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INSIGHTS FROM LAMPREY INTO DEVELOPMENT AND EVOLUTION OF GLIA AND CRANIAL SENSORY GANGLIA

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BIOLOGY

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ABSTRACT

During evolution, animal nervous systems have become increasingly complex. This complexity is based on the cumulative diversity of neural cells and the emergence of new nervous system structures. Among animals, vertebrates possess the most complex and specialized nervous system, which has evolved to contain diversified neural cell types that control organs with specialized functions. For the study of nervous system evolution, one outstanding question is how those novel cell types originated in the vertebrates. To address this question, this dissertation focuses on:

1) The evolution of one specific glial cell type in the vertebrate central nervous system (CNS)—oligodendrocytes, which form the myelin sheath in jawed vertebrate CNS;
2) The emergence of paired cranial sensory ganglia in the peripheral nervous system.

Myelin is a membrane protein that enables specialized cells to form an insulating layer around axons to speed up conduction along nerve cells. This protein and the cell types that form it (oligodendrocytes in the CNS and Schwann cells in PNS) are thought to be novel in jawed vertebrates. However, how these cell types evolved remains unclear. We asked whether the emergence of oligodendrocytes could only have occurred after the genetic "toolkit" required for this cell type appeared, or instead if the required genetic regulatory mechanism predates the evolution of the oligodendrocyte cell type itself. To address this question, I chose the basal jawless vertebrate—sea lamprey, since jawless vertebrates diverged from the jawed vertebrates prior to the evolution of oligodendrocytes. We used gene expression analyses coupled with

functional perturbation using CRISPR/Cas9 mediated genome editing of sea lamprey embryos. My results indicate that the core genetic mechanism required for oligodendrocyte development is present in this jawless vertebrate, even though they lack this cell type, suggesting a core network regulating gliogenesis arose in the last common vertebrate ancestor. Sea lampreys appear to use this ancestral regulatory mechanism to control development of motor root glia. After the divergence of jawless and jawed vertebrates, other genes including myelinating proteins, were recruited onto this ancestral genetic scaffold and triggered the evolution of oligodendrocytes.

Next, I focused on the evolution of cranial ganglia in the peripheral nervous system (PNS) of vertebrates. Among jawed vertebrates, the sensory neurons present in cranial ganglia are derived from two different cell populations—neurogenic placodes and neural crest. However, likely mechanisms directing development of cranial ganglia in the vertebrate common ancestor remain unclear. To study the evolution of vertebrate cranial sensory ganglia, I determined functional cellular contributions to cranial ganglia development in lamprey. I used gene expression analysis, cell lineage tracing and genome editing functional analysis, to show that lamprey cranial sensory neurons are likely purely derived from placodes, while neural crest surround each ganglia and function to pattern the neurons properly into each ganglion. My results suggest that in jawed vertebrates, neural crest replaced sensory neurons in the proximal portion of cranial ganglia while placode-derived neurons occupy the distal portion. Overall, these studies have implications for understanding the primitive scenario of placode and neural crest functions during early cranial ganglia evolution, after they first appeared in the

vertebrate common ancestor, and will lead to a better understanding of evolution of the vertebrate nervous system.

PREFACE

The purpose of this research project is to investigate the evolution of novel cell types and patterning mechanisms in the vertebrate nervous system. These cell types include glial precursor cells that may have given rise to oligodendrocytes, and cellular contributions to cranial sensory ganglia that form from ectodermal placodes and neural crest. I used the basal jawless vertebrate, sea lamprey (Petromyzon marinus) to investigate these questions, combining in situ hybridization, immunohistochemistry and cell linage tracking techniques to understand genetic mechanisms of gliogenesis in the lamprey central nervous system, and cellular contributions and functions during cranial ganglia development. These projects were conceived by me under the mentorship of Dr. David McCauley in the Biology department of The University of Oklahoma.

Chapter One is a comprehensive overview of evolution of the vertebrate nervous system. I wrote this chapter, with editorial assistance from Dr. McCauley and Joshua York, a fellow graduate student in the McCauley lab.

In Chapter Two, I investigate the genetic regulatory mechanism required for lamprey motor root glia development. This project was developed by me with input from my advisor, Dr. McCauley, and was improved through conversations with Joshua York. All of the experiments conducted in this project were conceived and initiated by me. I investigated the developmental expression of genes required for gliogenesis in lamprey in the context of oligodendrocyte precursor cell development in jawed vertebrates and used immunohistochemistry to identify glia in the lamprey. I also used CRISPR/Cas9 genome editing technology to generate mutant embryos, and measured the length of motor root glia in mutant and control groups. To analyze the significance

of this work, Joshua York assisted in performing statistical analyses to determine the significance of differences in motor root glia length between mutant and control embryos. I wrote the chapter, with editorial assistance from Dr. McCauley and Joshua York.

In Chapter Three, I investigate contributions of neural crest and epidermal placode cells to cranial sensory ganglia, with a focus on how sensory neurons are patterned into each ganglion. In this project, I performed in situ hybridization and immunohistochemistry to determine the developmental expression of neural crest markers in the context of cranial sensory ganglia formation. I used cell lineage tracing to investigate the contribution of neural crest and placode cells in lamprey cranial ganglia, and also CRISPR/Cas9 gene editing to delete the function of neural crest and placode regulatory genes. Using this technique, I eliminated both these cell populations, and then determined the function of each cell type during cranial ganglia development. This project was conceived by me, under the mentorship of Dr. McCauley. Most of this work was conducted by myself, with some assistance from Joshua York who cloned one of the neural crest marker genes, TwistA, and synthesized the RNA probe used for TwistA in situ hybridization. This chapter was also written by me, with editorial assistance from Dr. McCauley and Josh York.

CHAPTER 1: EVOLUTION OF THE VERTEBRATE NERVOUS SYSTEM

Tian Yuan, David W. McCauley

Keywords:

Central nervous system, peripheral nervous system, neural crest, oligodendrocytes, cranial ganglia

INTRODUCTION

The origin and diversification of the vertebrate nervous system is one of the great success stories of animal evolution (Holland, 2016). The advent of a highly complex nervous system enabled specialized behaviors that allowed vertebrates to distinguish themselves behaviorally, morphologically and physiologically from their closest invertebrate chordate relatives (Liebeskind et al., 2016). The vertebrate central nervous system (CNS) includes the brain and spinal cord, while the peripheral nervous system (PNS) includes sensory and motor neurons outside the spinal cord. Multiple types of neurons and supporting glial cells are present in both the CNS and PNS (Gottlieb, 1976). These different cell types perform highly specialized functions, and cooperate to accomplish complex neural activities in all vertebrate clades (Luskin, 1994). The molecular, genetic, cellular and developmental basis for both neurogenesis and gliogenesis in the vertebrate nervous system is a highly regulated process and changes to these developmental-genetic programs are thought to underlie the diversification of vertebrate nervous systems across almost half a billion years of evolution (Luskin, 1994; O'Connell, 2013). Compared to vertebrates, invertebrate nervous systems are relative simple. For example, while invertebrate chordates (e.g., amphioxus and tunicates) possess central and peripheral nervous systems, their brain region has smaller numbers of neural cells and fewer diversified cell types, while in the peripheral nervous system, invertebrate chordates lack cranial sensory ganglia (Holland, 2015; Holland, 2016; Mackie and Burighel, 2005; Wicht and Lacalli, 2005). How did the vertebrate nervous system evolve from the relatively simpler nervous systems of invertebrate chordates? What types of neural and glial cell types did the first vertebrates contain?

What was the ground state of gene regulatory networks (GRN) that drove neurogenesis and gliogenesis in early vertebrates, and how has the "rewiring" of these networks over time contributed to the evolution of novel cell types in the vertebrate CNS? Surprisingly, we still lack adequate answers to these fundamental questions. To address these issues, I have investigated the developmental and evolutionary origins of cell types in the vertebrate CNS and PNS, by using a primitively jawless vertebrate, the sea lamprey (*Petromyzon marinus*) that belongs to the sister taxon of all jawed vertebrates. The lamprey is the most appropriate model for these studies because they make up one of only two extant jawless vertebrates, and the only one for which embryos are easily accessible for experimental purposes. My first set of experiments (Chapter 2) focuses on the evolutionary origin of the oligodendrocyte, a glial cell type that forms the myelin sheath surrounding axons in the CNS of jawed vertebrates (Miron et al., 2011; Simons and Nave, 2015). In my second project (Chapter 3), I investigate the evolutionary origin of vertebrate cranial sensory ganglia and the roles of two cell populations, neural crest and placodes, from which they develop.

Vertebrate central nervous system development

Neurons in the central nervous system of jawed vertebrates

The vertebrate central nervous system consists of a tripartite brain in the anterior head region and a spinal cord that traverses the anteroposterior axis of the body. Both of these structures originate as a flattened, epithelial sheet of cells (i.e., neural plate) that rolls up to form a hollow tube, known as the neural tube, during the period of early development that is referred to as neurulation. The CNS in jawed vertebrates contains a

wide variety of cell types that include interneurons, motor neurons and four types of glial cells—astrocytes, oligodendrocytes, ependymal cells and microglia. In the neural tube, all of the neurons and glial cells are derived from the midline region of neuroepithelium known as the ventricular zone (Fig. 1.1). Once neurogenesis commences, these neuroepithelial cells will give rise to stem cell-like progenitors called radial glia, the major cell type in the vertebrate CNS capable of generating neurons, astrocytes and oligodendrocytes (Anthony et al., 2004; Gotz and Barde, 2005; Paridaen and Huttner, 2014; Wicht and Lacalli, 2005). Radial glial cells are referred to as "glia" because they superficially have many glia-like properties. For instance, they express a glial-specific intermediate filament protein-- glial fibrillary acidic protein (GFAP) (Mori et al., 2005). Newly born neurons and glia migrate toward the outer layers (mantle zone and marginal zone) and differentiate into the specific types of cells that define the vertebrate CNS (de Lahunta et al., 2016; Leclerc et al., 2012).

