* functioning within CEPsh glia lineages to control differentiation.

These results collectively suggest that *vab-3* functions in the CEPsh lineage, and probably in the CEPsh glia themselves

- **Different VAB-3 Domains May Regulate Expression of *hlh-17* and *ptr-10* in Dorsal and Ventral CEPsh Glia**

Although *vab-3*(*ns157*) mutants show a bias towards dorsal defects in *hlh-17*::GFP and *ptr-10*::myrRFP expression, the molecular lesion in these

animals may not completely eliminate *vab-3* function, and, thus, the phenotype of *vab-3*(*ns157*) animals may not represent the null phenotype of the gene. To test this, we examined CEPsh reporter transgene expression in animals homozygous for two previously described *vab-3* alleles, *e648* and *ju468*, that are likely to eliminate most, if not all activity of *vab-3* isoform A (Chisholm and Horvitz, 1995; Cinar and Chisholm, 2004). *vab-3*(*e648*) mutants have an opal stop mutation in exon 4 of *vab-3* isoform A, and *vab-3*(*ju468*) animals have an amber stop mutation in exon 10, which would truncate all VAB-3 protein isoforms produced by the locus (Figure 2.9B). We were unable to detect *hlh-17*::GFP expression in either dorsal or ventral CEPsh glia in these animals (Figure 2.4), suggesting that *vab-3* plays an important role in the differentiation of all CEPsh glia, and that *ns157* is indeed not a null allele of *vab-3*. A similar result was obtained for the *ptr-10*::myrRFP transgene, however, because *ptr-10* is expressed in several cells in the head, and *vab-3*(*e648*) and *vab-3*(*ju468*) mutants display defects in the positioning of some *ptr-10*-expressing cells, we were unable to unambiguously identify the CEPsh cells in these animals.

To test whether isoform B of *vab-3* may be important for glial differentiation, we examined animals containing the *bx23* allele, which contains a deletion in one of the two isoform B-specific exons (Figure 2.9B). As shown in Figure 2.4, no defects were seen in CEPsh glia expression of either *hlh-17*::GFP or *ptr-10*::myrRFP, suggesting that isoform A, but not isoform B, is relevant for glial

differentiation.

Since *vab-3* is required for the differentiation of both dorsal and ventral CEPsh glia, but *vab-3*(*ns157*) mutants, affecting the VAB-3 PD, are preferentially defective in dorsal CEPsh glial differentiation, we wondered whether the PD and HD of *vab-3* preferentially regulate differentiation of dorsal and ventral CEPsh glia, respectively. To test this, we first examined *hlh-17*::GFP and *ptr-10*::myrRFP expression in *vab-3*(*k143*), *vab-3*(*sy281*), and *vab-3*(*k109*) animals, all of which carry missense mutations in the VAB-3 PD. Contrary to our prediction, *vab-3*(*k143*) did not exhibit defects in the marker expression, and *vab-3*(*sy281*) exhibited weak defects in both dorsal and ventral CEPsh glia (Figure 2.4). Interestingly, however, we found that *vab-3*(*k109*) animals are preferentially defective in dorsal CEPsh glia, a bias similar to that of *vab-3*(*ns157*) mutants, indicating that the PD might have unique roles in these cells (Figure 2.4). To uncover the function of the VAB-3 HD, we then examined reporter expression in *vab-3*(*e1796*) animals, which carry two missense mutations in the VAB-3 HD (Figure 2.9B). Interestingly, we found that although *hlh-17* expression was abrogated in both dorsal and ventral CEPsh glia in these mutants, *ptr-10* expression was now preferentially absent in ventral CEPsh glia. Specifically, whereas 64% of animals had wild-type expression of *ptr-10*::myrRFP in their dorsal CEPsh glia, only 19% of animals had wild-type expression of the same marker in ventral CEPsh glia (Figure 2.4). Dorsal CEPsh glia expressing

ptr-10::myrRFP but not *hlh-17*::GFP still possessed normal posterior extensions ensheathing the nerve ring, consistent with our EM results (see above; Figure 2.6).

Taken together, our genetic studies of *vab-3* suggest two points. First, different domains of VAB-3 may be preferentially important for controlling ventral and dorsal CEPsh glia differentiation, suggesting that VAB-3 targets in these cells may be different. Second, the observation that *ptr-10* expression is relatively unaffected in dorsal CEPsh glia of *vab-3*(*e1796*) animals, whereas *hlh-17* expression is completely absent in these cells in these mutants, suggests that *vab-3* regulates *hlh-17* and *ptr-10* expression independently.

2.5 Isolation and Characterization of *hlh-17* Mutants

- ***hlh-17*, *hlh-31*, and *hlh-32* are Not Essential for the Development of CEPsh Glia**

In mice, mutation of *Olig* genes resulted in failure of development of motor neurons and OLPs in the spinal cord, and of all oligodendrocytes in the brain (Lu et al., 2002; Zhou and Anderson, 2002). Then what is the phenotype of *hlh-17* mutants? Complete loss of *hlh-17*::GFP expression, as occurs in *vab-3* mutants,

does not prevent normal development, suggesting that *hlh-17* is unlikely to be essential for organismal survival. However, a previous study suggested that a mutation, *ok487*, deleting portions of both *hlh-17* and the *hlh-17*-related gene, *hlh-31*, caused early larval arrest (McMiller and Johnson, 2005), suggesting a possible essential developmental role for *hlh-17*. To reconcile these observations, we first attempted to rescue the larval lethal phenotype of *hlh-17*(*ok487*) mutants. We found that neither the cosmid containing *hlh-17* and *hlh-31*, nor a 9 kb subclone of this cosmid containing both genes, was able to rescue the larval lethality (data not shown). Next, we examined animals heterozygous for the *ok487* allele and a deficiency, *sDf23*, which deletes the *hlh-17*/*hlh-31* locus (data not shown). We found that these animals were viable, suggesting that *ok487* is unlikely to be a loss-of-function mutation in *hlh-17*. Finally, by sequencing the *hlh-17*/*hlh-31* locus from *ok487* animals, we showed that the *ok487* lesion was not a deletion of the region, but rather an insertion of a portion of the *hlh-31* into *hlh-17*. This insertion leaves *hlh-31* intact, and may also allow *hlh-17* to be produced correctly (see Figure 2.11A for details). Taken together, these results suggest that the larval lethality in the *ok487* strain is not due to an *hlh-17* lesion, but is likely due to an unrelated linked lethal mutation.

To identify the role of *hlh-17*, we generated a new *hlh-17* mutation, *ns204*, using PCR followed by sib-selection (Jansen et al., 1997). Using both PCR and Southern analysis, we showed that *hlh-17*(*ns204*) contains a deletion removing

Figure 2.11

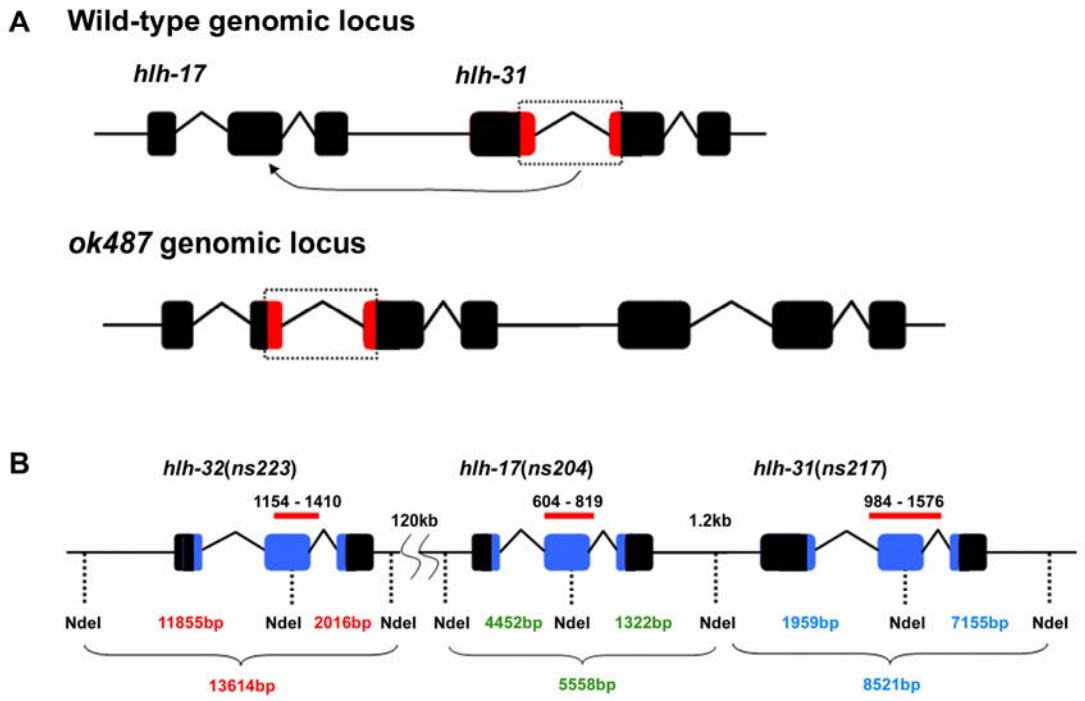

A
Wild-type genomic locus
hlh-17
hlh-31
ok487 genomic locus
B
hlh-32(ns223)
hlh-17(ns204)
hlh-31(ns217)
1154 - 1410
604 - 819
984 - 1576
120kb
1.2kb
NdeI
11855bp
2016bp
4452bp
1322bp
1959bp
7155bp
13614bp
5558bp
8521bp
C
1
2
3
13614bp
11855bp
8521bp
7155bp
5558bp
4452bp
2016/1959bp
1322bp
10.0kb
8.0kb
5.0kb
3.0kb
2.0kb
1.5kb
1.0kb
1: N2
2: hlh-17(ns204)
3: hlh-32(ns223)
hlh-17(ns204)
hlh-31(ns217)

Figure 2.11. Isolation of *hlh-17*, *hlh-31*, and *hlh-32* Deletion Alleles

(**A**) Diagrams depicting the wild-type *hlh-17*/*hlh-31* locus (top) and the alterations in the *ok487* strain (bottom). Boxes, exons. Λ-shaped lines, introns. The boxed red sequences in *hlh-31* (colored red) were duplicated and inserted into exon 2 of *hlh-17*.

(**B**, **C**) Genomic organization of *hlh-32*, *hlh-17*, and *hlh-31*. (**B**) Boxes, exons. Λ-shaped lines, introns. Locations of deletion alleles are indicated by red bars, with numbers indicating the precise deletion positions relative to the ATG in the genomic sequences of each gene. Blue areas encode the bHLH domains in each gene. The NdeI restriction enzyme was used to digest genomic DNA for the Southern blot shown in (**C**). The sizes of predicted NdeI digestion fragments are indicated with red, blue, or green numbers. Arrows in (**C**) indicate the sizes of each band. Each deletion deletes one NdeI site, yielding only three bands in the triple knockout strain (**C**, third lane). The blot in (**C**) was probed with labeled *hlh-17* cDNA, which can detect all the fragments depicted in the panel. The 1959 NdeI fragment of *hlh-31* and 2016 NdeI fragment of *hlh-32* are overlapping.

most of the bHLH domain of *hlh-17* (Figure 2.11B and 2.11C). Unfortunately, however, *hlh-17*(*ns204*) mutants did not display any obvious developmental defects. We have looked at *hlh-17*(*ns204*) animals in great detail, examining animals for gross morphological defects, defects in expression of *hlh-17*::GFP, defects in expression of *ptr-10*::myrRFP, and defects in axon guidance, but no obvious defects were observed (Figure 2.12)

Because *Olig2* functions redundantly with *Olig1* in some vertebrate settings, we wondered whether *hlh-17* might be acting redundantly with the *hlh-31* and *hlh-32* genes. To test this, we generated triple mutant animals, *hlh-32*(*ns223*) *hlh-17*(*ns204*) *hlh-31*(*ns217*), using PCR screening and sib-selection (Jansen et al., 1997). The mutations we generated delete essential portions of the bHLH domains of each gene (Figure 2.11B and 2.11C). To our disappointment, these triple mutants did not exhibit any obvious defects (Figure 2.13A-C). Thus, by implication, *hlh-17* mutants on their own are unlikely to show any defects, and functions of *hlh-17* remain unclear.

Figure 2.12

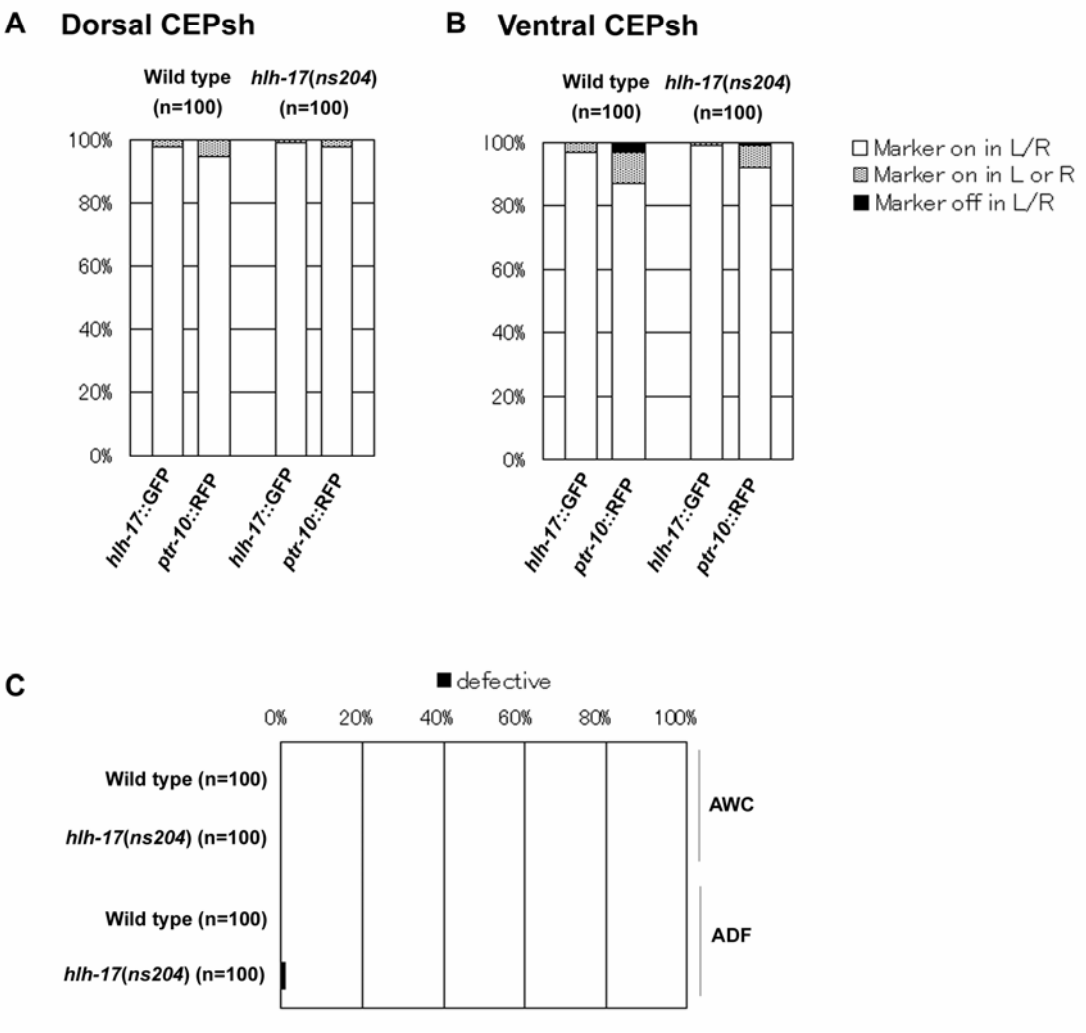

A Dorsal CEPsh
Wild type (n=100)
hlh-17(ns204) (n=100)
100%
80%
60%
40%
20%
0%
hlh-17::GFP
ptr-10::RFP
hlh-17::GFP
ptr-10::RFP
B Ventral CEPsh
Wild type (n=100)
hlh-17(ns204) (n=100)
100%
80%
60%
40%
20%
0%
hlh-17::GFP
ptr-10::RFP
hlh-17::GFP
ptr-10::RFP
Marker on in L/R
Marker on in L or R
Marker off in L/R
C
defective
0%
20%
40%
60%
80%
100%
Wild type (n=100)
hlh-17(ns204) (n=100)
Wild type (n=100)
hlh-17(ns204) (n=100)
AWC
ADF

Figure 2.12. *hlh-17*(*ns204*) Mutants Do Not Show Defects in CEPsh Development

(**A**) Histogram assessing *hlh-17*::GFP and *ptr-10*::myrRFP reporter transgene expression in dorsal CEPsh glia of animals of the indicated genotype. Vertical axis, % animals. White bars represent animals expressing reporters in both dorsal left and dorsal right CEPsh glia. Gray bars represent animals lacking expression of reporters in either dorsal left or dorsal right CEPsh glia. Black bars represent animals lacking expression in both dorsal left and dorsal right CEPsh glia. n, number of animals examined.