During early CNS development, radial glia express the transcription factor, Pax6. Following differentiation, Pax6 expression is down-regulated in most CNS cells, but is maintained in newly generated interneurons (Hack et al., 2004). Pax6 functions during early neurogenesis to induce the expression of the basic helix-loop-helix (bHLH) gene, Neurogenin, which, together with the transcription factor Mash1, promote neuron specification (Bertrand et al., 2002; Sansom et al., 2009). Other transcription factors including Sox2 and Hes1 interact with Pax6 to control the timing of neurogenesis as well as the renewal of neural stem cells (Sansom et al., 2009). The bHLH genes Math3 and NeuroD are also activated during maturation of neurons, although their role in the terminal step of neurogenic differentiation is still unclear (Kim, 2013; Lee et al., 1995;

Lee et al., 2000; Naya et al., 1997; Tomita et al., 2000). Members of the Sox gene family play diverse but important roles during vertebrate CNS development (Pevny and Placzek, 2005). For example, the SoxB genes, Sox1, Sox2 and Sox3, are expressed in neural progenitor cells and maintain neural progenitor identity, preventing premature differentiation (Bylund et al., 2003; Graham et al., 2003; Wegner, 2011). Sox21, on the other hand, functions to initiate neuronal differentiation (Sandberg et al., 2005). Moreover, gene knockdown experiments in amniotes indicate that Sox4 and Sox11 are necessary for the expression of pan-neural genes such as NF1, Lim2, Tuj1, SCG10 and Isl1/2 (Bergsland et al., 2006).

Jawed vertebrate central nervous system glial cells

In the vertebrate CNS, glial cells are more numerous than neurons. While neurons process and transmit electrical information, the primary function of glial cells is to protect and support neurons; to supply nutrients and oxygen to neurons; to insulate the axons; and remove pathogens and dead neurons.

Astrocytes: Astrocytes are named for their “star-shaped” appearance, and are the most numerous cells in the vertebrate CNS (Kettenmann, 2013). Astrocytes fill in the space among neurons and in doing so provide a supportive neural framework that helps to form and maintain the blood-brain barrier in the CNS (Kimelberg, 2010; Kimelberg and Nedergaard, 2010). They also play roles in the repair of neural tissue damage by forming scar tissue (Kimelberg and Nedergaard, 2010). During early gliogenesis within the ventricular zone, astrocyte precursor cells express Sox9 (Stolt et al., 2003). Later, as they migrate from the ventricular zone, astrocyte precursor cells can

be identified by the expression of several genes, including GLAST, FABP7/BLBP and FGFR3 (Owada et al., 1996; Pringle et al., 2003; Shibata et al., 1997). After migration, astrocyte precursors initiate a differentiation program by expressing GFAP, S100β, Aldh1L1, aquaporin 4 and AldoC (Gomes et al., 1999a; Gomes et al., 1999b; Gomes et al., 2003; Molofsky et al., 2012).

Oligodendrocytes: In jawed vertebrates, axons from the central nervous system are insulated by a fatty myelin sheath, which is produced from a subset of glial cells known as oligodendrocytes. Myelin is thought to have evolved independently in invertebrates and vertebrates, and is therefore considered non-homologous between these lineages (Hartline, 2008; Schweigreiter et al., 2006). In the vertebrate clade, however, myelin is a jawed vertebrate innovation, and myelin-producing oligodendrocytes are also believed to have first evolved in the jawed vertebrate lineage (Zalc et al., 2008).. Oligodendrocytes are derived from the motor neuron progenitor (pMN) domain in the ventral neural tube, and share the same neural precursors as motor neurons (Ravanelli and Appel, 2015). Motor neuron-oligodendrocyte precursors are identified by the unique expression of transcription factors such as Olig1 and Olig2 (Ravanelli and Appel, 2015). After motor neurons have been produced, Sox9 and nuclear factor 1 A (NFIA) convert the fate of remaining pMN precursors from motor neurons to oligodendrocytes (Deneen et al., 2006; Stolt et al., 2003). At this point, oligodendrocytes specifically express markers such as platelet-derived growth factor receptor alpha (PDGFRa), neural/glia antigen 2 (NG2), oligodendrocyte marker 4 (O4) and O1, which are commonly used as oligodendrocyte markers in the CNS. The expression of transcription factors Sox10 and Nkx2.2 mark the myelinating

oligodendrocyte stage. During myelination, Sox10 directly activates genes implicated in myelin protein production, including myelin basic protein (MBP) and proteolipid protein (PLP) (Lee et al., 2008). At this point, the plasma membrane of oligodendrocytes extends and tightly wraps around CNS axons to form a multi-layered insulating sheath, in which myelin-specific proteins located in the membrane are crucial for the integrity and maintenance of the myelin sheath. This insulating layer enables rapid transmission of signals along long axons of the vertebrate nervous system (Nave and Werner, 2014; Waxman et al., 1972).

Microglia: These cells are a type of modified immune cell in the CNS. Activated microglia serve as scavenger cells in the CNS and also function in neural tissue repair and neural regeneration (Kreutzberg, 1996). In mouse embryos, the development of microglia can be divided into three stages: early microglia, pre-microglia and adult microglia (Matcovitch-Natan et al., 2016). Different genes regulate microglia development and include, but are not limited to: Mcm5 and Dab2 in early-microglia, involved in cell cycle and differentiation, Csf1 and Cxcr2 upregulated in pre-microglia, and MafB, Cd14, Pmepa1 markers expressed in adult microglia (Hocevar et al., 2005; Matcovitch-Natan et al., 2016; Prunier and Howe, 2005).

Ependymal cells: Derived from radial glia, ependymal cells form the ependymal sheet that lines the ventricles of the neural tube and spinal cord (Spassky et al., 2005). These cells secrete cerebrospinal fluid in the brain and spinal cord to bathe neurons and glia, to maintain cerebral fluid balance (Del Bigio, 1995). Ependymal cells also induce neurogenesis and suppress gliogenesis by secreting the bone morphogenetic protein (BMP) inhibitor, Noggin, in the subventricular zone (Chmielnicki et al., 2004;

Lim et al., 2000). Ependymal cells express the glial markers S100β and GFAP.(Didier et al., 1986). A recent study has also shown that the forkhead box transcription factor FoxJ1 and homeobox gene Six3 are required for ependymal cell maturation (Jacquet et al., 2009; Lavado and Oliver, 2011; Yu et al., 2008).

Dorsal-ventral patterning of the CNS in jawed vertebrates

The neuroepithelium of the neural tube is divided into 13 regions that include the roof plate, dorsal progenitor domains pd1-pd6, ventral progenitor domains p0-p2, pMN, p3, and floor plate. Each of these is defined primarily by the types of cells they produce (Fig. 1.1). A bidirectional concentration gradient of bone morphogenetic protein (BMP) determines the dorsal neural tube neuronal subtype generation, while sonic hedgehog (shh) regulates ventral neural tube neuron differentiation (Briscoe et al., 2000; Ericson et al., 1997a). In the dorsal neural tube, six progenitor domains (dp1-dp6) give rise to dl1-dl6 neurons and two types of dorsal interneurons (Goulding, 2009). In the ventral half of the neural tube, p0-p2 and p3 domains give rise to V0-V2 and V3 interneurons (Goulding, 2009). Motor neurons are derived from the pMN progenitor region and are located in the ventral neural tube, where they control effector organs (muscle, glands, etc.) through projection of their axons into the PNS (Goulding, 2009; Ribes and Briscoe, 2009).

By activating and/or repressing different target gene batteries, the BMP and shh-bi-directional gradient controls the specification of different neural and glial subtypes that will eventually occupy and define discrete regions of the neural tube (Fig. 1.1) (Dessaud et al., 2008; Lee and Jessell, 1999; Timmer et al., 2002). Math1, Msx1, Msx2,

Lhx1 and Lhx2, are expressed in the dorsal-most roof plate and dl1 interneurons (Bermingham et al., 2001; Liu et al., 2004; Suzuki et al., 1997; Yeh et al., 1998). Paired box genes Pax7 and Pax3 are expressed in the dorsal half of the neural tube and regulate commissural neuron (V0 interneuron) development (Maczkowiak et al., 2010; Mansouri and Gruss, 1998). More ventrally, homeodomain proteins Dbx1 and Dbx2 are expressed more centrally, toward the middle of the neural tube, and specify the neural fate of V0 and V1 interneurons (Dessaud et al., 2008). Dbx1 and Dbx2 are both expressed in V0 interneurons, while only Dbx2 is expressed in V1 interneurons (Pierani et al., 1999; Pierani et al., 2001). This specification role is supported by experimental evidence in which mis-expression of Dbx1 resulted in the expansion of V0 interneurons into the p1 domain (Pierani et al., 1999). Pax6 is expressed throughout much of the ventral half of the neural tube, except the most ventrally located p3 domain and floor plate, and regulates the development of multiple types of neurons in the brain and spinal cord (Ericson et al., 1997b). Nkx6.1, a member of the Nkx class of homeobox genes, is expressed in neurons in the ventral neural tube, including motor neurons, V2 and V3 interneuron progenitors. Pax6 and Nkx6.1, functioning as repressors, prevent the expression of Nkx2.2, which inhibits motor neuron formation, and at the same time activates expression of transcription factors Olig1 and Olig2 (Briscoe and Novitch, 2008; Sander et al., 2000). Olig1 and Olig2 are pMN specific genes, and are critical for the development of motor neuron and oligodendrocyte development (Lu et al., 2002; Ravanelli and Appel, 2015). In zebrafish, loss of Olig2 prevents the development of motor neurons and oligodendrocytes, while over-expression of Olig2 results in formation of excess motor neurons and oligodendrocytes (Park et al., 2002). The most

ventral neural progenitor domain, p3, produces V3 interneurons. The homeobox Nkx class genes Nkx2.2 and Nkx2.9 are expressed in the p3 domain and are essential for V3 interneuron fate choice, as demonstrated by loss of V3 interneurons and expansion of motor neurons ventrally in mice lacking functional copies of both Nkx2.2 and Nkx2.9 (Holz et al., 2010). Taken together, a combinatorial set of transcription factors that are expressed along the dorsoventral axis of the neural tube are responsible for precisely regulating cell fates along the DV axis of the vertebrate CNS.

Neurogenesis and gliogenesis in the lamprey CNS

Lampreys are a group of primitively jawless (agnathan) vertebrates and are important evolutionary and developmental models for understanding the origin of many hallmark vertebrate traits, including the ancestral features of nervous system development. Surprisingly, the developmental mechanisms of neurogenesis and gliogenesis in the lamprey CNS are largely unexplored. Preliminary studies using gene expression analysis suggest that lamprey deploys some of the same molecular-genetic machinery for CNS development and patterning as seen in higher jawed vertebrates. For example, Pax6 is expressed in the developing neural tube, including the forebrain, hindbrain and spinal cord (Murakami et al., 2001). As in jawed vertebrates, Nkx2.2 is expressed in the ventral neural tube of the developing lamprey, along the anterior-posterior (AP) axis (Sugahara et al., 2011). Its expression domain is immediately dorsal to the floor plate, comparable to the pV3 region of jawed vertebrates (Holz et al., 2010; Sun et al., 2003). Taken together, these observations suggest that a GRN controlling development of the anterior neural tube and brain was present in the common vertebrate

ancestor prior to the divergence of jawless and jawed vertebrates, though it is unclear when this network arose (Glardon et al., 1998; Holland et al., 1998). Subsequent spatial expansion of this network led to regulation of neural development throughout the entire embryonic AP axis.

The lamprey CNS contains radial glial cells (Kettenmann and Ransom, 2005). Although glial markers such as GFAP were first thought to be absent from glia in lampreys, recent studies have shown that GFAP is expressed in their peripheral glia (Green, 2017). In addition to GFAP, radial glia also express a lamprey-specific intermediate filament keratin-like protein that is recognized by the lamprey-specific antibody, LCM29, according to Merrick et. al. (Merrick et al., 1995). That finding suggests several possible scenarios: 1) Radial glia in lamprey and jawed vertebrates may have evolved independently; 2) lamprey and jawed vertebrate radial glia are homologous, with a glial-specific keratin evolving only in lampreys; or 3) a radial glial cell that expresses a glial-specific keratin protein was present in the vertebrate ancestor but was lost in jawed vertebrates. Interestingly, and similar to observations in jawed vertebrates, radial glial cells in the lamprey possess long processes that emanate from the ventricular zone into the marginal zone. These cellular processes presumably function as a scaffold for newly specified neurons and glia as they migrate toward the marginal zone.

As a first step toward understanding possible evolutionary origins and diversification of molecular mechanisms that drove development of glia in the nervous system of early vertebrates, I have chosen to first study the evolutionary and developmental origins of one type of glial cell, oligodendrocytes. Toward this end, I

have used the sea lamprey as a model. Since lampreys diverged from jawed vertebrates c. 500 million years ago (MYA), comparisons of the mechanisms that control glial cell development in lampreys and jawed vertebrates are useful for determining how these cells evolved in the vertebrate common ancestor. Although this cell type, and its key cellular derivative--myelin-- is a novelty of the jawed vertebrate lineage, it is unknown if early vertebrates could have possessed a type of "proto-oligodendrocyte" cell type and/or if a general developmental-genetic program for an oligodendrocyte-like cell predates the evolution of true oligodendrocytes. Importantly, lampreys are thought to lack oligodendrocytes based primarily on the observation that lampreys lack a myelin sheath (Smith et al., 2013; Werner, 2013), but it is unclear if they possess the genetic architecture required for their specification. This makes the lamprey an ideal experimental model for probing the developmental and genetic basis for how a gene regulatory network controlling glial cell development in early vertebrates may have been adapted to give rise to oligodendrocytes. My first project focuses on investigating the expression patterns and functions of key genes present in lamprey that are homologous to oligodendrocyte marker genes present in the CNS of jawed vertebrates. In this work, I examine whether gene regulatory mechanisms involved in oligodendrocyte development may have pre-dated the divergence of jawless and jawed vertebrates, suggesting such mechanisms would most likely have performed a more general function in ancestral glial cell development.

Peripheral nervous system development in jawed vertebrates

The peripheral nervous system (PNS) refers to the part of the nervous system outside the brain and spinal cord. This includes both sensory-somatic and autonomic components. The sensory-somatic nervous system controls voluntary muscle movement while the autonomic nervous system regulates involuntary action such as internal-organ function (Bandler et al., 1991). The vertebrate PNS is primarily derived from the neural crest, except for the cranial sensory nervous system, that forms from two cell populations--neural crest and placodes. During vertebrate development, the future spinal cord begins as a neural tube that forms from the folding of the dorsally positioned neural plate. As neural folds fuse to form the neural tube, neural crest cells arise along the neural plate border along the AP axis (Fig. 2). Neural crest cells (NCCs) give rise to multiple cell lineages such as neurons, glia (satellite glia and myelinated Schwann cells), cranial cartilage, smooth muscle and pigment cells (Le Douarin, 2008). Placodes are epidermal thickened patches of cells that are restricted to the head region and arise from the ectoderm lateral to the anterior neural plate border. Both neural crest and placodes contribute to the formation of sensory neurons (neurogenic placodes) and other sensory organs in the head (olfactory epithelium, lens, inner ear) and for this reason are considered to have been a major driving force in the evolution of vertebrates (Gans and Northcutt, 1983b).

In the trunk, neural crest cells give rise to sensory neurons and satellite glia in dorsal root ganglia (DRG), as well as neurons and glia in the autonomic nervous system. DRGs are clusters of sensory neuron cell bodies, as well as the supporting glial cells (satellite glia), in the dorsal root of spinal nerves (Svenningsen et al., 2004). DRG sensory neurons transmit sensory information to the central nervous system (Sapunar et

al., 2012). The autonomic nervous system includes sympathetic, parasympathetic and enteric nervous systems, which act unconsciously and regulate body functions such as heart rate, digestion and respiratory rate (Janig, 1989). Schwann cells are peripheral glial cells that are derived from the neural crest and myelinate PNS axons, including the sensory root from DRGs and the peripheral part of the motor roots projecting from the ventral neural tube. Although different cell types have been shown to share a common gene regulatory "scaffold" during early development, there are also important cell-linage specific regulators that are necessary for cell type-specific roles during migration and differentiation.

Neural crest specification in jawed vertebrates

Neural crest cells are specified early, near the end of gastrulation (Basch et al., 2006) (Fig. 2). During neurulation, presumptive neural crest cells express a wide array of transcription factors, including Snail1/Snail2, FoxD3, Twist, AP-2, c-Myc and Sox9/Sox10 (Aybar et al., 2003; Cheung et al., 2005; Dottori et al., 2001; Honore et al., 2003; Knight et al., 2003; LaBonne and Bronner-Fraser, 2000; Lee et al., 2004; Meulemans and Bronner-Fraser, 2004; Montero-Balaguer et al., 2006; Sasai et al., 2001; Sauka-Spengler et al., 2007; Taylor and Labonne, 2005). After their specification, neural crest cells undergo an epithelial-mesenchymal transition (EMT) and detach from the dorsal neural tube. Cranial neural crest cells migrate dorsal-laterally to form craniofacial structures such as cartilage, neurons and glia. In the trunk, by contrast, neural crest cells either migrate dorsal-laterally to form melanocytes, or ventral-laterally to form smooth muscle, as well as neurons and glia in the PNS (Gilbert, 2000).

Neural crest-derived neural cell differentiation

As described above, neural crest cells generate a wide range of neural and glial PNS cell types in both the head and trunk of jawed vertebrates (Fig. 2), and the development of each of these cell types is governed by distinct gene regulatory modules.

The GRN for neuron development in the enteric nervous system includes the transcription factors Sox10, Pax3, Mash1, Phox2a/b, and the cell surface receptors RET, EDNRB (endothelin receptor-B) (Lang et al., 2000; Lang and Epstein, 2003; Leon et al., 2009; Pattyn et al., 1999). Sox10 directly interacts with Pax3 to activate RET expression and drive enteric neuron specification (Lang et al., 2000; Lang and Epstein, 2003). Similarly, Sox10 regulates the expression of EDNRB (Kim et al., 2003), whereas Mash1 upregulates expression of Phox2a, to indirectly activate RET (Hirsch et al., 1998; Lo et al., 1998). RET is a tyrosine kinase receptor for several types of ligands including GDNF (glial cell line-derived neurotrophic factor), neurturin, artemin and persephin, and is particularly important for enteric neuron development by inhibiting apoptosis and promoting differentiation of ENS precursors (Gianino et al., 2003; Hearn et al., 1998; Heuckeroth et al., 1998). Within the enteric nervous system, there are also distinct neural cell types that colonize specific regions along the embryonic AP axis. For example, receptor-ligand interactions between endothelin 3 and EDNRB are required for ENS development specifically in the colon (Gazquez et al., 2016; Woodward et al., 2000).

The sympathetic nervous system is a part of the autonomic nervous system and functions to stimulate the body's "fight or flight response" (McCorry, 2007). Neurons and glia comprising the sympathetic nervous system are entirely neural crest derived. The transcription factors expressed in neural crest cells that induce the sympathetic progenitor cell fate include paired homeodomain factor Phox2b, bHLH genes Mash1 and Hand2, as well as the zinc-finger transcription factor Gata2/3 (Lo et al., 1994; Stanke et al., 2004). After differentiation, Phox2b and Mash1 induce the expression of noradrenergic neurotransmitter marker genes tyrosine hydroxylase (TH), dopamine beta-hydroxylase and neuron-specific marker genes like Hu and neurofilament (Stanke et al., 2004).