(**B**) Same as (**A**), except that expression in ventral CEPsh glia is depicted.

(**C**) Axon guidance defects of AWC and ADF neurons in *hlh-17*(*ns204*) mutants. n, number of animals examined.

Figure 2.13

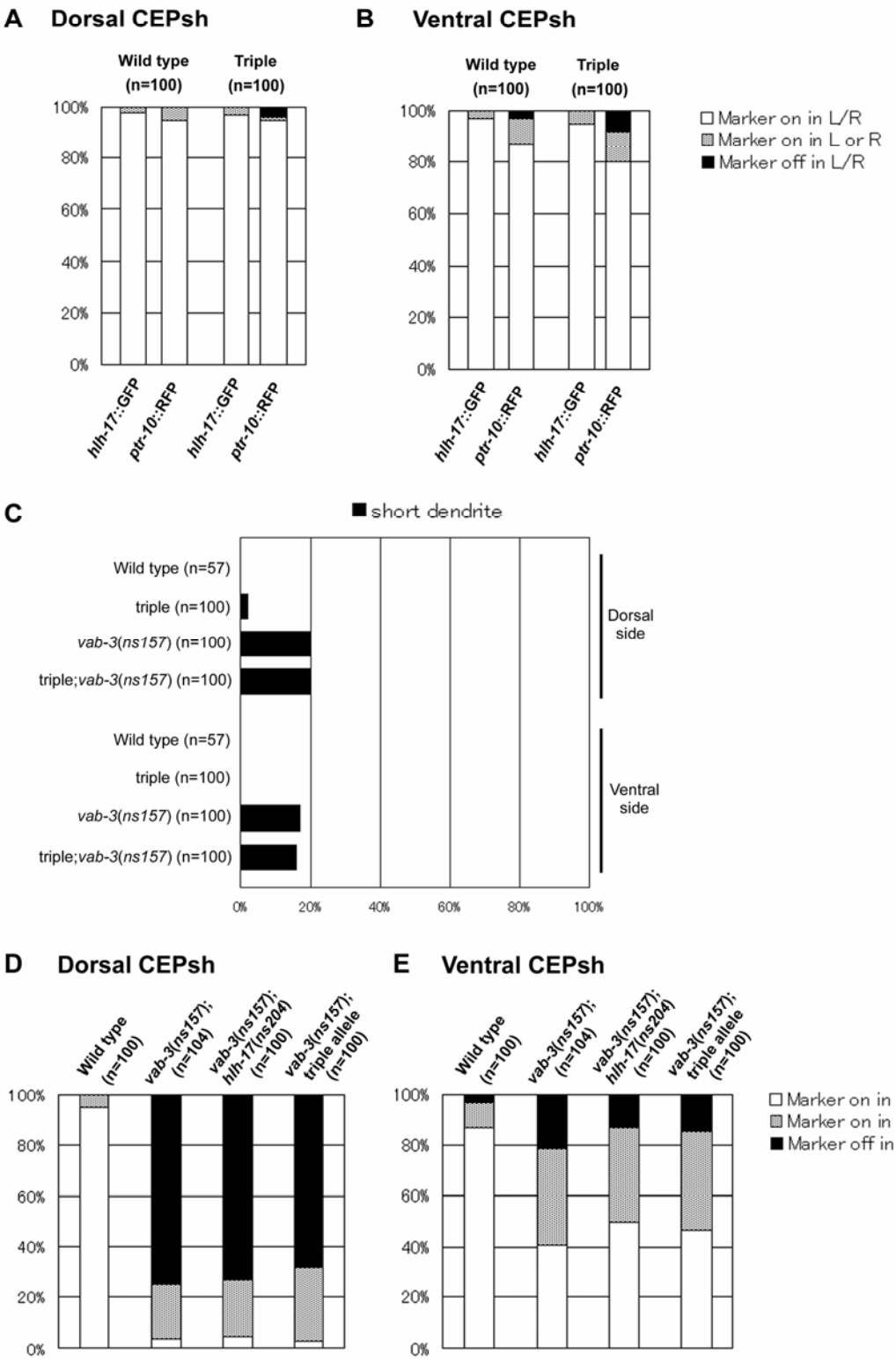

A Dorsal CEPsh
Wild type (n=100)
Triple (n=100)
100%
80%
60%
40%
20%
0%
hlh-17::GFP
ptr-10::RFP
hlh-17::GFP
ptr-10::RFP
B Ventral CEPsh
Wild type (n=100)
Triple (n=100)
Marker on in L/R
Marker on in L or R
Marker off in L/R
C
short dendrite
Wild type (n=57)
triple (n=100)
vab-3(ns157) (n=100)
triple;vab-3(ns157) (n=100)
Dorsal side
Wild type (n=57)
triple (n=100)
vab-3(ns157) (n=100)
triple;vab-3(ns157) (n=100)
Ventral side
0%
20%
40%
60%
80%
100%
D Dorsal CEPsh
Wild type (n=100)
vab-3(ns157); (n=104)
vab-3(ns157); hlh-17(ns204) (n=100)
vab-3(ns157); triple allele (n=100)
100%
80%
60%
40%
20%
0%
E Ventral CEPsh
Wild type (n=100)
vab-3(ns157); (n=104)
vab-3(ns157); hlh-17(ns204) (n=100)
vab-3(ns157); triple allele (n=100)
Marker on in L/R
Marker on in L or R
Marker off in L/R

Figure 2.13. A Triple Mutant of *hlh-17*, *hlh-31*, and *hlh-32* Has No Defects in Reporter Expression or CEP Dendrite Extension

(**A**) Histogram assessing *hlh-17*::GFP and *ptr-10*::myrRFP reporter transgene expression in dorsal CEPsh glia of animals of the indicated genotype. Vertical axis, % animals. White bars represent animals expressing reporters in both dorsal left and dorsal right CEPsh glia. Gray bars represent animals lacking expression of reporters in either dorsal left or dorsal right CEPsh glia. Black bars represent animals lacking expression in both dorsal left and dorsal right CEPsh glia. n, number of animals examined.

(**B**) Same as (**A**), except that expression in ventral CEPsh glia is depicted.

(**C**) Histogram depicting dendrite extension defects of strains of the indicated genotype.

(**D**, **E**) *ptr-10*::myrRFP reporter transgene expression in dorsal (**D**) or ventral (**E**) CEPsh glia, respectively, in the indicated mutant backgrounds.

2.6 Relationships Between *mls-2*, *vab-3*, and *hlh-17*

- ***vab-3*, but Not *mls-2*, is Capable of Inducing *hlh-17* Expression**

Exploration of the relationships between *vab-3*, *mls-2*, and *hlh-17* is essential for detailed understanding of the development of CEPsh glia. As a first step, we sought to examine whether *vab-3* was sufficient to regulate *hlh-17* expression. We ectopically expressed a transgene, consisting of the *vab-3* isoform A cDNA fused to a heat-inducible promoter, in 270-430 min embryos, by subjecting them to a 30 min heat pulse at 34˚C. Strikingly, GFP expression was induced throughout these embryos within 60 minutes of heat exposure (Figure 2.14). The induction was independent of *mls-2*, since *vab-3*-induced expression was still evident in *mls-2*(*ns156*) mutants (n>50, 2 lines observed). Thus, this result suggests that *vab-3* alone is able to induce *hlh-17* expression. Furthermore, the rapid appearance of GFP suggests that *vab-3* may directly regulate *hlh-17* expression, consistent, therefore, with the notion that *vab-3* normally regulates *hlh-17* expression within CEPsh glia. By deleting regions of the 1.9 kb *hlh-17* promoter region used to monitor GFP induction, we narrowed down the *vab-3*-responsive element to a region of 500 bp, immediately upstream of the *hlh-17* translation start site.

In chick embryos, overexpression of Nkx6.1 is sufficient to promote Olig2

Figure 2.14

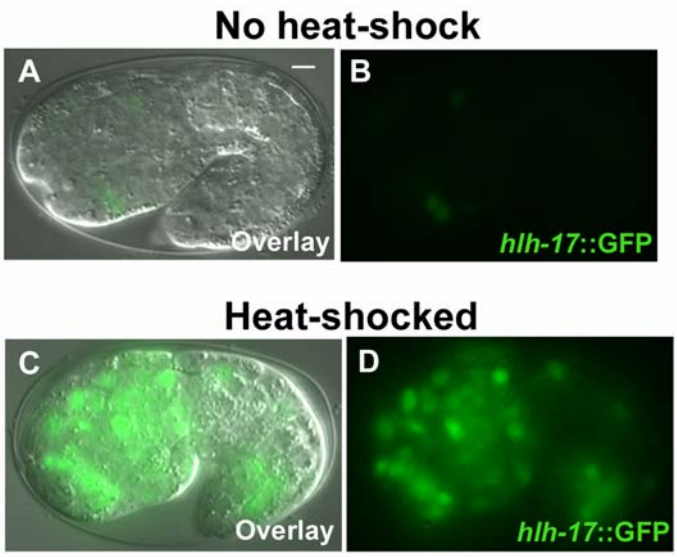


Figure 2.14. *hlh-17* is Induced by Overexpression of *vab-3*

(**A**, **B**) Induction of *hlh-17*::GFP reporter transgene expression by overexpression of *vab-3* isoform A cDNA using a heat-inducible promoter. Merged DIC and fluorescence image, and fluorescence image of an embryo not subjected to a heat-shock (**A** and **B**, respectively).

(**C**, **D**) Same as (**A**, **B**), respectively, with same exposure times, except that images were taken 90 min after a heat shock was administered. Note robust *hlh-17*::GFP expression.

expression at early developmental stages (Liu et al., 2003). To determine whether *mls-2* is sufficient to promote the expression of *hlh-17* and *ptr-10* in CEPsh glia, we performed another heat-shock experiment. We introduced a transgene containing the full-length *mls-2* cDNA, under the control of a *C. elegans* heat-shock promoter, into animals carrying either *hlh-17*::GFP or *ptr-10*::myrRFP reporters. We did not detect ectopic expression of the CEPsh reporters following heat-shock treatment of embryos at 34˚C for 30 minutes, suggesting that while *mls-2* is clearly required for *hlh-17* and *ptr-10* expression, it is likely to function together with other proteins to express these genes within CEPsh glia.

- ***vab-3* May Regulate Expression of *mls-2***

To gain some insights into the relationship between *vab-3* and *mls-2*, we introduced the heat-inducible promoter::*vab-3* cDNA transgene into animals containing the *mls-2* promoter:: gfp::*mls-2*::*mls-2* 3' end transgene. However, heat shock induction of VAB-3 failed to promote ectopic GFP expression (2 lines examined, 50 animals per line). We then examined the expression of *mls-2*::GFP reporters in *vab-3*(*ns157*) mutants. Surprisingly, we found that expression of *mls-2* in anterior cells of the embryo, including CEPsh glia and their precursors, around the 300 minute stage was greatly reduced or absent, however, expression at

around 450 minutes seemed normal (n>100). These results raised the possibility that, although *mls-2* is not required for *vab-3* function, *vab-3* might be regulating *mls-2* expression and thus functioning through *mls-2*.

We were unable to examine *vab-3* expression in *mls-2* mutants, because, as described above, the available reagents for *vab-3* did not allow us to unambiguously identify the CEPsh glia or their precursors. We note, however, that we did not detect any gross alterations in *vab-3* expression in *mls-2*(*ns156*) mutants. As shown in Figure 2.10C and 2.10D, we were able to detect VAB-3 protein with the anti-VAB-3 antibody in *mls-2* mutant embryos. This is in line with the idea that *vab-3* is acting upstream of *mls-2*.

- ***hlh-17* Cooperates with *vab-3* to Regulate Its Own Expression**

While *hlh-17*::GFP expression in ventral CEPsh glia was normal in *hlh-17*(*ns204*) mutants, we noticed that GFP expression levels were significantly reduced in *hlh-17*(*ns204*); *vab-3*(*ns157*) double mutants (Table 2.1). We observed a similar result using a different *hlh-17* allele we recently obtained (*tm2850*; Table 2.1). *ptr-10*::myrRFP expression levels were not affected in the double mutant backgrounds (Figure 2.13D and 2.13E). These results suggest that *hlh-17* may function together with *vab-3* to regulate its own expression.

Table 2.1. *hlh-17* and *vab-3* Together Regulate *hlh-17*::GFP Expression

Genotype[a]	Compared to[b]	Intensity of *hlh-17*::GFP			
		higher	same	lower	n
wild type[c]	wild type	12	20	9	41
hlh-17(*ns204*)	wild type	9	28	4	41
hlh-17(*tm2850*)	wild type	9	27	4	40
hlh-32(*ns223*) *hlh-17*(*ns204*) *hlh-31*(*ns217*)	wild type	10	27	4	41
vab-3(*ns157*)	wild type	4	28	13	45
vab-3(*ns157*)[d]	*vab-3*(*ns157*)	7	25	9	41
hlh-17(*ns204*); *vab-3*(*ns157*)	*vab-3*(*ns157*)	0	10	34	44
hlh-17(*tm2850*); *vab-3*(*ns157*)	*vab-3*(*ns157*)	0	7	34	41
hlh-32(*ns223*) *hlh-17*(*ns204*) *hlh-31*(*ns217*); *vab-3*(*ns157*)	*vab-3*(*ns157*)	0	4	36	40

[a]All animals also contained a genomically integrated *hlh-17*::GFP reporter transgene.
[b]Single animals of the indicated genotype were compared with either single wild-type or single *vab-3*(*ns157*) animals containing the same integrated *hlh-17*::GFP reporter transgene and relative fluorescence intensity levels compared.
[c]This comparison measures the variability of GFP expression within the *hlh-17*::GFP transgenic line in otherwise wild-type animals.
[d]This comparison measures the variability of GFP expression within the *hlh-17*::GFP transgenic line in *vab-3*(*ns157*) animals.