Although neural crest cells form along the entire length of the embryonic body axis, there are distinct subpopulations that form specific cell types. For example, in jawed vertebrates trunk neural crest can be divided into two specific populations of vagal and sacral neural crest, depending on the region where they originate along the axis (Ferguson and Graham, 2004). In amniotes, the vagal neural crest arises from somites three, four and five, whereas in mammals, the vagal neural crest emigrates from somites one through seven (Anderson et al., 2006; Chan et al., 2004; Epstein et al., 1994; Newgreen and Young, 2002; Tucker et al., 1986). Sacral neural crest cells are derived from the posterior neural tube and colonize the hindgut enteric nervous system (Wang et al., 2011). In birds, sacral neural crest originate from somite level 28 and posterior (Erickson and Goins, 2000). Several transcription factors have been shown to function in enteric nervous system development. For instance, knockout of Phox2b and Sox10 caused loss of enteric neurons throughout the gastrointestinal tract in mouse

embryos (Kapur, 1999; Pattyn et al., 1999; Southard-Smith et al., 1998). In addition, both Pax3 and Mash1 are necessary for the formation of enteric ganglia (Guillemot et al., 1993; Lang et al., 2000).

Dual contributions to the cranial sensory nervous system

Cranial sensory neurons in the head are derived from two separate cell populations: neurogenic placodes and cranial neural crest (Barlow, 2002; D'Amico-Martel and Noden, 1983; Steventon et al., 2014). Cranial placodes include the adenohypophyseal, olfactory, lens, otic, lateral line (in aquatic vertebrates), trigeminal and epibranchial placodes (Schlosser, 2006). Neurogenic placodes are those that give rise to cranial sensory neurons and cranial ganglia, e.g. trigeminal, lateral line and epibranchial placodes (Baker and Bronner-Fraser, 2001; Grocott et al., 2012; Schlosser, 2010). Cranial neural crest arising from the anterior neural plate border give rise to sensory neurons and glia, as well as branchial cartilage in the head (Bronner-Fraser, 1998; Fish and Schneider, 2014; Gammill and Bronner-Fraser, 2002; LaBonne and Bronner-Fraser, 1998). Both of these cell populations (neural crest and placodes) contribute to cranial ganglia. Vertebrate cranial sensory ganglia include the trigeminal ganglion (V), facial ganglion (VII), vestibulocochlear ganglion (VIII), petrosal ganglion (IX), and fish and amphibians also possess a lateral line ganglion (Alexandre and Ghysen, 1999; Streit, 2004; Winklbauer, 1989). Amniotes contain separate proximal and distal ganglia in V, VII, IX and X nerves, while in anamniotes, those proximal and distal portions are fused (Barlow, 2002). The distal ganglia are formed from neurogenic placodes, while neurons in the proximal ganglia are formed from neural crest. In

anamniotes, by contrast, proximal and distal portions of each ganglia are fused, with the placodes mainly contributing neurons in the distal portion, whereas neural crest give rise to neurons in the proximal portion.

Transcription factors that regulate placode development include Six homeobox, genes (Six 1-6), Eyes absent (Eya) genes 1-4, Pax genes, Dlx3/5 and FoxI1 (Heller and Brandli, 1999; Hirsch and Harris, 1997; Pandur and Moody, 2000; Pohl et al., 2002). Six and Eya proteins are co-factors, with Six directly binding to DNA, whereas Eya interacts with Six transcription factors to form a transcriptional complex (Ikeda et al., 2002; Ohto et al., 1999; Pignoni et al., 1997). Mutations to Six1 and Eya1 in vertebrate animal models (e.g. mice, zebrafish) result in deficiencies in various placodes (Schlosser, 2007). For example, in Six1 and Six4 compound mutant mice, the olfactory placode failed to form (Chen et al., 2009). Neuroblasts in the VIIth ganglion are degenerated in $Eya1^{-/-}$ and $Six1^{-/-}$ mice (Zou et al., 2004). Furthermore, ectopic expression of mouse Six3 in medaka fish resulted in ectopic lens formation (Oliver et al., 1996). Among Pax gene sub-families, Pax2/5/8, Pax3/7 and Pax6 from the Pax4/6 group are expressed in placodes. For example, Pax8 is expressed in the early otic placode while Pax2 expression is detected in early otic and epibranchial placodes (Baker and Bronner-Fraser, 2000; Burton et al., 2004; Hans et al., 2004; Mackereth et al., 2005; Torres et al., 1996). Pax3 is specifically expressed in the ophthalmic branch of the trigeminal ganglion (opV), and Pax6 is critical for the development of lens and olfactory placodes (Altmann et al., 1997; Bhattacharyya and Bronner-Fraser, 2004; Buckiova and Syka, 2004; Epstein et al., 1991; Gehring and Ikeo, 1999; Tremblay et al., 1995).

Although bona fide placodes are considered to be a vertebrate innovation, there is evidence suggestive of a type of neurogenic placode (proto-placode) in the invertebrate chordate, *Ciona intestinalis* (Abitua et al., 2015). *Ciona* embryos have an ectodermal region surrounding the anterior neural tube that expresses Six and Eya homologs, similar to the condition in vertebrate placodes (Abitua et al., 2015). Strikingly, cells from this region give rise to sensory cells in the larval head. However, unlike the placodes characteristic of vertebrates, these placode-like sensory cells are scattered in the head region and do not cluster to form ganglia (Abitua et al., 2015).

There is also evidence that invertebrate chordates could have rudiments of the neural crest, since there appears to be a cell lineage in ascidians that is derived from a neural plate border and expresses a number of neural crest specifier genes, including Id, Snail and FoxD (Abitua et al., 2012b; Imai et al., 2006; Jeffery et al., 2008; Russo et al., 2004; Squarzoni et al., 2011; Wada and Makabe, 2006). This cell lineage (a9.94) is able to give rise to melanocytes, a recognized neural crest derivative in vertebrates (Le Douarin, 2008; Nitzan et al., 2013; Sommer, 2011). However, there is no evidence that the rudimentary neural crest-like cells in tunicates such as *Ciona* can contribute to any part of a crude cranial sensory nervous system, as occurs in vertebrates.

Lamprey peripheral nervous system development

Lamprey is one of two living jawless vertebrates (hagfish being the other) that possesses neural crest and placodes (McCauley and Bronner-Fraser, 2003; Medeiros, 2013; Modrell et al., 2014; Sauka-Spengler and Bronner-Fraser, 2008b). Like jawed vertebrates, lamprey neural crest derives from a neural plate border and migrates to

form derivatives that include melanocytes, pharyngeal cartilage and components of the peripheral nervous system including dorsal root ganglia (McCauley and Bronner-Fraser, 2003). A recent study also suggests that trunk neural crest in lamprey gives rise to an enteric nervous system, just as in jawed vertebrates (Green et al., 2017). However, the lamprey lacks a number of neural crest derivatives present in jawed vertebrates, including sympathetic chain ganglia, jaws, and myelinating glia as described above (Haming et al., 2011). In addition, lampreys do not appear to have a population of vagal neural crest (Green et al., 2017). In jawed vertebrates, vagal neural crest cells give rise to the neurons that innervate the entire gastrointestinal tract (Allan and Newgreen, 1980; Tucker et al., 1986; Young et al., 2000). Instead, lamprey enteric neurons are derived from neural crest exclusively in the posterior trunk (Green et al., 2017).

The gene regulatory network for lamprey neural crest development appears to be highly conserved with the NC GRN in jawed vertebrates, with the exception of some NC specification and effector genes (Sauka-Spengler and Bronner-Fraser, 2008b). For example, lamprey utilizes many of the same neural crest specification genes that are used in jawed vertebrates, including Id, AP-2, Snail, c-myc, FoxD-A and SoxE. However, the Ets1 and Twist genes that play important roles during early NC specification and migration in jawed vertebrates are not expressed in pre-migratory and migratory neural crest in the lamprey (Hopwood et al., 1989; Linker et al., 2000; Meyer et al., 1997; Sauka-Spengler et al., 2007; Tahtakran and Selleck, 2003). One possible explanation is that Ets1 and Twist were co-opted for NC specification after the divergence of jawless and jawed vertebrates.

Lamprey cranial ganglia and the cell contributions

Similar to jawed vertebrates, lamprey possesses placodes that arise from non-neural ectoderm in the head. Previous cell lineage tracing coupled with immunostaining experiments indicate that lamprey neurogenic placodes give rise to sensory neurons in opV (ophthalmic trigeminal), mmV (maxillomandibular trigeminal), lateral line and epibranchial ganglia (Modrell et al., 2014). Lamprey pax3/7 is specifically expressed in the opV, but not mmV, placode-derived cranial neurons. This feature is similar to cranial ganglia development seen in jawed vertebrates (Baker et al., 2002; Dude et al., 2009). Lamprey neural crest cells also contribute to cranial ganglia, but do not overlap with expression of the pan-neural marker HuC/D, and have been considered to be presumptive glial cells (Modrell et al., 2014). Thus, whether neural crest cells contribute to cranial sensory neurons in the ganglia, and the role of neural crest in ancestral vertebrate cranial ganglia development are still unknown.

To better understand possible evolutionary origins of the cranial sensory nervous system in vertebrates, I used the sea lamprey as a model to study the development of cranial ganglia, examining the contribution of neural crest and placode cells to these structures in this primitively jawless vertebrate. The results of these studies have implications for understanding the ancestral scenario of placode and neural crest functions during early cranial ganglia evolution. This study reveals changes in developmental processes of cranial ganglia formation in jawed vertebrates after their divergence from jawless vertebrates.

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FIGURE LEGENDS

Figure 1.1. Summary of neural progenitor regions in the developing vertebrate neural tube. Glial cells and neurons in vertebrates share progenitor regions. Pd1-6 (dorsal progenitor) domains produce interneurons and glial cells in the dorsal neural tube. P0-p3 (ventral progenitor) domains produce interneurons and glial cells in the ventral neural tube. pMN (motor neuron) domain produce motor neurons and oligodendrocyte precursors. CC, central canal; FP, floor plate; ML, mantle layer; MN, motor neurons; MZ, marginal zone; NVZ, neuroepithelial ventricular zone; OPC, oligodendrocyte precursor cells; RP, roof plate.

Figure 1.2. Neural crest origin from neural plate border {Modified from (York et al., 2017)}. (A-C) illustrate the cross sections of vertebrate embryos. (A) medial neural plate (Venkatesh et al.) is flanked with neural plate border (red) and lateral epidermal ectoderm (purple). (B) Pre-migratory neural crest are specified from neural plate border when neural tube begin folding. (C) Neural crest go through EMT and migrate ventrally and differentiated into different cell types as illustrated in (D).

FIGURES

Figure 1.1. Summary of neural progenitor regions in the developing vertebrate neural tube.

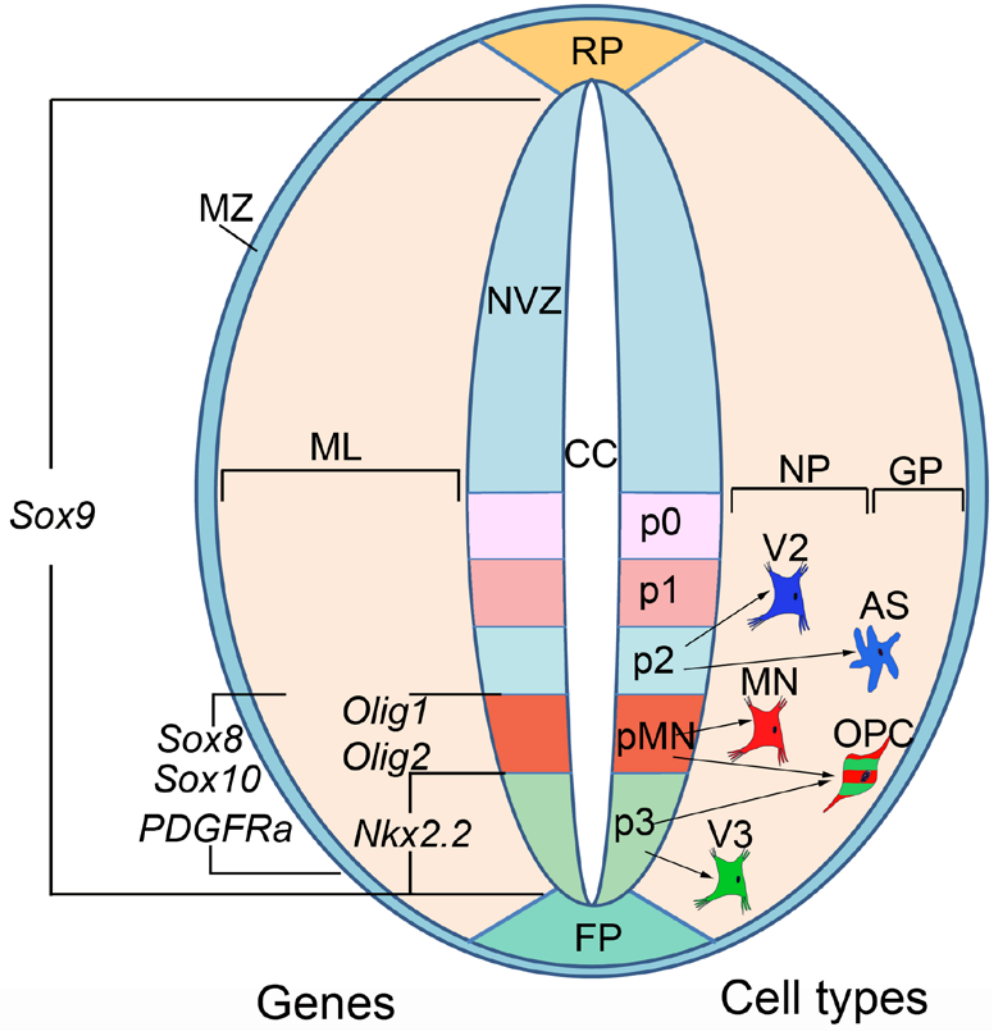
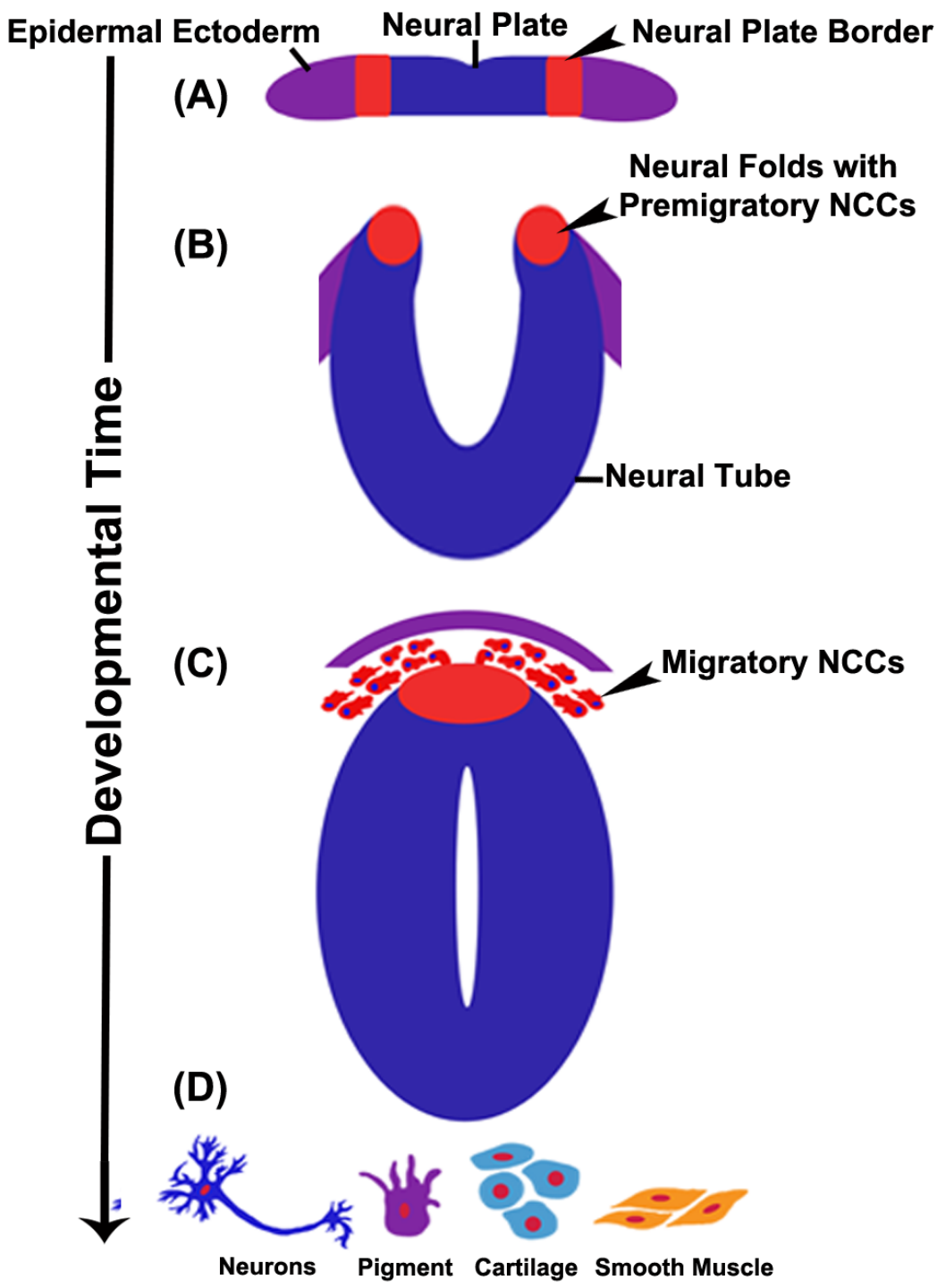


Figure 1.2. Neural crest origin from neural plate border



CHAPTER 2: GLIOGENESIS IN LAMPREYS SHARES GENE REGULATORY INTERACTIONS WITH OLIGODENDROCYTE DEVELOPMENT IN JAWED VERTEBRATES

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ABSTRACT

Glial cells in the nervous system regulate and support many functions related to neuronal activity. Understanding how the vertebrate nervous system has evolved demands a greater understanding of the mechanisms controlling evolution and development of glial cells in basal vertebrates. Among vertebrate glia, oligodendrocytes form an insulating myelin layer surrounding axons of the central nervous system (CNS) in jawed vertebrates. Jawless vertebrates lack myelinated axons but it is unclear when oligodendrocytes or the regulatory mechanisms controlling their development evolved. To begin to investigate the evolution of mechanisms controlling glial development, we identified, in a primitively jawless vertebrate, the sea lamprey, key genes required for the differentiation of oligodendrocytes in gnathostomes, including *Nkx2.2, SoxE* genes, and *PDGFR*. We analyzed their expression, and used CRISPR/Cas9 genome editing to perturb their functions. We show that orthologs required for oligodendrocyte development in jawed vertebrates are expressed in the lamprey ventral neural tube, in similar locations where gnathostome oligodendrocyte precursor cells (OPC) originate. In addition, they appear to be under the control of conserved mechanisms that regulate OPC development in jawed vertebrates and may also function in gliogenesis. Our results suggest that although oligodendrocytes first emerged in jawed vertebrates, regulatory mechanisms required for their development predate the divergence of jawless and jawed vertebrates.

INTRODUCTION

Animal nervous systems are composed of neurons and glia. Neurons transmit electrical impulses that coordinate sensory and motor responses by the animal to its environment. Glia provide structural, nutritive, and maintenance support for neurons (Jakel and Dimou, 2017). Recently, there has been increasing interest in glial cells as it has become clear that they participate in many important aspects of activity within the nervous system (Banerjee and Bhat, 2007; Barres, 2008; Min and Nevian, 2012; Nave and Trapp, 2008; Sakry et al., 2015), as reflected by a marked increase in the glia/neuron ratio during animal evolution. For example, glial cells make up only 10% of the *Drosophila* nervous system, whereas they may contribute nearly 50% of cells present in the human brain (Azevedo et al., 2009; Herculano-Houzel, 2014; Ito et al., 1995). Therefore, understanding mechanisms regulating development and evolution of glial cells will be instructive for explaining the evolution of animal nervous systems.