2.7 Discussion

- **Conserved Features of a Transcriptional Program Promoting Ensheathing Glia Development**

The development of the vertebrate spinal cord has been extensively studied, and a host of transcriptional regulators promoting neuronal and glial cell fates in this structure have been described (Jessell, 2000; Nicolay et al., 2007; Rowitch, 2004). Many of these regulators function in response to a gradient of the Sonic Hedgehog (Shh) signaling molecule. For example, five proteins, *Pax6*, *Pax7*, *Irx3*, *Dbx1*, and *Dbx2*, are repressed by Shh signaling, and two proteins, *Nkx6.1* and *Nkx2.2*, are induced by Shh, to subdivide the ventral aspect of the neural tube into five, molecularly distinct, domains (Briscoe and Ericson, 2001). Recent work has begun to reveal molecular programs underlying patterning and specification of neuronal cell types in the dorsal cord as well, although less is known here (Caspary and Anderson, 2003; Lee and Jessell, 1999).

Studies of the developing neural tube demonstrated that early generation of ensheathing glia of the spinal cord, the oligodendrocytes, occurs in the pMN area of the ventral neural tube (Richardson et al., 2000). Oligodendrocytes express the Olig2 transcriptional regulator (Lu et al., 2000; Zhou et al., 2000), and both Olig2 expression and oligodendrocyte formation are regulated by the Shh-dependent

expression of the Pax6 and Nkx6 transcription factors (Liu et al., 2003; Mizuguchi et al., 2001; Novitch et al., 2001; Vallstedt et al., 2001). Recent work has demonstrated that oligodendrocyte production is not constrained to the ventral cord, and can occur at later stages of development in dorsal regions as well (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). Although the regulatory events promoting oligodendrocyte formation and Olig2 expression in dorsal areas are not well known, the oligodendrocytes that are generated do not require Nkx6, and express Pax7. Thus, ensheathing glia in the neural tube arise from at least two molecularly and spatially distinct domains.

The studies we describe here demonstrate an intriguing similarity between the development of the CEPsh glia of *C. elegans*, and oligodendrocyte development in the vertebrate spinal cord (Figure 2.15). First, although myelin is not present in *C. elegans* (Ward et al., 1975; White et al., 1986), CEPsh glia, like oligodendrocytes, ensheath neuronal processes: both dendritic tips of the CEP sensory neurons, and axons of many neurons that make up the animal's central nervous system equivalent, the nerve ring.

Second, as we demonstrate here and as has been previously described (McMiller and Johnson, 2005), CEPsh glia express the HLH-17 transcription factor, which is the *C. elegans* protein most highly-related to Olig2.

Third, although all four CEPsh cells express HLH-17 and ensheath neurons, the cell lineages giving rise to dorsal and ventral CEPsh glia are distinct

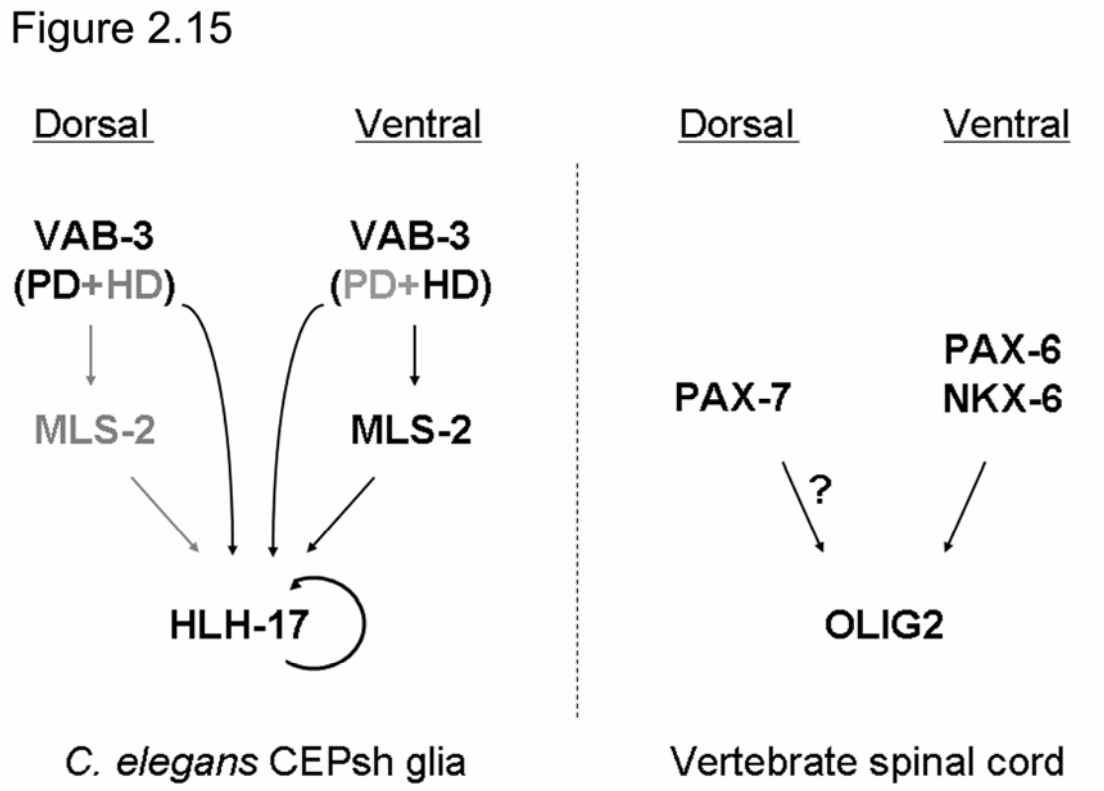


Figure 2.15. Models for Transcriptional Control of Ensheathing Glia Formation

In ventral *C. elegans* CEPsh glia and in ventral verterbrate spinal cords, Nkx-family and Pax6-related proteins regulate *Olig*-related gene expression. In dorsal *C. elegans* CEPsh glia and in the dorsal vertebrate spinal cords, a Pax7-related protein may promote Olig expression. Gray shading of VAB-3 PD and HD reflects increased VAB-3 PD requirement in dorsal CEPsh glia for *hlh-17* expression. The homeodomain of VAB-3 is preferentially required in ventral glia for expression of *ptr-10*, suggesting a possible similar preference for *hlh-17*. HLH-17, together with VAB-3, regulates its own expression.

and unrelated, suggesting that dorsal and ventral CEPsh glia may arise by different mechanisms. Indeed, studies of the UNC-6/Netrin protein revealed that it is asymmetrically expressed only in ventral CEPsh cells, supporting a distinct molecular origin for these cells.

Fourth, here we have shown that expression of HLH-17 is differentially regulated in dorsal and ventral CEPsh glia, reminiscent of Olig2 expression in the vertebrate spinal cord. Expression of HLH-17/Olig in ventral CEPsh glia requires the MLS-2 transcription factor, as well as the homeodomain of the VAB-3 transcriptional regulator, whereas HLH-17/Olig expression in dorsal CEPsh cells requires mainly VAB-3, and, specifically, the Paired domain of this protein.

MLS-2 is similar to Nkx superfamily proteins and is distantly related to Nkx6. VAB-3 is the *C. elegans* protein most similar in sequence and domain structure to Pax6 and Pax7, although VAB-3 lacks an octapeptide sequence present in Pax7 (Jostes et al., 1990). Thus, in both *C. elegans* and vertebrates, glia that ensheath central nervous system neurons express an Olig bHLH transcription factor, and expression of this protein is regulated differently in dorsal and ventral regions. Specifically, ventral regions require Nkx-related, and Pax6-related transcription factors for Olig protein expression, whereas dorsal expression of Olig proteins may require a Pax7 protein.

Interestingly, in Pax6 mutant mice, oligodendrocyte precursor cell generation is delayed (Sun et al., 1998), suggesting that Pax6 may control Nkx6.

We observed a similar relationship between VAB-3 and MLS-2, demonstrating that VAB-3 acts at early time points to control MLS-2 expression.

Are Nkx-Olig and Pax-Olig programs fundamental to all metazoans? Studies of glia in *Drosophila melanogaster* suggest that development of these cells is mainly controlled by the *glial cells missing* (*gcm*) gene, encoding a Zn-finger transcription factor. Thus, it is possible that glia development in *Drosophila* is fundamentally different from glia development in *C. elegans* and vertebrates. However, *Drosophila* possesses an *Olig*-related gene, *Oli*, that is expressed extensively in the animal's brain (Brody et al., 2002). While the details of the expression pattern are not known, it is possible that *Oli* is expressed in glia. Furthermore, at least some *C. elegans* glia express the Zn-finger transcription factor LIN-26, which is required for terminal differentiation of these cells (Labouesse et al., 1996); and, as in *Drosophila gcm* mutants, some glia in *lin-26* mutants adopt neuronal fates. Finally, the vertebrate Zn-finger transcription factor *Zfp488* can cooperate with *Olig2* to control oligodendrocyte differentiation (Wang et al., 2006). Thus, it is possible that the mechanisms regulating glial differentiation and function are indeed conserved among all multicellular animals.

The molecular similarities in glial development between *C. elegans* CEPsh glia and vertebrate glia suggest that other components of these developmental programs may be conserved as well. These components could be identified using the powerful genetic tools available in *C. elegans* (see also Chapter 4).

- **Notable Differences Between the Development of *C. elegans* and Vertebrate Glia**

Although our results hint at possible similarities between CEPsh glia development in *C. elegans* and the development of vertebrate oligodendrocytes, they revealed clear differences as well. Importantly, mice carrying mutations in *Olig2* have significantly fewer oligodendrocytes than are present in wild-type animals (Zhou and Anderson, 2002), suggesting primary developmental roles for this gene. However, *hlh-17* mutations in *C. elegans* do not appear to grossly perturb the generation or differentiation of CEPsh glia. One explanation for this may be redundancy. In vertebrates, *Olig2* and *Olig1* function together to control the number of oligodendrocytes (Lu et al., 2002; Zhou and Anderson, 2002). Indeed, we identified two genes encoding proteins highly related to HLH-17. Unfortunately however, mutants carrying deletions in all three genes did not exhibit any obvious defects in CEPsh glia, suggesting that HLH-17 may be redundant with other factors.

What could be the function of HLH-17 and its related proteins? It is possible to consider that they primarily regulate post-developmental aspects of CEPsh function. The persistence of *hlh-17* gene expression in CEPsh glia through larval development and in adult animals is consistent with this possibility. It is of note that both *Olig2* and *Olig1* continue to be expressed in maturing oligodendrocyte

lineage cells (Ligon et al., 2004; Lu et al., 2000), and perhaps even in multipotent progenitor cells of the adult brain (Aguirre et al., 2004; Hack et al., 2004). They are thought to have post-developmental functions, and may be involved in nervous system repair following injury (Ligon et al., 2006). It is tempting to speculate that HLH-17 and the related *C. elegans* proteins may have such roles as well.

- **Heterogeneity Among *C. elegans* Glia**

It is becoming evident that glial cells within a same subtype can be phenotypically diverse. Astrocytes, for instance, express different type of receptors and transporters depending on the brain regions (Matthias et al., 2003). In the spinal cord, they can be classified into three distinct subtypes by the combinatorial expression of certain genes (Hochstim et al., 2008). Moreover, the phenotypic heterogeneity of glia is generated, at least in part, by incorporating diverse developmental pathways for each subtype. Indeed, the specification of the three astroglial subtypes in the spinal cord requires three independent transcriptional programs (Hochstim et al., 2008). Similarly, although the phenotypic diversity of oligodendrocytes is less studied, their Nkx-Olig and Pax-Olig developmental programs established in the spinal cord may not be

universal. For example, the generation of ventral oligodendrocytes in the spinal cord depends on Nkx6, while this homeodomain protein in the anterior hindbrain instead suppresses oligodendrocyte specification (Vallstedt et al., 2005). Even within the spinal cord, the Pax-Olig program may not be functioning in the caudal region (Novitch et al., 2001). These observations together suggest that several distinct genetic programs are required to specify a subtype of glial cells.

In this study, we have provided evidence for the heterogeneity of *C. elegans* glia by demonstrating that the development of dorsal and ventral CEPsh glia are differentially regulated. Moreover, we also noticed that glial defects in *mls-2* mutant animals are restricted to the four sheath cells of CEP sensilla, and glial cells of other head sensilla remained apparently intact. Thus other *C. elegans* glia are developmentally distinguishable from CEPsh glia, and require distinct regulatory factors for their differentiation. One of the candidates for such regulatory factors is *lin-26*, which has been indicated to specify the fates of non-neuronal ectodermal cells including some sheath and socket cells (Labouesse et al., 1996). It is tempting to further study the genetic programs underlying the differentiation of other 46 *C. elegans* glia, which may provide additional insights into the mechanism of metazoan gliogenesis (see also Chapter 4).

Chapter 3

Functional Analysis of CEPsh Glia

CEPsh Glia-Dependent Neurite Extension and Guidance

Summary

In the previous chapter, we described results suggesting that *mls-2* and *vab-3* play important roles early in the development of the CEPsh glia. Unfortunately, roles of *hlh-17* remained unclear, but at least the gene provided very nice reporter for CEPsh glia. Because mutations in *mls-2* and *vab-3* affect early aspects of glial differentiation, we reasoned that by examining *mls-2* and *vab-3* mutants for neuronal defects, we may be able to uncover previously uncharacterized roles for CEPsh glia in neuronal development. In this chapter, we will first describe dendrite-extension defects we observed in *mls-2* and *vab-3* mutants. Involvement of CEPsh glia in these defects was confirmed by targeted cell ablation experiments. We also demonstrate that CEPsh glia function in controlling axon branching and guidance in the nerve ring. Similar steps were taken to describe their role in this process, taking advantage of *mls-2* and *vab-3* mutants as well as the cell ablation technique. Furthermore, we provide evidence that at least some of the axon guidance functions are mediated by the UNC-6/Netrin protein. These observations indicate that there are intensive glia-neuron interactions *in vivo* during *C. elegans* nervous system development.