A key evolutionary innovation of the vertebrate nervous system is the formation of myelinating glia that surround and insulate axons to increase the speed of electrical conductivity, facilitating rapid responses by animals. All jawed vertebrates (gnathostomes) possess myelinated axons in both the central and peripheral nervous systems, but myelin is absent in living basal jawless (agnathan) vertebrates (Bullock et al., 1984). In gnathostomes, the myelin sheath is formed by two different glial cell types. Oligodendrocytes originating in the ventral neuroepithelium of the neural tube myelinate axons within the central nervous system (CNS) (Compston et al., 1997; Nave, 2010; Sherman and Brophy, 2005), while neural crest-derived Schwann cells ensheath axons of peripheral neurons (Kettenmann and Ransom, 1995).

Oligodendrocytes are considered to be unique to jawed vertebrates and are derived from precursor cells in the ventral neural tube. Oligodendrocyte precursor cells (OPCs) arise primarily from the pMN and p3 ventral neuroepithelial domains along the neural tube (Fu et al., 2002; Kessaris et al., 2008) from which motor neurons and V3 interneurons respectively are also derived (Fig. 2.1) (Briscoe and Ericson, 2001; Zhuang and Sockanathan, 2006). Newly formed OPCs migrate from the pMN and p3 domains toward the marginal zone, where cells from both domains express key regulatory factors including the SoxE genes (*Sox8*, *Sox9*, and *Sox10*), *Olig1*, *Olig2* and *Nkx2.2* (Fu et al., 2002). *Olig1* and *Olig2* also regulate differentiation of motor neurons while *Nkx2.2* regulates the differentiation of V3 interneurons and perineurial glia (Holz et al., 2010; Zhou and Anderson, 2002). On the other hand, functions of SoxE genes in the CNS, particularly *Sox8* and *Sox10*, are restricted to OPCs (Pozniak et al., 2010; Stolt et al., 2004; Stolt et al., 2005). *Sox9* expression in presumptive neural stem cells throughout the neuroepithelial ventricular zone specifies glial fate and blocks neurogenesis in the CNS (Stolt et al., 2003). *Sox9* deletion in the mouse model reduces numbers of OPCs, while mice lacking *Sox10* have normal numbers of OPCs that fail to mature, indicating a terminal differentiation role for *Sox10* in oligodendrocyte development (Stolt et al., 2002). Finally, platelet derived growth factor receptors (*PDGFR*) respond to PDGF signals to promote OPC survival; in mice, the survival and migration of OPCs largely depends on PDGF-AA in the CNS (Baroti et al., 2016; Stolt et al., 2006). These findings identify a core conserved set of transcription factors and an intercellular signaling system that together coordinate the development of OPCs in jawed vertebrates (Fig. 2.1).

Our understanding of both central and peripheral gliogenesis in lampreys is rudimentary. Peripheral glial cells have been described in lampreys and were found to surround the lateral line system, yet these cells do not form myelin sheaths (Gelman et al., 2009). In the lamprey CNS, radial glial cells express keratin-like proteins that have been observed in both larval and adult lampreys (Merrick et al., 1995). Other studies have suggested that myelinating glial cells, such as oligodendrocytes, are not present in lamprey (Bullock et al., 1984). Although the origin of myelinating glia represents a key step in early vertebrate evolution, how these cells evolved is unknown. Mechanisms regulating the development and differentiation of glia in lamprey have not been explored and therefore may provide key insights into how vertebrates acquired myelinating glia.

As a starting point, we chose to investigate whether mechanisms controlling the development of oligodendrocytes are spatiotemporally conserved in lamprey. To understand if the evolutionary origin of glial cells such as oligodendrocytes predated agnathan-gnathostome divergence, we asked if genes controlling OPC development in gnathostomes are expressed in similar locations of the developing CNS in lamprey. Alternatively, the regulatory circuitry controlling CNS glial cell fates may have arisen independently in these groups.

To test our hypothesis, we examined the developmental expression and function of homologs to genes in lamprey that are known to regulate oligodendrocyte development in jawed vertebrates. While we have been unable to identify *Olig1* or *Olig2* homologs in lamprey, our results show that lamprey SoxE genes and *Nkx2.2* are expressed in the ventral neural tube, similar to gnathostome homologs expressed in

pMN and p3 domains where OPCs originate. Later in development, *PDGFRab* and *SoxE1* are detected in putative glial cells lateral to the neural tube and notochord. Functional perturbations using CRISPR/Cas9 genome editing suggest genetic interactions similar to what have been observed in jawed vertebrates may control development along the lamprey ventral neural tube, and are suggestive of gliogenic function. We discuss our findings in the context of developmental mechanisms controlling the evolution of glial differentiation in early vertebrates.

MATERIALS AND METHODS

Embryo collection and fixation

Spawning adult sea lamprey, *Petromyzon marinus*, were collected from streams near Hammond Bay Biological Station, Millersburg, MI, and shipped to the University of Oklahoma. Animals were held in a recirculating water system maintained at 14°C. Eggs were manually stripped from individual females and fertilized *in vitro* with sperm obtained from mature males. Embryos were held in 0.05X Marc's Modified Ringer solution (MMR) at 18°C under constant flow (Lakiza et al., 2011). Embryos at selected stages (T22-26) (Tahara, 1988), were fixed in a 4% MEMFA solution (0.1 M MOPS pH7.4, 2mM EGTA, 1mM MgSO4 and 4% Paraformaldehyde) overnight at 4°C, dehydrated into methanol, and stored at -20°C for later use (McCauley and Bronner-Fraser, 2002a). All experiments were conducted according to the University of Oklahoma, Institutional Animal Care and Use Committee, protocol R15-027.

Isolation of gene sequences and phylogenetic analysis

Searches of the *Petromyzon marinus* genome on the UCSC Genome Browser, Sep 2010 WUGSC 7.0/petMar2 assembly (Smith et al., 2013) were cross referenced with Nkx2.2 sequence (AB583552) from the Arctic lamprey *Lethenteron camtschaticum* (formerly *Lethenteron japonica*) to obtain a putative Nkx2.2 DNA sequence from *P. marinus.* Exons were predicted using the GENSCAN Web Server. Sequences were amplified from an embryonic cDNA library (kindly provided by J. Langeland) or from cDNA synthesized from mRNA isolated from embryos. *P. marinus* Nkx2.2 was PCR amplified using specific primers (see Table 2.2). PDGFRab sequence was obtained

from the March 2007 *P. marinus* genome on the UCSC Genome Browser Gateway (WUGSC 3.0/petMar1). A partial cDNA fragment was amplified by PCR and both gene sequences were cloned into the pGEM T-easy vector (Promega) for sequencing and identification. Oligonucleotide sequences are shown in Table 2.2. Predicted amino acid sequences were aligned with related proteins using ClustalW in MEGA 6.0 (Tamura et al., 2013). Accession numbers for amino acid sequences used in our alignments are provided in the supplementary information. Maximum Likelihood analysis performed in MEGA 6.0 was supported by bootstrapping with 1000 replicates.

CRISPR/Cas9 and Microinjection

Guide RNA (gRNAs) were synthesized following protocols described previously (Hwang et al., 2013a; Hwang et al., 2013b; Square et al., 2015; Zu et al., 2016a). Deletion sites for each gene targeted are listed in Table 2.1. To minimize off target effects, target sequences were BLAST searched against the sea lamprey genome to ensure the 10 nucleotides proximal to the PAM site had no absolute matches with other locations in the genome that were similar to the gRNA sequence. Target sites were chosen to be close to the 5' end of coding sequence. Two random nucleotide sequences not present in the lamprey genome (RgRNA1, RgRNA2) were used to control for toxicity effects (Table 2.1), and were used as negative controls in knockout experiments. Cas9 protein (PNA Bio) was prepared according to manufacturer's instructions. Zygote stage embryos were injected with a ~ 5 nl cocktail containing 800 pg gRNA,1000 pg Cas9 protein, and 10% fluorescein dextran lineage tracer (Square et al., 2015). Embryos

were screened for fluorescence as an indicator of successful injection and uninjected embryos were discarded.

Genotyping

Post hoc PCR validation of CRISPR/Cas9 gene editing was performed on individual embryos following *in situ* hybridization (ISH) (York et al., 2017). After ISH and whole mount imaging, regions of interest were vibratome sectioned, and the remainder of each embryo was held in lysis buffer (200 mM NaCl, 100 mM Tris-HCl, pH 8.5, 5 mM EDTA 0.2% sodium dodecyl sulfate, 100 μg ml^{-1} proteinase K) overnight at 50°C. Genomic DNA was extracted from individual embryos and purified by ethanol precipitation. To validate CRISPR deletions, primer sets flanking CRISPR gRNA target sites (Table 2.3) were used to PCR-amplify deletion sites, which were confirmed by DNA sequencing.

For validate CRISPR/Cas mutation efficiency, 5 embryos at stage T26 were randomly picked from each injected batch. Genomic DNA was extracted as described above. DNA fragments flanking the CRISPR gRNA target sites were PCR-amplified and subcloned into the pGEM T-easy vector. Twenty clones from each putative mutant were selected and sequenced (see Supplementary Information).

Gene expression analysis and microscopy

Fixed embryos held in 100% methanol were rehydrated into PBST and ISH performed as described (Nikitina et al., 2009b). Following ISH, embryos were

embedded in 5% agarose and vibratome sectioned (20 μm) for imaging and analysis. Note: in early stage embryos, the notochord ventral to the neural tube is sometimes missing in transverse sections as a result of sectioning artifacts (see Figs. 2.2, 2.3, 2.4, 2.5).