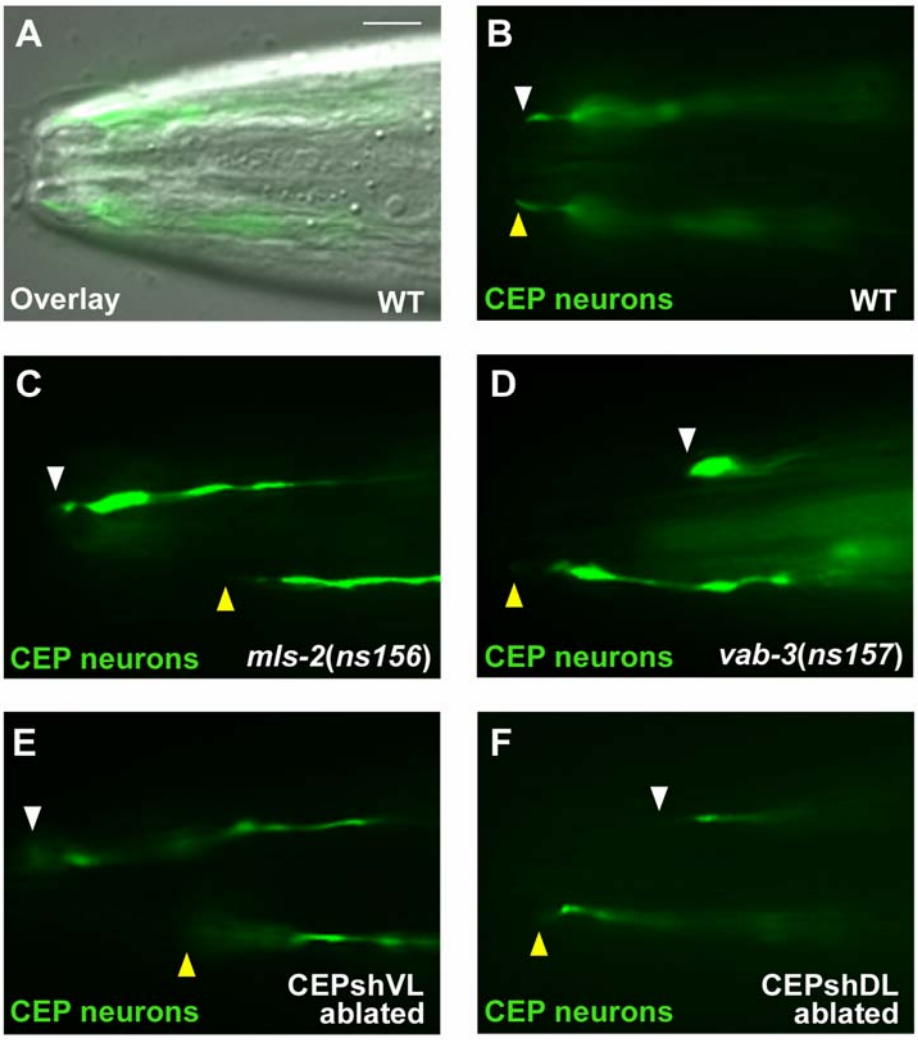
3.1 Role of CEPsh Glia in Dendrite Extension

- ***mls-2* and *vab-3* Mutants Exhibit Defects in CEP Neuron Dendrite Extension**

The CEPsh glia send processes towards the tip of the *C. elegans* nose, and are tightly associated with the CEP sensory neurons, ensheathing them at their ciliated dendritic endings (Figure 1.2A). As shown in Figure 3.1, dendrite extension was defective in both *mls-2*(*ns156*) and *vab-3*(*ns157*) animals. Specifically, whereas dendrites of CEP neurons normally extend to the tip of the nose, as can be seen in animals expressing the *dat-1*::GFP reporter transgene, which specifically labels the CEP neurons (Nass et al., 2005), dendrites in *mls-2* and *vab-3* mutants were shorter, and failed to reach the nose tip (Figures 3.1A-D). Mutant dendrites still possessed cilia at their tips (e.g. Figure 3.1C), suggesting that the defects observed in these mutants were specific to dendrite length.

Consistent with the defects in expressing the *hlh-17* and *ptr-10* reporters, CEP neuron dendritic defects in *mls-2*(*ns156*) mutants were mostly restricted to ventrally situated dendrites: 95% of ventral CEP dendrites were inappropriately short, whereas only 21% of dorsal dendrites were short. In contrast, despite their dorsally biased defects in CEPsh marker expressions, *vab-3*(*ns157*) animals exhibited relatively weak defects in both sides: only 20% and 17% of dendrites of

Figure 3.1


A
Overlay
WT
B
CEP neurons
WT
C
CEP neurons
mls-2(ns156)
D
CEP neurons
vab-3(ns157)
E
CEP neurons
CEPshVL
ablated
F
CEP neurons
CEPshDL
ablated

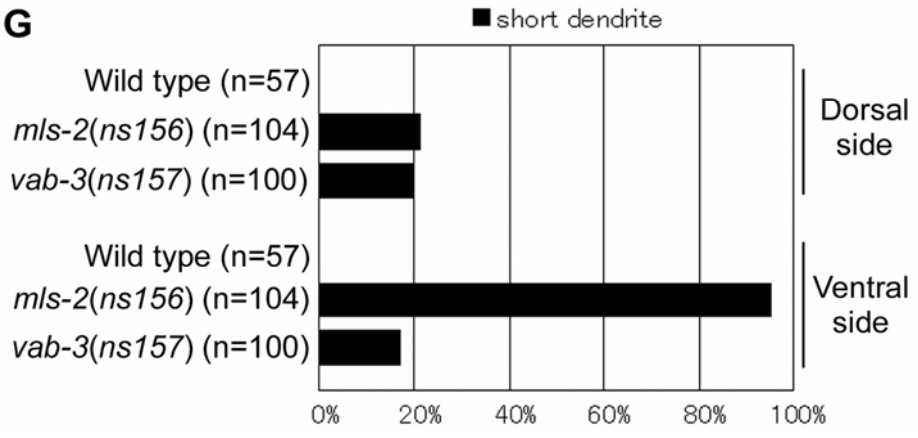

G
short dendrite
Wild type (n=57)
mls-2(ns156) (n=104)
vab-3(ns157) (n=100)
Wild type (n=57)
mls-2(ns156) (n=104)
vab-3(ns157) (n=100)
Dorsal side
Ventral side
0%
20%
40%
60%
80%
100%

Figure 3.1. CEPsh Glia are Required for CEP Neuron Dendrite Extension

(**A**) Merged DIC and fluorescence image, and (**B**) fluorescence image of a wild-type adult expressing *dat-1*::GFP in CEP neurons. The dorsal left and ventral left dendrite tips are indicated by white and yellow arrowheads, respectively. (**C**, **D**) Fluorescence images of *mls-2*(*ns156*) and *vab-3*(*ns157*) adults, respectively, expressing *dat-1*::GFP in CEP neurons and showing defects in the extension of ventral and dorsal dendrites, respectively.

(**E**, **F**) Fluorescence images of *dat-1*::GFP-expressing adult animals lacking either ventral left (**E**) or dorsal left (**F**) CEPsh glia. Note that extension of ventral and dorsal dendrites is defective, respectively.

(**G**) Histogram depicting dendrite extension defects of strains of the indicated genotype.

dorsal and ventral CEP neurons were short, respectively (Figure3.1G). These results suggest that *vab-3*(*ns157*) mutants may have a weaker defect in CEPsh glia development than do *mls-2*(*ns156*) mutants, consistent with our EM studies,

- **CEPsh Glia are Responsible for the Extension of CEP Neuron Dendrite**

To test whether CEPsh glia are playing any role in the process of CEP dendrite extension, we used a laser microbeam to ablate the direct precursors of CEPsh glia in animals expressing the *dat-1*::GFP reporter transgene, and examined these glia-less animals for defects in CEP dendrite length. Four of four animals in which the direct precursor of the ventral left CEPsh cell, ABplpaaapap, was ablated, had a shortened ventral left CEP neuron dendrite. Similarly, four of four animals in which the direct precursor of the dorsal left CEPsh cell, ABarpaaaapp, was ablated, displayed a short dorsal left CEP neuron dendrite (Figures 3.1E and 3.1F). These observations strongly suggest that CEPsh glia play key roles in regulating CEP dendrite length in *C. elegans*. Thus our observation that *vab-3*(*ns157*) only weakly perturbs dendrite length determination may be suggesting that *vab-3*(*ns157*) mutants have a weaker defect in CEPsh glia development than do *mls-2*(*ns156*) mutants and the mutation is unlikely to eliminate all CEPsh glia functions. Furthermore, these observations support

cell-autonomous roles for *mls-2* and *vab-3* in regulating dendrite length, consistent with our studies of these genes in the context of *hlh-17* and *ptr-10* reporter transgene expression.

3.2 Role of CEPsh Glia in Axon Guidance and Branching

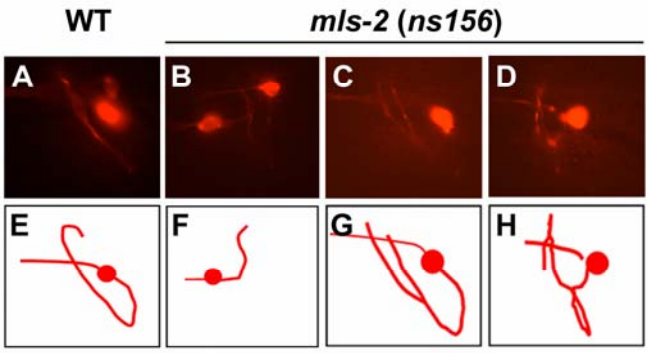
- ***mls-2* and *vab-3* Mutants Exhibit Defects in Axon Guidance and Branching**

It has been previously speculated that the CEPsh glia play a role in directing axon growth within the nerve ring (Wadsworth et al., 1996), since these glial cells ensheath the outer surface of the nerve ring, and extend fine processes within it. However, to date, no direct evidence to support this assertion has been garnered. To assess possible roles for CEPsh glia in axon development and guidance, we examined the processes of three different neurons of the amphid sensilla, AWC, AFD, and ADF, using the cell-specific reporters *odr-1*::RFP (L'Etoile and Bargmann, 2000), *ttx-1*::DsRed (Satterlee et al., 2001), and T08G3.3::RFP (Sagasti et al., 1999), respectively. The axons of all three neurons enter the nerve ring through the ventrally directed amphid commissure. While axon shapes and lengths are highly regular in wild-type animals, we observed clear defects in these

processes in *mls-2* and *vab-3* mutants. Defects ranged from lack of ventrally directed processes to abnormal branching. Major classes of defects are depicted in Figures 3.2A-H. Although defects were observed in the axons of all three neurons we examined, these were clearly more severe in AFD and AWC than in ADF. For example, in *mls-2*(*ns156*) mutants, defects were observed in 99%, 80%, and 39% of AFD, AWC, and ADF neurons examined, respectively. In *vab-3*(*ns157*) mutants, defects were also observed, but these tended to be less severe than in *mls-2* mutants (Figure 3.2I; also see Zallen et al., 1999). Nonetheless, a similar bias towards defects in AFD and AWC was observed. These results suggest that axon guidance and development in the nerve ring is differentially regulated with respect to individual neurons.

To gain insights into the role of CEPsh glia in the process of axon growth, we took advantage of the observation that *vab-3*(*ns157*) mutants frequently show defects in *hlh-17*::GFP expression in ventral CEPsh glia (Figure 2.4). Interestingly, as shown in Figure 3.2I, for all three neurons we examined, defects were more severe if *hlh-17*::GFP expression was also disrupted in ventral CEPsh glia. For example, whereas only 22% of *vab-3*(*ns157*) mutants carrying the *hlh-17*::GFP reporter exhibited AFD axon defects when ventral CEPsh glia expressed *hlh-17*::GFP, 54% of AFD neurons were defective when ventral CEPsh glia failed to express *hlh-17*::GFP. These results are consistent with the idea that normal CEPsh cell development is required for axon development.

Figure 3.2


WT
mls-2 (ns156)
A
B
C
D
E
F
G
H

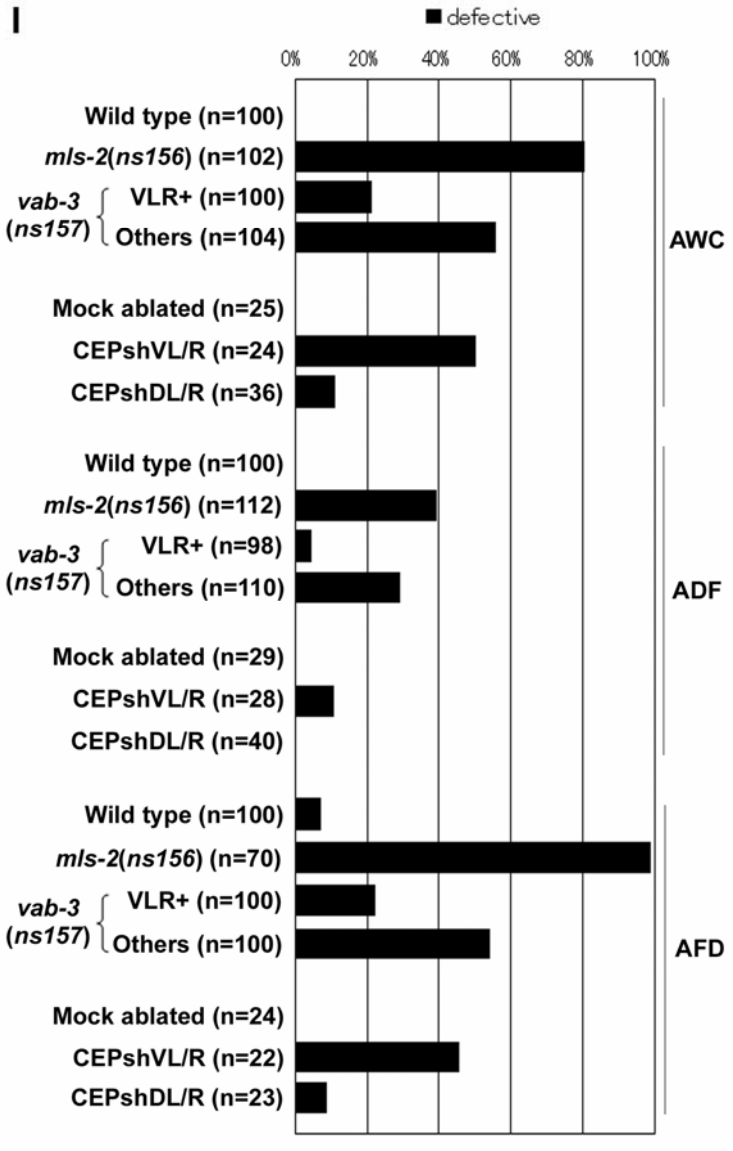

I
defective
0%
20%
40%
60%
80%
100%
Wild type (n=100)
mls-2(ns156) (n=102)
vab-3 (ns157)
VLR+ (n=100)
Others (n=104)
AWC
Mock ablated (n=25)
CEPshVL/R (n=24)
CEPshDL/R (n=36)
Wild type (n=100)
mls-2(ns156) (n=112)
vab-3 (ns157)
VLR+ (n=98)
Others (n=110)
ADF
Mock ablated (n=29)
CEPshVL/R (n=28)
CEPshDL/R (n=40)
Wild type (n=100)
mls-2(ns156) (n=70)
vab-3 (ns157)
VLR+ (n=100)
Others (n=100)
AFD
Mock ablated (n=24)
CEPshVL/R (n=22)
CEPshDL/R (n=23)

Figure 3.2. *mls-2* and *vab-3* Mutants Exhibit Axon Guidance Defects in the Nerve Ring

(**A-H**) AWC axon defects observed in *mls-2*(*ns156*) adult animals. Fluorescence images (**A-D**) and corresponding schematic drawings (**E-H**) of AWC neurons expressing an *odr-1*::RFP reporter transgene. (**A**, **E**) Wild-type morphology of AWC axon. (**B-D**, **F-H**) AWC position and axonal defects frequently observed in *mls-2*(*ns156*) adults. In panels (**A**) and (**B**), reporter expression in the AWB neuron is also seen. Similar types of defects are seen in *vab-3*(*ns157*) mutants and in AFD neurons of both *mls-2*(*ns156*) and *vab-3*(*ns157*) animals.

(**I**) Histogram depicting AWC, ADF, and AFD axonal defects of strains of the indicated genotypes and of animals in which the indicated CEPsh glia have been ablated. *vab-3*(*ns157*) animals were categorized as VL/R+ animals, expressing *hlh-17*::GFP in both ventral left and right CEPsh glia, or Others, animals lacking expression of *hlh-17*::GFP in one or both ventral CEPsh glia. Black bars, defective axons. n, number of animals observed. In *mls-2*(*ns156*) mutants, some animals fail to express AWC reporter transgenes. We only scored animals in which robust reporter transgene expression was evident.