Immunohistochemistry

Immunohistochemistry was performed either as a monolabel or after whole mount *in situ* hybridization in co-labeling experiments. Monolabeling for all antibodies was performed using standard procedures (described below), with an added proteinase K digestion step (20μg ml^{-1} for 10 mins) for LCM29 to enhance antibody penetration. Sectioned embryos were blocked (1 hr at room temperature) in TBS (12 mM Tris pH 7.5, 150 mM NaCl) supplemented with 2 mg ml^{-1} BSA, 0.1% (v/v) Triton-X-100, and 20% heat inactivated normal goat serum, followed by overnight incubation at 4°C in primary antibody diluted as described (LCM29, 1:500, gift from M. Selzer, Temple University; RMO270 anti-NFM, 1:300, Invitrogen; anti-HuC/D,1:300, Invitrogen; anti-HB9,1:10, Developmental Studies Hybridoma Bank). Sections were then washed in TBST (TBS with 0.1% Tween-20) three times for 10 min each at room temperature. Alexa 546 or horseradish peroxidase (HRP) conjugated secondary antibodies were diluted in blocking solution (1:500) and incubated for 2 hours at RT then washed 3X5 min in TBST. HRP-conjugated secondary antibody was developed in diaminobenzidine solution (Thermo Fisher) and the color reaction was monitored visually, then halted by replacing DAB with TBST. Alternately, sections labeled using Alexa 546-conjugated secondary antibodies were either washed and counterstained with 4′,6-diamidino-2-

phenylindole (DAPI, 0.5μg ml^{-1} in TBST), or imaged immediately after washing. For whole mount IHC, following 1 hour blocking as described above, embryos were incubated in the primary antibody overnight, followed by 3X30 min washes in TBST and a second 1 hour incubation in blocking solution at RT. Incubation in secondary antibodies (goat-anti mouse Alexa 546, goat anti mouse-HRP) (overnight at 4°C) was followed by washes (3X30 min) in TBST and embryos were then developed in DAB solution for coloration, or were imaged immediately using a Zeiss #43 Filterset (Alexa 546). Images were collected using a Zeiss Axioimager Z1 equipped with an Apotome structured illumination module and Zeiss Axiovision v 4.6.1. Image stacks were rendered as maximum intensity projections using the Inside 4D module of the Axiovision software package.

Statistical Analysis

The length of IF-keratin+ M_V cells was measured on 10 sections at different axial levels from 3 wildtype or CRISPR mutants, using the measurement function of the AxioVision software. Statistical analysis was performed using R (Team, 2013) and significance determined by one-way analysis of variance (ANOVA) with Tukey's Honestly Significant Difference multiple comparison post hoc test.

RESULTS

In jawed vertebrates, SoxE genes (*Sox8*, *Sox9* and *Sox10*) have been shown to regulate oligodendrocyte development (Finzsch et al., 2008; Stolt et al., 2002; Wegner, 2008). To begin to investigate gliogenesis in the developing lamprey, we first examined expression of lamprey SoxE orthologs within the CNS. In the CNS of gnathostomes, *Sox8* and *Sox10* are primarily expressed in oligodendrocyte precursors in the ventral neural tube, but not in neurons (Stolt et al., 2004). In zebrafish, *Sox10* is also reportedly expressed in a small population of motor exit point glia (MEP glia) (Smith et al., 2014). *Sox9* is expressed in both OPCs and in radial glia (Stolt et al., 2003). Thus, SoxE genes act as important regulators specific to OPC glial development in jawed vertebrates. Among the lamprey SoxE homologs, *SoxE1, SoxE2* and *SoxE3* (Lakiza et al., 2011; McCauley and Bronner-Fraser, 2006), *SoxE2* is orthologous with gnathostome *Sox10*, *SoxE3* groups with *Sox9* (Lee et al., 2016; McCauley and Bronner-Fraser, 2006), but the orthology of *SoxE1* remains uncertain (Lee et al., 2016; but see Zhang et al., 2006).

To examine SoxE expression in the context of a putative role in gliogenesis, we first determined *SoxE1* expression along the trunk (Fig. 2.2A-D). Transverse sections revealed *SoxE1* transcripts localized both internal and external to both sides of the basal surface along the ventral neural tube (arrows in Fig. 2.2B) in the Tahara late stage T24 embryo (10 days post fertilization; E10) (Tahara, 1988). At T26, *SoxE1* expression is visible as segmental dorsoventral stripes along the anteroposterior axis (arrow in Fig. 2.2C), that in transverse sections reveal expression adjacent to the neural tube and notochord (arrows in Fig. 2.2D).

In the early stage T24 embryo (embryonic day 9), *SoxE2* is expressed in the ventrolateral neural tube (black arrows in Fig. 2.2F) and also immediately outside the ventral neural tube (double small arrows in Fig. 2.2F). Dorsally, *SoxE2* is expressed in dorsal root ganglia (DRG) and inside the neural tube adjacent to DRG (red arrow in Fig. 2.2F). Its expression in the neural tube is comparable to *Sox10* expression in oligodendrocytes of gnathostomes, along the marginal zone of the neural tube that will myelinate motor and sensory axons of the CNS (Park et al., 2002; Stolt et al., 2005). In the whole mount T24 embryo (Fig. 2.2E), *SoxE2* expression is visible in the otic vesicle (black arrow) and in the epibranchial region along the pharynx (black arrowheads), as well as segmentally along the trunk (red arrows) in developing DRGs. By T25, *SoxE2* expression is absent from the neural tube (asterisks in Fig. 2.2H) but remains in DRGs along the trunk region (Fig. 2.2G, H).

SoxE3 is expressed in the ventral half of the neuroepithelium (black arrow in Fig. 2.2J) as well as in migrating neural crest (arrowheads in Fig. 2.2I, J) at stage T23, with continued neuroepithelial expression at T24 (arrow in Fig. 2.2L), whereas neural crest expression is diminished at this stage (Fig. 2.2K,L) (see also Zhang et al., 2006). *SoxE3* expression is comparable to *Sox9* in the neuroepithelium of the developing mouse CNS (Finzsch et al., 2008; Stolt et al., 2003).

Nkx2.2 is a key regulator of OPC and V3 interneuron specification and differentiation among jawed vertebrates (Briscoe et al., 2000; Fu et al., 2002; Qi et al., 2001; Yang et al., 2010). Previous work in the Arctic lamprey (*Lethenteron camtschaticum,* formerly *Lethenteron japonica*) showed that *Nkx2.2* is expressed in the ventral telencephalon, but trunk expression was not described (Sugahara et al., 2011).

Accordingly, we amplified a 716 bp DNA sequence from cDNA that was identified as a putative *Nkx2.2* gene fragment (Fig. 2.9A) and determined its early developmental expression in the nervous system. At stage T22, *Nkx2.2* is expressed ventrally along the anterior neural tube (arrows in Fig. 2.2M, N). At T23, expression extends caudally into the trunk region but has not expanded dorsally from its ventral neural tube domain (Fig. 2.2O, P). Later (T25), lamprey *Nkx2.2* continues to be expressed in the ventral neural tube (Fig. 2.2Q, R). Overall, lamprey *Nkx2.2* expression in the ventral neural tube appears similar to the p3 expression domain of *Nkx2.2* in jawed vertebrates (compare p3 region highlighted in Fig. 2.1 with *NKX2.2* expression domains illustrated in Fig. 2.2U, V).

Platelet-derived growth factor receptor-a (*PDGFR*a) is important for OPC development in jawed vertebrates, where it binds the PDGF-AA ligand in the CNS (Pringle et al., 1992; Pringle and Richardson, 1993). To determine if a *PDGFR* gene is expressed in the lamprey ventral neural tube, we isolated a single *PDGFR* sequence from a *P. marinus* cDNA library (kindly provided by J. Langeland). Our phylogenetic analysis places this gene as a basal homolog in the vertebrate *PDGFR*a and *PDGFR*b clade; accordingly we have named it *PDGFRab* (Fig. 2.9B). Our analysis does not eliminate the possible existence of other *PDGFRab* genes in the lamprey genome. Developmental expression of *PDGFRab* revealed that it is not expressed in the neural tube, as has been described in jawed vertebrates (Pringle and Richardson, 1993), but interestingly, at stage T25 its expression flanks the neural tube and notochord, similar to expression of *SoxE1* at T26, (Fig.2.2, compare arrow in 2T with 2D; see also Fig. 2.2U). Strong segmental expression of *PDGFRab* is also present lateral to each dermatome

(arrowheads in Fig. 2.2S, T), though the significance of this expression has not been established.

A comparative summary of expression patterns in lamprey and a gnathostome (mouse) for genes involved in oligodendrocyte development in the neural tube is shown in Figs. 2U,V. In sum, lamprey *Nkx2.2*, *SoxE1*, *SoxE2* and *SoxE3* are expressed in the ventral neural tube, similar to the expression domains of their gnathostome homologs involved in oligodendrocyte development in jawed vertebrates. *SoxE1* and *PDGFRab* are also expressed lateral to the neural tube and notochord at later stages (stage T25 and T26). Note that our comparative expression summary analysis does not include *Olig1* or Olig 2 since the lamprey orthologs to these genes have not been identified.