- ***hlh-17* May Function Redundantly with *hlh-17*-Related Genes and *vab-3***

Since *vab-3* functions together with *hlh-17* to control *hlh-17* transcription, we wondered whether *hlh-17*(*ns204*); *vab-3*(*ns157*) double mutants might display enhanced axon guidance defects. However, these animals displayed the same extent of axon defects as the *vab-3*(*ns157*) single mutant alone. Interestingly, however, we did find an enhancement in defects in *hlh-32*(*ns223*) *hlh-17*(*ns204*) *hlh-31*(*ns217*); *vab-3*(*ns157*) mutants. For example, whereas only 21 of 100 (21%) *vab-3*(*ns157*) mutants, in which the ventral CEPsh expressed *hlh-17*::GFP, had AWC axonal defects, 22 of 39 (56%) *hlh-32*(*ns223*) *hlh-17*(*ns204*) *hlh-31*(*ns217*); *vab-3*(*ns157*) mutants expressing *hlh-17*::GFP in ventral CEPsh glia had AWC axonal defects. These results support the possibility that *hlh-17* may function redundantly with *hlh-17*-related genes and *vab-3* to regulate ventral CEPsh functions. However, since we could not detect expression of the *hlh-17*-related genes in CEPsh glia, it is also possible that they may control axon guidance through a different mechanism. Consistent with this possibility, we could not find an enhancement in the dendritic defects in *hlh-32*(*ns223*) *hlh-17*(*ns204*) *hlh-31*(*ns217*); *vab-3*(*ns157*) mutants (Figure 2.13C).

- **Ventral CEPsh Glia Play Essential Role in Axon Guidance and Branching in the Nerve Ring**

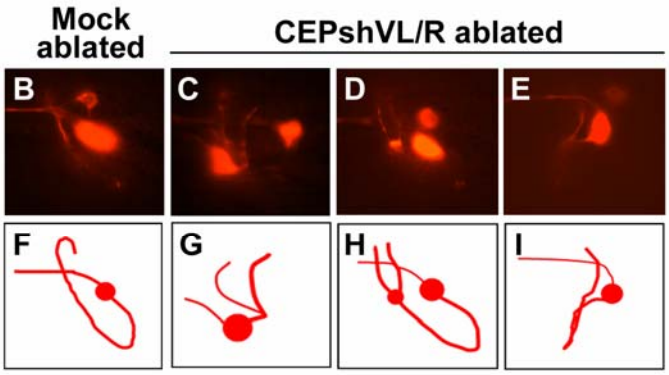
The observations of defects in mutant animals suggest that CEPsh glia, specifically ventral CEPsh glia, affect axon guidance and development in the nerve ring. To confirm that the axon defects we observed were a consequence, at least in part, of defects in CEPsh glia, we took two approaches.

First, we performed a mosaic analysis using the *mls-2* gene. We generated *mls-2* mutants carrying integrated *hlh-17*::GFP and *ttx-1*::DsRed reporter transgenes to label the CEPsh and AFD neurons, respectively. Into these mutants, we then introduced the *mls-2* rescuing cosmid, C39E6, and another AFD-specific reporter, *nhr-38*::GFP, both carried on an unstable extrachromosomal array. The presence of the *mls-2* cosmid array in ventral CEPsh glia was scored by the appearance of ventral *hlh-17*::GFP expression, and the presence of this array in the AFD neurons was scored by expression of *nhr-38*::GFP. As shown in Figure 3.3A, when the array was absent from both CEPsh glia and AFD, 30/30 animals displayed axon defects. However, when the array was present in both AFD and CEPsh glia, 32/39 animals had no axonal defects. The *ttx-1*::DsRed reporter we used had a weak effect on AFD axon guidance on its own (about 10% of AFD axons were abnormal in wild-type animals carrying the *ttx-1*::DsRed integrated reporter transgene). These results support the notion that the axonal defects

Figure 3.3

A

Cell type	Transgene	Axonal defect +	Axonal defect –	n
CEPshVL/R AFD	– –	30	0	30
CEPshVL/R AFD	+ +	7	32	39
CEPshVL/R AFD	+ –	12	18	30
CEPshVL/R AFD	– +	23	7	30



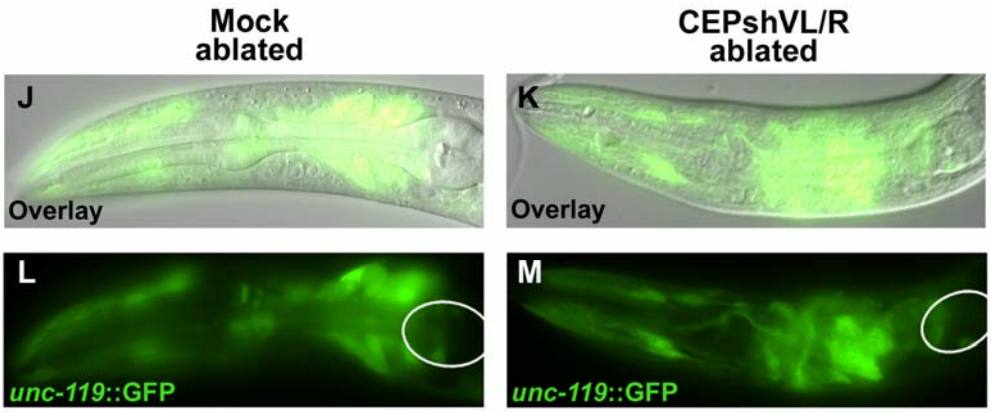


Figure 3.3. CEPsh Glia are Important for Axon Guidance in the Nerve Ring

(**A**) Table summarizing the results of mosaic studies of *mls-2* (see text and Experimental Procedures for details). Axonal defect +, AFD axon defect seen. Axonal defect -, no defect seen. Transgene +, transgene present in indicated cell. Transgene -, transgene inferred not to be present in indicated cell. n, number of animals examined.

(**B-I**) AWC axonal defects observed in adult animals in which the ventral left and ventral right CEPsh glia were ablated using a laser microbeam. Fluorescence images (**B-E**) and corresponding schematic drawings (**F-I**) of AWC neurons expressing an *odr-1*::RFP reporter transgene. (**B**, **F**) Wild-type morphology of AWC neuron in a mock-ablated animal. (**C-E**, **G-I**) AWC positional and axonal defects frequently observed in operated animals. In all panels, reporter expression in the AWB neuron is also seen.

(**J**) Merged DIC and fluorescence image, and (**L**) fluorescence image of a wild-type L1 animal expressing an *unc-119*::GFP reporter in all neurons. The white oval indicates the position of the posterior bulb of the pharynx. Green mass near the oval consists of axons composing the nerve ring as well as neuronal cell bodies.

(**K**, **M**) Same as (**J**) and (**L**), respectively, except that a growth-arrested L1 animal in which the ventral left and ventral right CEPsh glia were ablated is shown. Note that neuronal processes and cell bodies composing the nerve ring are disorganized and anteriorly mispositioned. Abnormal anteriorly directed axons are evident as well.

observed in *mls-2* mutants are indeed caused by the *mls-2* lesion.

We then examined animals in which the array was present in one or the other cell type, but not in both. We found that in 18/30 animals in which the array was present in ventral CEPsh glia, but was absent from AFD neurons, axon guidance was restored to normal. However, in animals in which the array was present in AFD but absent from ventral CEPsh glia, only 7/30 animals had wild-type axons. These results strongly suggest that *mls-2* functions within the ventral CEPsh lineage, probably within the ventral CEPsh cells themselves, to regulate AFD axon guidance and morphology. The observation that *mls-2* presence in the AFD lineage could weakly restore wild-type axon morphology, suggests that *mls-2* may also function within this lineage. Alternatively, and perhaps more likely, *mls-2* function in these animals could have been restored to CEPsh glia, but was not sufficient for robust *hlh-17* expression.

Although *mls-2* is likely to function within CEPsh glia to regulate axon development, some of the defects seen may also have non-glial origins. To determine precisely the contributions of CEPsh glia to axon development, we used the cell-ablation technique as our second approach. We ablated the direct precursor cells of CEPsh glia using a laser microbeam, and analyzed the effects of CEPsh absence on the morphologies of the AWC, ADF and AFD axons (Bargmann and Avery, 1995). We note first that in our ablation studies, we never observed the death of any neurons physically associated with, or that showed any

other interaction with CEPsh glia. Specifically, of 206 operated animals containing reporters for one of the neurons AWC, ADF, AFD, or CEP neurons, 205 animals showed normal neuronal marker expression that persisted throughout the life-time of the animal. We do not know why one of the 206 animals failed to express the reporter transgene, but lack of marker expression can also be seen on occasion in unablated transgenic animals.

As shown in Figure 3.2I, when precursors of both ventral CEPsh glia were ablated, 50% and 45% of animals exhibited defects in AWC and AFD axons, respectively, whereas only about 5% of ADF axons were defective. Major classes of defects observed in ablated animals are depicted in Figures 3.3B-I.These results are consistent with our examination of *mls-2* and *vab-3* mutants, in which specific defects in AWC and AFD were noted, but defects in ADF were much less frequently observed. One possibility for the differential effects of ventral CEPsh glia on different axons may be that at certain points within the nerve ring, some axons may be in close proximity to ventral CEPsh glial processes, whereas others are not. To determine whether such regions of the nerve ring exist, we examined previous EM reconstructions of the nerve ring (White et al., 1986). As shown in Figure 3.4, axons of the AWC and AFD neurons at two different locations along their length are situated adjacent to CEPsh glia processes, on the outer surface of the nerve ring and ventral ganglion. By contrast, ADF axons are located more towards the interior of the nerve ring, where CEPsh glial processes are absent.

Figure 3.4

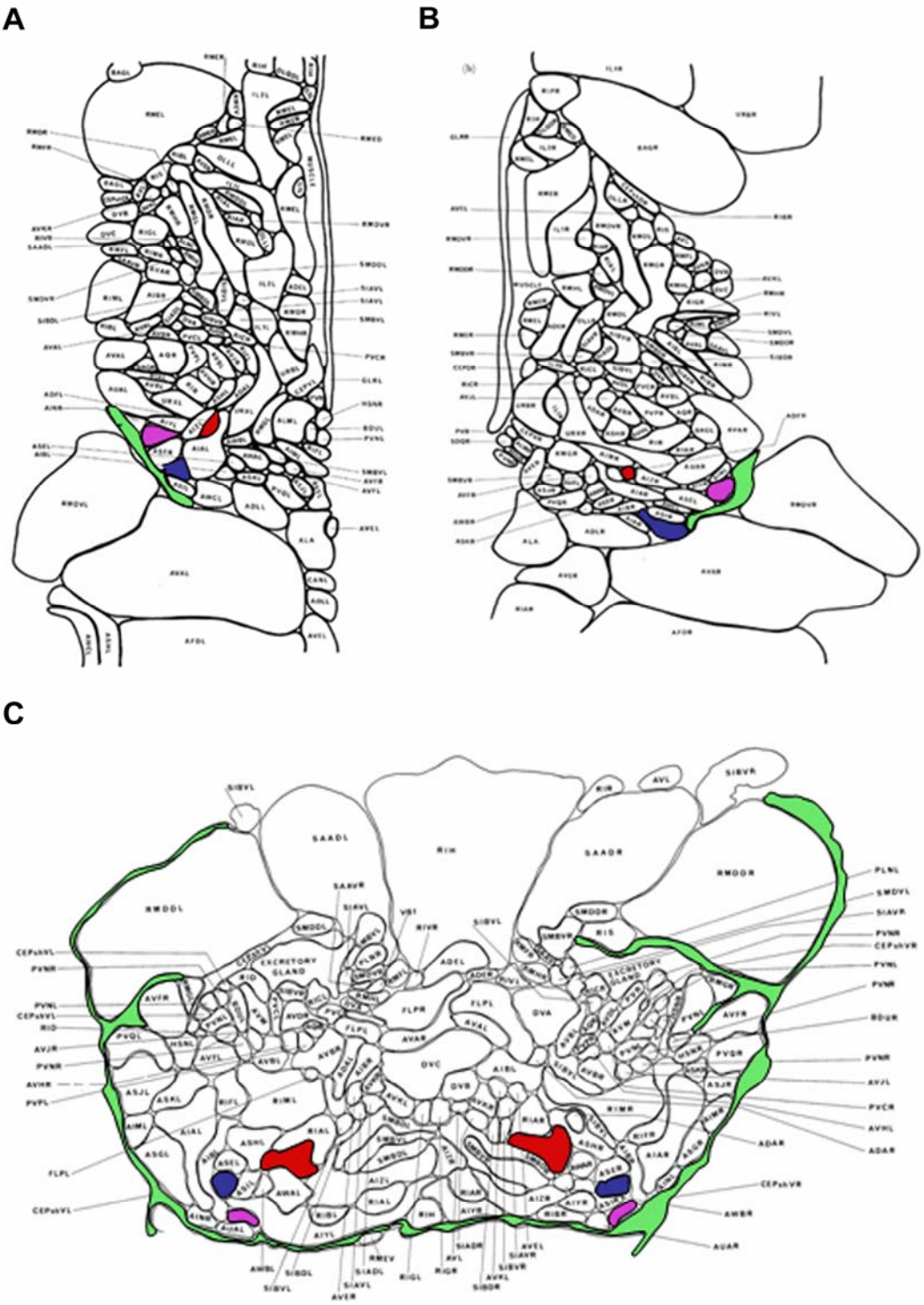


Figure 3.4. AWC and AFD Neurons Contact CEPsh Glia in the Nerve Ring and Ventral Ganglion

(**A**) Left lateral cross section of the nerve ring. Image reproduced from White et al., 1986. Green, CEPshVL glia process. Blue, AWCL neuron. Purple, AFDL neuron. Red, ADFL neuron.

(**B**) Same as (**A**) except that a right lateral cross section is shown. Green, CEPshVR glia process. Blue, AWCR neuron. Purple, AFDR neuron. Red, ADFR neuron.

(**C**) Transverse section through the anterior region of the ventral ganglion. Image reproduced from White et al., 1986. Green, CEPshVL/R glia process. Blue, AWCL/R neurons. Purple, AFDL/R neurons. Red, ADFL/R neurons.

These observations are at the very least consistent with the possibility that proximity to CEPsh glia may be necessary for the guidance and branching decisions of some axons.

Guidance and branching defects were generally observed in AWC, AFD, and ADF only when ventral CEPsh glia precursors were ablated. Ablation of dorsal CEPsh glia had only a very weak effect on these neurons (Figure 3.2I). These results suggest that CEPsh glia function in a spatially-restricted manner to regulate axon development.

During the course of our ablation studies, we noticed that 17 of 91 (19%) of the animals in which ventral CEPsh glia were ablated arrested development in the L1 larval stage. This arrest was not observed in mock-ablated animals, but was seen on rare occasions when cells near the CEPsh precursors were ablated (1 of 26 ablated animals). AFD, ADF, and AWC neurons in these arrested larvae had severe defects in axon projections (data not shown), suggesting a global defect in nerve ring assembly. To examine the integrity of the nerve ring in ablated animals, we ablated ventral CEPsh glia precursors in animals carrying the *unc-119*::GFP pan-neuronal reporter. Six of 30 animals examined arrested as L1 larvae and exhibited highly disorganized nerve rings, often separated into multiple anteriorly-displaced axon bundles, as well as anteriorly-displaced neuronal cell bodies (Figures 3.3J-M). Interestingly, 38% of *mls-2*(*ns156*) mutants (n=577), arrested as L1 larvae, and frequently displayed similar nerve ring defects.

Furthermore, we observed severe defects in neuronal cell body positioning and process positioning as well as larval arrest in strong *vab-3* mutants, consistent with previous observations (Chisholm and Horvitz, 1995).

Together, these results suggest that the CEPsh glia may be important not only for the guidance and branching of specific axons within the nerve ring, but also for the assembly of the entire structure.

- **UNC-6/Netrin Mediates Glia-Dependent Axon Guidance**

What is the molecular mechanism underlying CEPsh glia-dependent axon guidance? CEPsh glia could control axon guidance in the nerve ring by expressing specific axon guidance cues. Indeed, it was previously reported that the *C. elegans* netrin gene, *unc-6*, is expressed in ventral CEPsh glia as well as other glial cells in the head of the animal (Wadsworth et al., 1996). To determine whether UNC-6 may mediate CEPsh glia-dependent axon guidance, we first examined the effects of a strong loss-of-function mutation in the *unc-6* gene (*ev400*) on the guidance of the AWC neurons. Previous studies have shown that 29% of AWB neurons display axonal guidance defects in *unc-6*(ev400) animals (Zallen et al., 1999). We found that the AWC neuron exhibits even more pronounced defects, with 54/102 (53%) neurons displaying guidance defects,

suggesting a role for *unc-6* in the guidance of these axons.

To determine in which cells *unc-6* normally functions to promote AWC axon guidance, we generated *unc-6*(*ev400*) animals containing the integrated AWC reporter *odr-1*::RFP (see above), as well as an unstable extrachromosomal array consisting of the *hlh-17*::GFP transgene, and plasmid ∆pSM containing the *unc-6* promoter driving the *unc-6* cDNA. The latter construct is able to efficiently rescue the uncoordinated locomotion defects of *unc-6* mutants (Colon-Ramos et al., 2007). We found that 24/25 (96%) animals in which *hlh-17*::GFP was expressed in ventral CEPsh glia had normal AWC axon outgrowth. By contrast, 12/23 (52%) animals in which *hlh-17*::GFP was expressed in dorsal CEPsh glia but not in ventral CEPsh glia had normal AWC axon outgrowth. The severity of AWC defects was, therefore, indistinguishable from the severity of defects in animals lacking any rescuing construct.

During the period of nerve ring formation, UNC-6 is expressed exclusively in glia of the head (Wadsworth et al., 1996). While most sheath glia are derived from the ABa progenitor cell, four glial cells, including the two ventral CEPsh glia, are derived from the ABp progenitor. Furthermore, the only bilaterally symmetric glial pair generated from ABp are the ventral CEPsh glia. Taken together, therefore, the UNC-6 expression studies, together with our mosaic studies strongly suggest that UNC-6 functions within ventral CEPsh glia to control AWC axon guidance.

3.3 Discussion

- **CEPsh Glia Control Axon Guidance and Branching**

The observation that ventral CEPsh glia, as well as other *C. elegans* glia express the UNC-6/Netrin guidance molecule has led to the suggestion that these glia may control axon guidance (Wadsworth et al., 1996). We have shown, using studies of *mls-2* and *vab-3* mutants, as well as cell ablation studies, that CEPsh glia indeed play a key role in regulating axon guidance. Furthermore, we showed that much of this role can be attributed to expression of UNC-6/Netrin in these glia, since the axon guidance defects of AWC neurons in *unc-6* mutants can be rescued by expressing the gene in ventral CEPsh glia.

Our results are consistent with the notion that UNC-6 might function to regulate physical interactions between AWC or AFD neurons and CEPsh glia, since both neurons showed pronounced axon guidance defects in animals missing CEPsh glia, whereas the ADF neuron, which does not contact CEPsh glia, showed only minor defects. Indeed, short-range functions for Netrin-related proteins have been previously described (Baker et al., 2006), consistent with this possibility. Interestingly, Netrin is present on periaxonal myelin of oligodendrocytes in the spinal cord, supporting an adhesive role, and bolstering the hypothesis that CEPsh glia possesses similarities in both their transcriptional

program of development and in differentiated features to oligodendrocytes (Manitt et al., 2001).

Our studies raise the intriguing question of whether the synaptogenesis defects recently reported (Colon-Ramos et al., 2007) in mutants lacking UNC-6 or its receptor, UNC-40, are due to a direct role for these proteins in synapse formation between the AIY and RIA neurons studied, or due to a subtle guidance defect of the AIY axon, altering the microenvironment of the axon, thus preventing the initiation of synapse formation.

In addition to UNC-6, other axon guidance/branching proteins in the nerve ring are known, including SAX-3/Robo (Zallen et al., 1999), VAB-1/Eph (George et al., 1998; Zallen et al., 1999) and UNC-40/DCC (Chan et al., 1996). Whereas SAX-3 function in peripheral *C. elegans* neurons is regulated by SLT-1/Slit ligand, the nerve ring defects of *sax-3* mutants might reflect redundant interactions of SAX-3 with SLT-1 and a different ligand (Hao et al., 2001). *sax-3* defects include anteriorly displaced nerve rings and cell bodies, anterior axon projections, and defects in ventral projections and axon elongation (Zallen et al., 1999). Although most animals lacking ventral CEPsh glia show only axon guidance and branching defects, about 20% arrest development in the L1 stage, exhibiting anteriorly displaced nerve rings and cell bodies (Figure 3.3). It is possible, therefore, that CEPsh glia also secrete a SAX-3/Robo ligand regulating nerve ring positioning, assembly and axon guidance. The weak penetrance of the *sax-3*-like defects we

observed might reflect our inability to ablate all four CEPsh glia simultaneously.

- **Glial Regulation of Dendrite Extension**

Our laser ablation experiments, as well as studies of *mls-2* and *vab-3* mutants, have conclusively shown that CEPsh glia are intimately involved in the process of CEP dendrite extension. Importantly, we never observed the death of CEP neurons in our ablation studies, indicating that *C. elegans* neurons can live normally in an intact organism in the absence of their associated glia. We have previously made similar observations in amphid sensilla, the largest sensory organ in *C. elegans*. In the absence of amphid sheath glia, amphid neurons did not die, but displayed morphological abnormalities at their dendrites (T. Bacaj and S. Shaham, unpublished observations). Thus, the role in dendrite extension may be universal among most, if not all, *C. elegans* sheath glia.

In *C. elegans* as well as in other model organisms, less is known, compared to axonal outgrowth and pathfinding, about the molecular mechanisms underlying dendrite extension and development. In mammals, several extrinsic and intrinsic factors have been shown to be involved in the process of dendrite growth (Jan and Jan, 2003), but it is unclear whether similar factors play role in the dendrite specification in *C. elegans*. Studies in our lab have recently demonstrated that the

secreted extracellular factors DEX-1 and DYF-7 act cooperatively for the development of amphid sensory dendrites (M. Heiman and S. Shaham, unpublished observations). Mutants of both *dex-1* and *dyf-7* genes exhibit dendrite extension defects, similar to the ones we observed in our CEPsh ablation experiments. Although we have not examined CEP dendrites in *dex-1* and *dyf-7* mutants, similar genes are likely to be acting in the process of their extension. It is tempting to study relationships between these genes and sheath glia during the development of *C. elegans* nervous system.

Chapter 4

Conclusions and Future Directions

4.1 Conclusions

Although glia outnumber neurons in the vertebrate CNS, they remain enigmatic in many ways. One obstacle to studying glia and their interactions with neurons has been their trophic support of neurons. Thus, manipulation of glia, *in vivo* or *in vitro*, often leads to the death of associated neurons, precluding detailed studies of glial effects on neuronal function. Roles for glia in regulating neuronal survival (and *vice versa*) have also been shown in *Drosophila* (e.g. reviewed in Shaham, 2005). One approach to circumvent this difficulty has been to culture neurons in the presence of survival factors, some of which are glia-derived, and then examine the consequence of adding back glia. This scheme has been successful in demonstrating an important function for glia-produced thrombospondin in synapse formation between vertebrate neurons (Christopherson et al., 2005). Furthermore, these experiments suggest that additional glial factors must be required for synaptic function. However, demonstrating the validity of these studies *in vivo* has been challenging.

Our studies have revealed that the CEPsh glia of *C. elegans*, while possessing morphological, functional, and molecular similarities to vertebrate glia, differ from their vertebrate counterparts in that they are not required for the survival of associated neurons. This observation has allowed us to explore the functions of glia *in vivo* in a well-characterized, genetically manipulable animal,

and to demonstrate key roles for these cells in dendrite, and axon extension and guidance. Our results open up the possibility of using *C. elegans* to identify conserved mechanisms by which glia contribute to synapse formation, synaptic function, neuronal ensheathment, ionic regulation, and permeability barriers in the nervous system. Furthermore, because *C. elegans* possesses 46 additional glial cells that resemble CEPsh glia in structure and in expression of specific proteins (such as HLH-17 and PTR-10), studies of these other cells are likely to reveal additional layers of glial influences on the nervous system.

4.2 Future Directions

- **Identification of New Molecular Components in the Glial Developmental Pathway**

As discussed in section 1.3 of Chapter 1, a number of genes are required for the specification and terminal differentiation of glia. The molecular similarities in glial development between *C. elegans* CEPsh glia and vertebrate glia suggest that other components of these developmental programs may be conserved as well. The powerful genetic tools available in *C. elegans* may facilitate identification of novel components in the developmental pathway. We have only characterized

four of thirteen alleles we isolated in the screen (Figure 2.3A), and further characterization of the rest of the mutants may reveal a great detail about glia development in *C. elegans*.

We have also performed a genetic screen to identify upstream regulators of *ptr-10* (see Appendices). Identification of these regulators of *Patched*-related genes may elucidate molecular pathways leading to lumen morphogenesis (Perens and Shaham, 2005). Interestingly, *ns2870* mutant animals isolated from the screen do not express *ptr-10*::RFP specifically in CEPsh glia. Moreover, *hlh-17*::GFP is expressed at extremely low level in these animals, suggesting that the gene affected in this mutant may also regulate *hlh-17*, and encode another transcription factor that functions within the pathway shown in Figure 2.15.

It is also possible to take a candidate approach to identify components involved in the *C. elegans* glia development. We have already examined mutants of *cog-1*, which encodes *C. elegans* ortholog of the mammalian Nkx6.1, but did not find any obvious defects in CEPsh glia. *C. elegans* possesses orthologs of many of the genes expressed in the developing spinal cord and analysis of available mutants may provide additional insights.

- **Role of bHLH Proteins in *C. elegans* Gliogenesis**

The bHLH proteins form a large superfamily of transcriptional regulators that are found in almost all eukaryotes (Ledent and Vervoort, 2001; Massari and Murre, 2000). This family is defined by the bHLH domain, which consists of approximately 60 amino acids with two functionally distinct regions. The basic region, located at N-terminal end of the domain, consists of ~15 amino acids with a high number of basic residues, and binds to DNA at a consensus hexanucleotide sequence known as the E-box (5'-CANNTG-3') (Ferre-D'Amare et al., 1993; Ma et al., 1994). Different bHLH proteins recongnize different E-box consensus sequences. At the C-terminal end of the domain is the HLH region, which facilitates interactions with other protein subunits to form homo- and heterodimeric complexes (Ferre-D'Amare et al., 1994; Murre et al., 1989). Many different combinations of dimeric structures are possible, each with different binding affinities to E-boxes. Consequently, bHLH proteins exhibit diverse developmental functions, particularly in neurogenesis (Lee, 1997), myogenesis (Weintraub et al., 1991), heart development (Srivastava and Olson, 1997), and hematopoiesis (Begley et al., 1989).

Gliogenesis also requires functions of a number of bHLH proteins, including members of Olig, Hes, and Mash. In the telencephalon, OLPs might develop from the region in which the expression of Mash1 and Olig1/2 overlap (reviewed in

Rowitch, 2004). Misexpression of *Hes1* and *Hes5* increases generation of Muller glia (Furukawa et al., 2000; Hojo et al., 2000). Therefore, considering the similarities in the glial developmental pathway we observed, it is possible that bHLH proteins play essential role in development of CEPsh and other *C. elegans* glia.

In this study, we demonstrated that a bHLH factor *hlh-17*, which is most highly-related to vertebrate *Olig2*, is expressed in most, if not all, of the *C. elegans* glia, but its functions remain obscure. It seems rather unlikely that Oligs and other glial bHLH proteins act alone in a given cell type. Olig2, for instance, can act both as a homodimer and a heterodimer, and one of the bHLH proteins that can heterodimerize with Olig2 is E47 (Lee et al., 2005). The astroglial bHLH factor SCL is also known to genetically interact with Olig2 (Muroyama et al., 2005). Therefore, identification of other glial-expressing bHLH factors, which are possible candidates of *hlh-17* dimerization partners, may provide additional insights into their roles in *C. elegans* gliogenesis. We have already examined mutants of *C. elegans* gene *hlh-2*, which encodes a bHLH protein orthologous to E47, but could not identify any obvious defects at least in CEPsh glia. To be more comprehensive, all forty-two *C. elegans* bHLH genes need to be examined for their expression patterns and also for any glial defects. This can be performed using RNAi against these genes, perhaps in animals with a sensitized background, carrying *hlh-17*(*ns204*) or *hlh-32*(*ns223*) *hlh-17*(*ns204*) *hlh-31*(*ns217*) in addition to glial

markers such as the *hlh-17*::GFP. Using these sensitized backgrounds, one may also design a simple genetic screen similar to the one performed in this study to identify genes that genetically interact with *hlh-17*.

- **Postembryonic Functions of CEPsh Glia**

In this study, we have focused on the embryonic functions of CEPsh glia, and demonstrated their roles in dendrite extension and axon guidance. Several observations indicate that these cells may play important roles postembryonically. First, electron microscopic images of adult animals show that CEPsh glia send fine processes to several synaptic sites within the nerve ring, which may modulate synaptic activity (White et al., 1986). Secondly, in mature vertebrate brains, Olig1/2-expressing cells may play crucial roles during the remyelination following injury (Ligon et al., 2006), indicating that CEPsh glia, in which the persistent *hlh-17* expression is observed, may have similar postembryonic functions.

Colón-Ramos *et al.* recently provided evidence that CEPsh glia promote local synaptogenesis in the nerve ring (Colon-Ramos et al., 2007), indicating potential interactions between the CEPsh processes and synapses. Thus it is especially tempting to investigate postembryonic functions of CEPsh glia at synaptic sites. However, it is difficult to directly measure activities of a single

synapse in *C. elegans*, partly because electrophysiology is not compatible with the size of the animals. Instead, a proper readout needs to be identified in order to monitor changes in synaptic activity.

Menachem Katz, a postdoctoral fellow in our lab, is now studying behavioral defects of animals in which CEPsh glia are genetically ablated postembryonically. He has found that body movements of ablated animals are sluggish compared to wild-type animals (unpublished observations). He further confirmed that similar behavioral defects are observed in animals in which all four CEPsh glia are ablated using a laser microbeam. It would be interesting if he can demonstrate that these behavioral defects are attributable to the loss of synaptic modulatory functions of CEPsh glia. Additionally, *ns2870* mutant animals described above may be useful in his studies, because the mutation is likely to affect postembryonic development of CEPsh glia. Thus any other defects identified in these mutant animals may be indicative of postembryonic functions of these glia.

- **Massive Identification of Glial Expressing Genes**

Examination of the gene expression profile of CEPsh glia may help to further elucidate their function and to identify molecular components conserved between *C. elegans* glia and vertebrate glia. Indeed, quantitative analysis of gene

expression in oligodendrocytes and their precursor cells has uncovered several previously uncharacterized genes that are highly upregulated as oligodendrocytes differentiate (Dugas et al., 2006).

Taking advantage of the *hlh-17* promoter which expresses highly specifically in CEPsh glia in adult stage animals, we may be able to perform CEPsh-specific microarray analysis using the mRNA-tagging technique (Kunitomo et al., 2005; Roy et al., 2002; Von Stetina et al., 2007). This experiment can be done not only in wild-type animals but also in our collection of mutants. It would be especially intriguing to compare the gene expression profiles between CEPsh glia of wild-type animals and *ns2870* mutants, because it may help to reveal potential targets of *hlh-17* and to understand its function.

- **Identification of Other Genetic Programs Involved in *C. elegans* Gliogenesis**

In mammals, specification of different glial subtypes requires different transcriptional programs (Hochstim et al., 2008; Muroyama et al., 2005; discussed in section 2.7 of Chapter 2). Several observations suggest that *C. elegans* glia and their developmental pathways are also diverse. For example, *mls-2* mutants appear to have no defects in glial cells of inner and outer labial sensilla located in

the head of the animal. Thus, although CEPsh glia and glia of inner and outer labial sensilla share expression of *hlh-17* and *ptr-10*, differentiation of the latter glia may require a different set of genes. Moreover, we have never observed expression of *hlh-17*::GFP in sheath glia of the amphid, the largest sensory organ in *C. elegans*. Since *hlh-17*::GFP expression weakens very early in the development in cells other than CEPsh glia (discussed in section 2.1 of Chapter 2), we may simply be missing its expression in amphid sheath glia. Alternatively, CEPsh and amphid sheath glia may be significantly different in their gene expression profiles. Indeed, amphid sheath glia are the only glia in the head of the animal that do not express *ptr-10*, although they express a related gene *ptr-7*/*daf-6* (Perens and Shaham, 2005).

Considering these observations, we may be able to gain new insights into glial development by identifying genes involved in specification of *C. elegans* glia other than CEPsh glia. Amphid sheath glia are probably the easiest to work on because we have very specific reporters to visualize these cells (Perens and Shaham, 2005). Mutants defective in the development of amphid sheath glia may be isolated using a simple forward genetic strategy, screening for animals in which the expression of their reporters is constantly diminished.

Appendices

Appendix 1. Regulation of *ptr-10* Expression by bHLH Factors

As discussed in Chapter 4, bHLH proteins bind to DNA at a consensus sequence known as the E-box. During our course of study, we recognized that the 300 bp *ptr-10* promoter sequence we used to make the *ptr-10*::myrRFP construct contained two E-box consensus sequences, which we designated E-box1 and E-box2 (Figure 1A). To determine whether these E-boxes are functional *in vivo*, we introduced three point mutations into each E-box that were supposed to eliminate functions of the consensus sequence (Lee et al., 2005). Surprisingly, the glial expression of *ptr-10*::myrRFP reporter was completely abolished when the E-box2 was mutated (Figures 1B-E), while mutations in the E-box1 apparently had no effect on the reporter expression. These observations suggest that at least E-box2 is functional *in vivo* and that *ptr-10* is regulated, at least partially, by bHLH transcription factors.

To test whether *hlh-17* regulate *ptr-10*, we ectopically expressed a transgene, consisting of the full-length *hlh-17* cDNA fused to a heat-inducible promoter, in 270-430 min embryos carrying *ptr-10*::myrRFP reporter, by subjecting them to a 30 min heat pulse at 34˚C. Interestingly, L1 larvae hatched from the heat-shocked embryos expressed *ptr-10*::myrRFP at a much higher intensity than L1 larvae hatched from non-heat-shocked embryos (Figures 1F-K). Following heat-shock induction, however, we did not observe expression of RFP in cells that

Figure 1

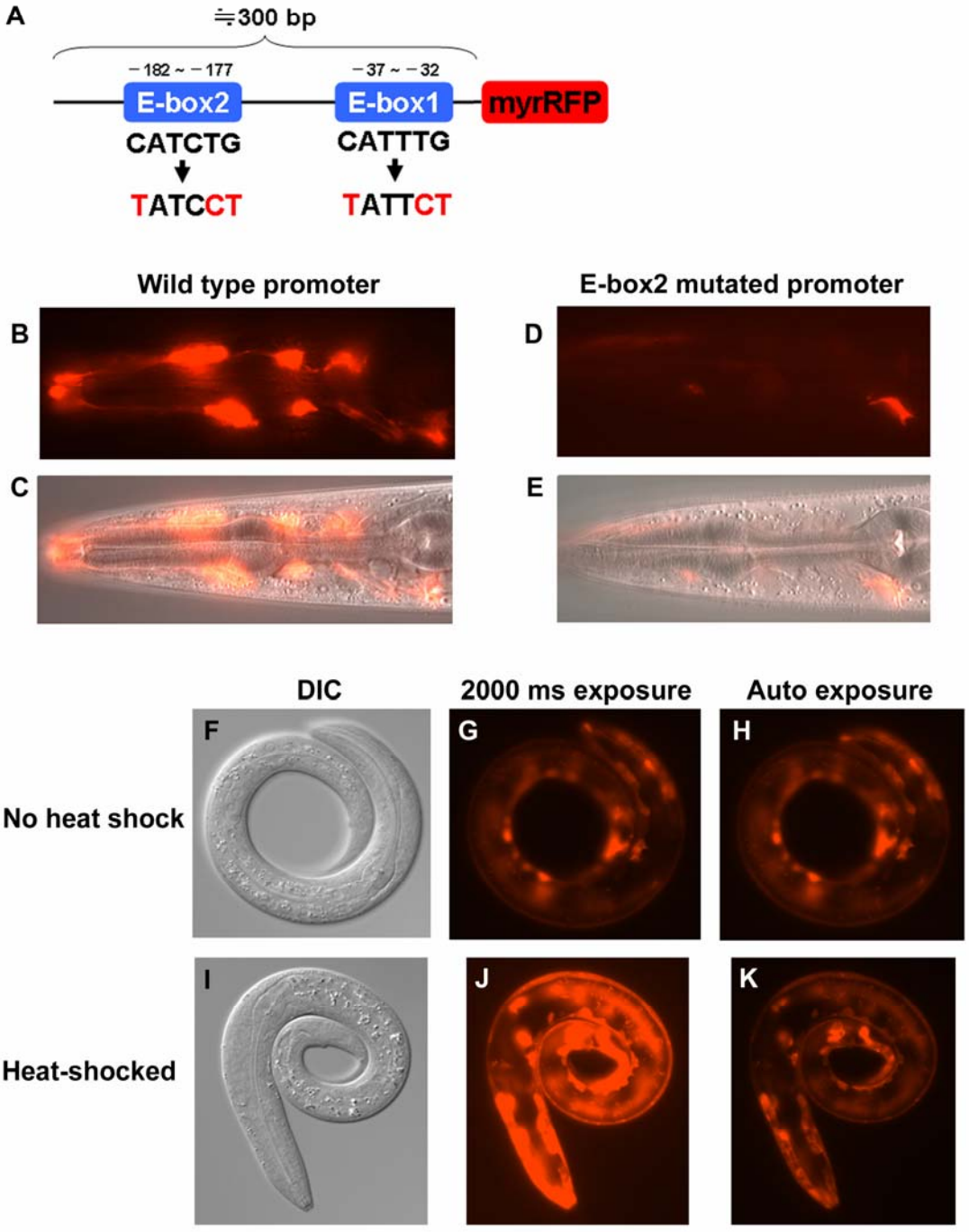

A
≒300 bp
−182 ~ −177
−37 ~ −32
E-box2
E-box1
myrRFP
CATCTG
CATTTG
TATCCT
TATTCT
Wild type promoter
E-box2 mutated promoter
B
C
D
E
DIC
2000 ms exposure
Auto exposure
No heat shock
Heat-shocked
F
G
H
I
J
K

Figure 1. bHLH Transcription Factors May Regulate *ptr-10* Expression

(**A**) A diagram depicting the structure of *ptr-10*::myrRFP construct. A 300 bp DNA sequence upstream of the *ptr-10* translation start site was ligated to the myrRFP gene. The 300 bp fragment contained two E-box sequences which are indicated by blue boxes. Numbers indicate the precise positions of the E-boxes relative to the first ATG in the genomic sequence of *ptr-10*. CATTTG sequence of E-box1 and CATCTG sequence of E-box2 was mutated to TATTCT and TATCCT, respectively.

(**B**, **C**) Fluorescence image (**B**) and merged DIC and fluorescence image (**C**) of an adult animal expressing *ptr-10*::myrRFP transgene with wild-type promoter sequence.

(**D**, **E**) Fluorescence image (**D**), and merged DIC and fluorescence image (**E**) of an adult animal expressing *ptr-10*::myrRFP transgene with mutations in the E-box2. Note that RFP expression is abolished in glia but not in the excretory system.

(**F-H**) Induction of *ptr-10*::myrRFP reporter transgene expression by overexpression of *hlh-17* cDNA using a heat-inducible promoter. DIC image (**F**), and fluorescence images (**G**, **H**) of an L1 larva hatched from an embryo not subjected to a heat-shock. To compare the intensity of RFP expression, exposure time was fixed at 2000 ms for (**G**).

(**I-K**) Same as (**F-H**), respectively, except that an L1 larva hatched from a heat-shocked embryo is shown. Note that the intensity of RFP expression is higher in (**J**) compare to (**G**).

normally do not express *ptr-10*::myrRFP (compare Figure 1H and 1K). These observations together indicate that *hlh-17* can act upstream of *ptr-10*, but is not sufficient by itself to induce the expression of *ptr-10*.

Although *hlh-17* is a good candidate for *ptr-10* regulatory factors, it is not essential for *ptr-10* expression because neither the *hlh-17*(*ns204*) nor the *hlh-32*(*ns223*), *hlh-17*(*ns204*); *hlh-31*(*ns217*) mutant animals exhibited defects in *ptr-10*::myrRFP expression pattern (Figures 2.12 and 2.13). The fact that *vab-3*(*e1796*) mutants fail to express *hlh-17*::GFP, but not *ptr-10*::myrRFP (Figure 2.4), further indicate that expressions of *hlh-17* and *ptr-10* are regulated independently of one another.

Appendix 2. Identification of *ptr-10*-Off Mutants

The previous work in our lab has demonstrated that the *Patched*-related gene *daf-6* (also know as *ptr-7*) is required for lumen formation in *C. elegans* (Perens and Shaham, 2005). During our course of study, we confirmed by electron microscopy that *ptr-10*(*tm2580*) mutant animals also exhibit defects in lumen formation, indicating that *ptr-10* may play a role similar to that of *daf-6* within CEPsh glia.

To identify regulators of *ptr-10* expression, we mutagenized animals carrying

a genomically-integrated *ptr-10*::myrRFP reporter transgene with ethyl methanesulfonate (EMS; see Experimental Procedures), and scanned resulting F2 progeny for alterations in the expression pattern of the reporter using a dissecting microscope equipped with epifluorescence, to visualize RFP. From 30,000 mutagenized haploid genomes examined, we recovered six candidate mutants exhibiting abnormal reporter transgene expression. Mutant allele *ns2870* bred true, and was characterized further. Over 50 % of *ns2870* adult animals do not express *ptr-10*::myrRFP specifically in CEPsh glia and nearly all animals express the *hlh-17*::GFP reporter at an extremely low level (Figure 2), suggesting that the gene affected in this mutant may also regulate *hlh-17*.

To identify the gene affected by the *ns2870* mutation, we used single nucleotide polymorphism differences between the *ns156* and CB4856 *C. elegans* strains (Wicks et al., 2001) to map the mutation to a 8.19 map unit interval on the III chromosome between cosmids C40H1 and T28D6. Additional mapping is necessary to identify the gene mutated in this mutant.

Figure 2

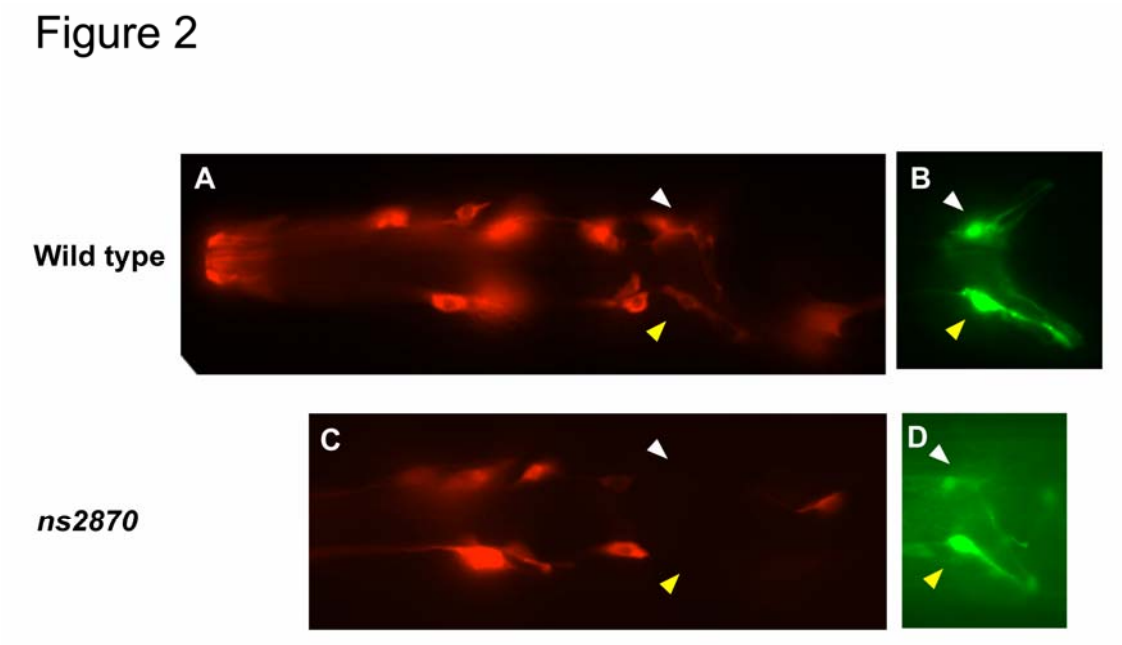


Figure 2. Expression Patterns of *hlh-17*::GFP and *ptr-10*::myrRFP Reporter Transgenes in *ns2870* Mutant Animals

(**A**, **B**) Fluorescence images of a wild-type adult expressing *ptr-10*::myrRFP (**A**) and *hlh-17*::GFP (**B**) reporter transgenes. The expression in dorsal and ventral CEPsh glia is indicated by white and yellow arrowheads, respectively. The two images were taken in different animals.

(**C**, **D**) Same as (**A**, **B**), respectively, except that an *ns2870* adult is shown. Exposure time of (**D**) is about 4 seconds, which is about 20 times longer than that of (**B**). Note that *ptr-10*::myrRFP reporter expression is missing in dorsal and ventral CEPsh glia.

Experimental Procedures

- **General Methods and Strains**

Strains were handled using standard methods (Brenner, 1974). All strains were maintained and scored at 20˚C unless otherwise indicated. The wild-type strain used was *C. elegans* var. Bristol (N2). The following strains and integrated transgenes were used: LGI: *nsIs105* (*hlh-17*::GFP); LGIV: *nsIs136* (*ptr-10*::myrRFP), *hlh-17* (*ns204*, *ok487* (McMiller and Johnson, 2005)), *hlh-31* (*ns217*), *hlh-32* (*ns223*), *sDf23* (Ferguson and Horvitz, 1985); LGV: *oyIs17* (*gcy-8::GFP*) (Satterlee et al., 2001), *oyIs44* (*odr-1::RFP*) (Lanjuin et al., 2003), *oyIs51* (T08G3.3::RFP) (Lanjuin et al., 2006), *nsIs145* (*ttx-1*::RFP), *otIs45* (*unc-119*::GFP) (Altun-Gultekin et al., 2001); LGX: *mIs-2* (*ns156*, *ns158*, *ns159*, *cc615* (Jiang et al., 2005)); *vab-3* (*ns157*, *e648* (Hodgkin, 1983), *e1796* (Hedgecock et al., 1987), *ju468* (Cinar and Chisholm, 2004), *bx23* (Baird et al., 1991), *k143* and *k109* (Nishiwaki, 1999), *sy281* (Chisholm and Horvitz, 1995)); *nsIs108* (*ptr-10*::myrRFP). *egIs1* (*dat-1*::GFP) (Nass et al; 2002), *nIs60* (*vab-3* promoter::GFP, gift from Andrew Chisholm), *zuIs178* (*his-72*::H3.3-GFP) (Ooi et al., 2006), and *ruIs32* (*pie-1*::H2B-GFP) (Praitis et al., 2001) insertions are unmapped. *nT1 qIs51* IV; V (Siegfried et al., 2004) was used as a balancer for *hlh-17* (*ok487*) and *sDf23*.

The following extrachromosomal arrays were used: *nsEx646* [*hlh-17*::myrGFP + *lin15*(+)], *nsEx725* [*hlh-31* promoter::RFP + *lin15*(+)], *nsEx729*

[*hlh-32* promoter::RFP + *lin15*(+)], *nsEx1420* [C39E6 + *rol-6* (*su1006*)], *nsEx1419* [*mls-2* promoter::*mls-2* + *rol-6* (*su1006*)], *nsEx1404* [F14F3 + *rol-6* (*su1006*)], *nsEx1463* [heat-shock promoter::*mls-2* + *rol-6* (*su1006*)], *nsEx1464* [heat-shock promoter::*vab-3* + *rol-6* (*su1006*)], *nsEx1577* [*mls-2* promoter::*vab-3*::*mls-2*::*mls-2* 3' UTR + *rol-6* (*su1006*)], *nsEx1717* [C39E6 + *nhr-38*::GFP + *elt-2*::GFP].

- **Isolation of Mutants and Mapping**

mls-2 and *vab-3* mutants: *nsIs105* (*hlh-17*::GFP) animals were mutagenized with 30 mM ethyl methanesulfonate (EMS) as described previously (Sulston and Hodgkin, 1988). Animals were then propagated on 9 cm NGM agar plates for 5 days. Gravid F1 adults were individually plated on 1600 agar plates and allowed to self-fertilize for 5 days. F2 progeny on each plate were screened as early adults and mutants displaying CEPsh glia defects were isolated. Mutants were mapped by crossing to the polymorphic CB4856 strain, followed by isolation of recombinant homozygous mutant animals as described by Wicks et al. (2001).

ns2870: *nsIs108* (*ptr-10*::myrRFP) animals were mutagenized with 30 mM ethyl methanesulfonate (EMS) as described previously (Sulston and Hodgkin, 1988). Animals were then propagated on 9 cm NGM agar plates for 5 days. Gravid F1 adults were harvested, bleached and their progeny incubated overnight

in M9 buffer. F2s were plated onto 9 cm plates and were screened as early adults for alterations in the expression pattern of the reporter.

hlh-17, *hlh-31*, and *hlh-32* deletions: Deletion alleles were isolated using the method of Jansen et al. (1997) and including a poison primer. The following primer pairs were used for screening:

hlh-17: poison primer, 5'GCATGACTTAAACGAGGCACTTGACG3'; outer primers, 5'ATGGGGTCCCTGGGGACTC3', 5'CCGATTTCCGCTTCAACTGGGAG3'; inner primers, 5'TCCCTGGGGACTCTCCTCG3', 5'CGATTTTTGCTGCTAATGGG CAACAC3'.

hlh-31: poison primer, 5'GCATGACTTAAACGAGGCACTTGACG3'; outer primers, 5'CAGTCCGGATGGAATGAACAAAAGGG3', 5'CTACATGGTCGCTTGATGGCTTCAC3'; inner primers, 5'TTGCAGCCAACTCAAAGTTGGGTC3', 5'GGGAGACCAATACACTGAGCTCC3'. *hlh-32*: poison primer, 5'GCATGACTTAAACGAGGCACTTGACG3'; outer primers, 5'GCCTCTGGTAGTCTACGGC3', 5'CTAATCTCCTTCGGATGGTGTTGACACGG3'; inner primers, 5'GCTTCCGTTTTTGGGAAACAAGAG3', 5'CTTAGCTCTTCGATTGCTTTTGCCTG3'.

- **cDNA Isolation**

cDNAs for *hlh-17*, *hlh-31*, *hlh-32*, *mls-2* and *vab-3* were isolated by amplification using PCR of a plasmid-based cDNA library (Schumacher et al., 2002) using primers in the vector and within the genes.

- **Plasmid Constructions**

hlh-31 promoter::RFP: we amplified by PCR a 1.5 kb DNA fragment from cosmid *hlh-31* containing a 1.2 kb sequence upstream of the *hlh-31* first ATG as well as the coding sequence for the first 32 amino acids. The resulting amplicon was ligated to the DsRed (RFP) gene digested with *Sph*I and *Bam*HI. *hlh-32* promoter::RFP: we amplified by PCR a 3 kb DNA fragment sequences upstream of the *hlh-32* translation start site from the Yeast Artificial Chromosome Y105C5 containing. The resulting amplicon was ligated to the DsRed gene digested with *Sph*I and *Bam*HI. *hlh-17*::GFP: we amplified by PCR a 2.7 kb DNA fragment from cosmid *hlh-31* containing 1.9 kb of sequences upstream of the *hlh-17* translation start site, as well as sequences encoding the first 58 amino acids. The resulting amplicon was ligated to pPD95.69 (Miller et al., 1999) digested with *Sph*I and *Bam*HI. *hlh-17*::myrGFP: we amplified by PCR a 4 kb DNA fragment from cosmid

F38C2 containing sequences upstream of the *hlh-17* translation start site. The resulting amplicon was ligated to the myrGFP gene (Adler et al., 2006) digested with *Sph*I and *Bam*HI. *ptr-10*::myrRFP: we amplified by PCR a 300 bp DNA fragment from cosmid F55F8 containing genomic sequence upstream of the *ptr-10* translation start site. The resulting amplicon was ligated to the myrRFP gene (Adler et al., 2006) digested with *Sph*I and *Xma*I. *mls-2* promoter::GFP::*mls-2*::*mls-2* 3' UTR: a construct similar to that described in Jiang et al. (2005) was generated. *mls-2* promoter::*vab-3*::*mls-2*::*mls-2* 3' UTR: *vab-3* isoform A cDNA was digested with *Sal*I and ligated into the *mls-2* promoter::gfp:: *mls-2*::*mls-2* 3' UTR construct described above and digested with *Sal*I, replacing the gfp moiety with *vab-3*. Heat-shock promoter::*mls-2*/*vab-3*: *mls-2* or *vab-3* isoform A cDNAs were digested with *Age*I and *Eco*RI and ligated into the *Age*I and *Eco*RI restriction sites of the vector pPD95.75 (Miller et al., 1999). We then amplified by PCR the heat-shock promoter from the vector pPD49.78 (Fire et al., 1990). The resulting amplicon was digested with *Sph*I and *Bam*HI and ligated into the *Sph*I and *Bam*HI restriction sites of the above described cDNA vectors. 8.3 kb subclone of cosmid C39E6: we amplified by PCR an 8.3 kb DNA fragment from cosmid C39E6 containing 5.5 kb of sequences upstream of the *mls-2* ATG, as well as the coding sequence for *mls-2* and 300 bp immediately downstream of the endogenous *mls-2* stop codon. The resulting amplicon was digested with *Pst*I and *Eco*RI and ligated into the *Pst*I and *Eco*RI restriction sites of the vector pPD95.75

(Miller et al., 1999). *nhr-38*::GFP was a gift from Piali Sengupta (Miyabayashi et al., 1999).

- **Transgenic Strain Constructions**

Germline transformations were carried out using standard protocols (Mello and Fire, 1995). *hlh-17*::GFP (50 ng/ul) and *ttx-1*::RFP (3 ng/ul) were injected alone into N2 animals. *ptr-10*::myrRFP (50 ng/ul) was injected into *lin-15*(*n765*) animals with plasmid pJM23 (20 ng/ul), containing wild-type *lin-15* (Huang et al., 1994). The resulting extrachromosomal transgenes were stably integrated by treatment with 4,5',8-trimethylpsoralen (TMP) followed by identification of animals homozygous for insertion events (Yandell et al., 1994), to establish stably transmitting lines.

For *mls-2* rescue experiments, cosmid C39E6 and the 8.3 kb subclone of this cosmid were injected into *mls-2*(*ns156*) animals at a concentration of 1 ng/ul. For *vab-3* rescue experiments, cosmid F14F3 or a 24 kb *Asc*I-*Pme*I fragment digested from this cosmid were injected into *vab-3*(*ns157*) animals at a concentration of 1 ng/ul. The *mls-2* promoter::*vab-3*:: *mls-2*::*mls-2* 3' UTR construct was injected into N2 animals with plasmid pRF4 (40ng/ul), containing the dominant marker *rol-6* (*su1006*) (Mello et al., 1991), at a concentration of 20

ng/ul. Other GFP or RFP reporter constructs were injected into animals at a concentration of 30-50 ng/ul with either pRF4, pJM23, or *elt-2*::GFP (Fukushige et al., 1998) as transformation markers (30-40 ng/ul).

- **Mosaic Analysis**

C39E6, *nhr-38*::GFP, and *elt-2*::GFP were co-injected at concentrations of 1 ng/ul, 50 ng/ul, and 20 ng/ul, respectively, into a strain of genotype *nsIs105* (*hlh-17*::GFP); *nsIs145* (*ttx-1*::RFP); *mls-2*(*ns156*). Animals harboring the extrachromosomal array were selected using *elt-2::*gfp expression under a fluorescence dissecting microscope, mounted for observation on a compound microscope in M9 medium, and assessed for appearance of *hlh-17*::GFP and *nhr-38*::GFP expression in the ventral CEPsh glia and AFD neurons, respectively.

For the *vab-3* mosaic studies, we co-injected the *vab-3* containing cosmid F14F3, *ptr-10*::myrRFP, and *rol-6* at concentrations of 1 ng/ul, 50 ng/ul, and 20 ng/ul, respectively, into a strain of genotype *vab-3*(*ns157*); *nsIs105* (*hlh-17*::GFP).

For the *unc-6* mosaic studies we used an *elt-2*::GFP reporter (Fukushige et al., 1998) to follow transgenic animals.

- **Cell Ablation Studies**

The strains used for ablation contained either *hlh-17*::GFP or *ptr-10*::myrRFP, and one other marker expressed in neurons of interest (*dat-1*::GFP for CEP, *odr-1*::RFP for AWC, T08G3.3::RFP for ADF, and *gcy-8*::GFP for AFD) to facilitate scoring of CEPsh glia fate and axonal defects. In some cases, ablated strains contained the *unc-119*::GFP reporter (Maduro and Pilgrim, 1995). Direct precursor cells of CEPsh glia were ablated in embryos at the 250-300 min stage in a drop of S-basal on 5% agar pads using a micropoint laser set up and standard methods (Bargmann and Avery, 1995). Precursors of CEPsh cells were identified by following the cell division patterns of embryos starting at 100 minutes post-fertilization as described previously (Sulston et al., 1983). Ablations were scored as successful if the CEPsh cells were absent two days later, as determined by fluorescence microscopy.

- **Microscopy**

Animals were examined by epifluorescence using either a fluorescence dissecting microscope (Leica), an Axioplan II compound microscope (Zeiss), or a spinning disc confocal microscope (Zeiss) equipped with a Perkin-Elmer

UltraView spinning disk confocal head. For the compound microscope, images were captured using an AxioCam CCD camera (Zeiss) and analyzed using the Axiovision software (Zeiss). For the spinning disk confocal microscope, images were captured using an EMCCD (C9100-12) gain camera (Hamamatsu) and analyzed using the MetaMorph software (UIC)

- **Heat-Shock Studies**

Heat-shock constructs were injected at a concentration of 20 ng/ul with pRF4 (40 ng/ul) as the transformation marker. Animals were placed at 34˚C for 30 min, allowed to recover at 20˚C, and scored for induction of reporters of interest 60-150 min later.

- **Whole-Mount Antibody Staining**

Permeabilization, fixation, and antibody staining of embryos followed published procedures (Finney and Ruvkun, 1990). Anti-VAB-3 antibody was a gift from David Greenstein and was diluted 1:1000. The secondary antibody was goat anti-rabbit IgG conjugated with Texas Red (Abcam), diluted 1:200. After

incubation with the secondary antibody, embryos were mounted using SlowFade Antifade Kit (Invitrogen). Mounted embryos were immediately observed and photographed with an Axioplan II compound microscope (Zeiss).

- **Southern Hybridizations**

Preparation of genomic DNA, agarose gel electrophoresis, and Southern blotting were performed using standard techniques (Ausubel et al., 1989). Probes were prepared from *hlh-17* cDNA using PCR.

- **Lineage Analysis**

Lineage tracing was performed essentially as described by Murray et al. (2006). 3D time-lapse image series were collected for wild-type (n=2), *mls-2*(*ns156*) (n=4) and *vab-3*(*ns157*) (n=4) embryos carrying a nuclearly localized *his-24*::GFP reporter using a Zeiss LSM510 confocal microscope. We used the program StarryNite (Bao et al., 2006) to automatically trace the lineage from the images, and the program AceTree (Boyle et al., 2006) to identify and edit errors in the StarryNite annotations. Lineages were followed through the 350-cell stage and

selectively traced the CEPsh-producing lineages (ABarpa, ABplpaa, ABprpaa) through the birth of the CEPsh cells. Because StarryNite makes significantly more errors at and beyond the 350-cell stage than it does in earlier stages, we followed each cell by eye in each lineage throughout its lifespan and corrected all errors. Tree displays and 3D projections were generated in AceTree.

Acknowledgements

We thank Piali Sengupta for the *nhr-38*::GFP plasmid, Shohei Mitani for the *hlh-17*(*tm2850*) mutant, Daniel Colón-Ramos and Kang Shen for the *unc-6* rescuing plasmid, David Greenstein for the anti-VAB-3 antibody. Some nematode strains were provided by the Caenorhabditis Genetics Center, which is supported by the National Institutes of Health. Shai Shaham is a Klingenstein fellow in the neurosciences and a Monique Weill-Caulier Scholar.

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