As shown above, *SoxE1* is expressed outside the ventral neural tube adjacent to its expression inside the neural tube at T24 (Fig.2.2B) and at this stage has been downregulated in neural crest (but see Uy et al., 2012). This raises the possibility that *SoxE1*-positive cells at this stage are derived from the CNS and migrate peripherally. One possibility is that *SoxE1* may play a role in motor axons or development of the glial cells that accompany them (e.g. motor exit point and perineural glia), since motor roots are known to extend peripherally through a ventrolateral trajectory (Clark et al., 2014; Lewis and Kucenas, 2014; Smith et al., 2014). To further characterize ventral expression of *SoxE1* along the trunk, neural tubes were dissected from stage T24 embryos (Fig. 2.3A) to reveal expression in punctate domains positioned basally along both sides of the ventrolateral neural tube (arrows in Fig. 2.3A), suggesting its expression in these domains may be related to positioning of motor axons exiting the neural tube, since motor neurons extend axon projections peripherally in a segmental

manner. To determine if *SoxE1* is localized to motor neurons, we examined its co-expression with the motor neuron marker, HB9 (Fig. 2.3B) (Arber et al., 1999). HB9-labelled cells are located in the ventral neural tube, but do not overlap with *SoxE1* (Fig. 2.3B; see also Fig. 2.1), suggesting *SoxE1*-expressing cells are unlikely to be motor neurons. Further, the lack of overlap between *SoxE1* and a pan-neuronal marker, HuC/D (Lin et al., 2002) confirms the non-neuronal identity of these cells (Fig. 2.3C-E).

Since *SoxE1* does not appear to be expressed neuronally, we considered whether *SoxE1*-positive cells lying adjacent to the neural tube and notochord cells may be glial (Fig. 2.2D). To test this notion, we next examined expression of vertebrate and lamprey-specific glial markers. Glial fibrillary acidic protein (GFAP) was shown recently to be expressed along lateral line glia (Green et al., 2017). We found that GFAP expression is present in radial glial cells along the neural tube, but does not appear to be expressed in cells that accompany sensory and motor axons in the developing lamprey (data not shown). A lamprey-specific monoclonal antibody, LCM29, was previously found to label keratin specific to intermediate filaments (IF) within glia, but not neurons, in larval and adult lampreys (Merrick et al., 1995; Uematsu et al., 2004; Zhang et al., 2014). We examined IF-keratin during embryogenesis (Fig. 2.4). At stage T24, IF-keratin localizes to motor roots that appear to extend beyond the ventrolateral surface of the neural tube, beneath the somitic mesoderm (arrowhead in Fig. 2.4A) and is also observed in the CNS (arrow in Fig. 2.4A). By T26, IF-keratin localizes to radial glia in the CNS, as has been described in larvae and adults (Merrick et al., 1995), along the trigeminal ganglion medial and ventral to the otic vesicle (OV) (Fig. 2.4B), as well as along other cranial sensory ganglia (Fig. 2.4C). At the same

stage, IF-keratin is expressed in both ventral (motor) and dorsal (sensory) roots along the trunk (Fig. 2.4D-F). Motor roots diverge into a dorsal branch flanking the neural tube (M_D) and a ventral branch flanking the notochord after they exit the neural tube (M_V) (Fig. 2.4E). This pattern is also confirmed by neurofilament expression (M_D and M_V in Fig. 2.4J). Note that motor and sensory axons do not merge into a single tract in lampreys, as they do in other vertebrates, but instead alternate within each somite along the trunk (Fig. 2.4D) (Matesz and Szekely, 1978) (Green et al., 2017) such that the dorsal branch of motor roots (M_D in Fig. 2.4E) can be distinguished from more laterally positioned dorsal root ganglia (DRG in Fig. 2.4F).

While LCM29-positive IF-keratin has been shown to be glial-specific in larval and adult sea lamprey (Merrick et al., 1995; Uematsu et al., 2004; Zhang et al., 2014), its restriction to glia during embryogenesis has not been established. Since glia and axons are tightly associated, and the observed expression pattern of IF-keratin in motor roots is similar to neurofilament expression in motor axons, we determined whether IF-keratin and neurofilament localize to separate glial and neuronal cell populations respectively in developing lamprey larvae. Co-labeling of neurofilament (NF) and IF-keratin expression reveals that along motor roots, keratin-positive cells accompany, but do not overlap with NF-positive axons (Fig. 2.4G-I), suggesting that IF-keratin is localized to glia, but is excluded from axons, during lamprey development.

To determine if *SoxE1* expression is associated with glia at locations where motor axons exit the neural tube, we examined co-expression of *SoxE1* and IF-keratin. At stage T24, segmental expression of *SoxE1* on the ventral neural tube (Fig. 2.3A) overlaps with IF keratin-positive motor root glia at MEPs (Fig. 2.4K), in support of the

suggestion that *SoxE1* expression in the ventral neural tube identifies MEPs. Taken together with our observation above that *SoxE1* expression does not overlap with a motor neuron marker (Fig. 2.3B), this further supports the possible glial identity of *SoxE1*-positive cells. To further test this notion, we examined co-expression of *SoxE1* and IF-keratin at stage T26, when *SoxE1* expressing cells are positioned adjacent to the neural tube and notochord (Fig. 2.2D). Their overlapping expression (lower arrow in Fig. 2.4L) suggests a possible glial identity of *SoxE1*-positive cells in motor roots, but due to technical limitations of double ISH/IHC chromogenic labeling that prevented achieving single cell resolution, we cannot exclude the possibility that *SoxE1* is expressed in both motor axons and glia, since they are so closely associated at this stage.

Next, we asked whether lampreys possess gene regulatory interactions among, *Nkx2.2, PDGFR,* and SoxE genes that could play roles in the context of putative gliogenesis. In jawed vertebrates, *Nkx2.2* and *PDGFR*a regulate *Sox10* expression to control OPC differentiation (Zhu et al., 2014). *Sox9,* on the other hand determines the glial fate of CNS stem cells in the neuroepithelium, including that of *Sox10*-positve OPCs (Stolt et al., 2003). Accordingly, we used the CRISPR/Cas-9 system to delete sequences from *Nkx2.2, PDGFR,* and SoxE genes in the lamprey embryo (Hwang et al., 2013a; Hwang et al., 2013b; Mali et al., 2013; Square et al., 2015; York et al., 2017) and examined SoxE and IF-keratin expression in mutant embryos (Figs. 2.5, 2.7). In *Nkx2.2* mutants, *SoxE1* expression is diminished outside the neural tube at T24 (asterisks in Fig. 2.5D) as compared with negative control embryos (arrowhead in Fig. 2.5A). At stage T26, *SoxE1* expression in *Nkx2.2* mutants is lost from regions flanking

the neural tube and notochord (compare arrow in Fig. 2.5B with asterisk in 2.5E). Since *SoxE1* expression overlaps with IF-keratin in glia that accompany motor axons (Fig. 2.4L), and is lost from this region in *Nkx2.2* mutants at T26 (Fig. 2.5E), we examined effects on glial-specific keratin expression along motor roots in *Nkx2.2* mutants. Notably, IF-keratin positive glia that support dorsal motor branches (M_D) are absent (asterisk in Fig. 2.5F) as compared to the control (M_D in Fig. 2.5C), while those along ventral motor branches (M_V) are significantly shorter compared to M_V in the wildtype (arrows in Fig. 2.5C,F). Since *Nkx2.2* expression is restricted to the ventral neural tube at all stages examined (Fig. 2.2M-R), this result suggests the regulation of IF-keratin positive glia that accompany motor axons outside the neural tube may be linked to gene expression that originates in the CNS. Although lamprey *PDGFRab* is not expressed in the ventral neural tube (Fig. 2.2T), we examined whether a possible regulatory link exists between *PDGFRab* and *SoxE1* in the lamprey PNS. Deletion of *PDGFRab* activity did not affect *SoxE1* expression at stage T24, where it remained localized to cells on both sides of the motor exit points along the ventrolateral neural tube (Fig. 2.5G). This result is not unexpected since *PDGFRab* expression is not observed in the lamprey neural tube. However, *SoxE1* and IF-keratin expression were absent from regions flanking the neural tube and notochord in *PDGFRab* mutants at T26 (compare arrows in Fig. 2.5B, C with the same region in 2.5H, I). Taken together, the observation that *PDGFRab* expression flanks the neural tube and notochord at T25 (Fig. 2T), coupled with loss of *SoxE1* and IF-keratin from this region in *PDGFRab* mutants (Fig. 2.5H, I) suggest this gene may play a role in regulating development of glia that accompany motor axons outside the neural tube. *SoxE3* is expressed in the ventral

neuroepithelium at T23 (Fig. 2.2I, J), and its deletion resulted in loss of *SoxE1* expression from the ventral neural tube at T24 (asterisk in Fig. 2.5J), and from cells flanking the neural tube at T26 (Fig. 2.5K; compare with Fig. 2.5A, B), as well as elimination of IF-keratin expression along motor roots (Fig. 2.5L), further supporting the possibility that gene interactions that control gliogenesis in jawed vertebrates may also play a role in this process in the lamprey. To determine if changes in *SoxE1* expression that we observed among CRISPR mutants could be related to gliogenesis, we examined IF-keratin expression in *SoxE1* CRISPR mutants (Fig. 2.5N). Similar to observations in *Nkx2.2*, *SoxE3* and *PDGFRab* knockout embryos (Fig. 2.5F,I,L), keratin-positive glia in the dorsal branch of motor roots (M_D) are eliminated in *ΔSoxE1* mutants, whereas the glial cells along the ventral branch of motor roots (M_V) are reduced in length (Fig. 2.5M, N). This result lends additional support to a putative role for *SoxE1* in gliogenesis. Taken together, these observations suggest that *Nkx2.2*, *PDGFRab,* and *SoxE3* may function in a regulatory network that mediates gliogenesis in motor roots, by regulating the expression of *SoxE1*. Specific embryos shown that contained a putatively mutant phenotype, as determined by gene expression analysis (Fig. 2.5) were also sequenced to link gene expression phenotypes with individual genotypes. Embryos shown in Fig. 2.5 contained only mutant alleles for each gene that was examined (Fig. 2.11). Additional validation of CRISPR deletion efficiency is described below.

To quantify mutant effects on glial cells in the ventral branch of motor roots, we measured the length of ventral root glia in *Nkx2.2*, *PDGFRab*, *SoxE1* and *SoxE3* mutants (Fig. 2.5M). Maximum intensity projections were made from image stacks for

each mutant, and the length of 10 ventral roots from 3 embryos of each mutant class (30 ventral roots measured in total for each mutant) was determined by measuring the distance that IF-keratin-positive glia emerged from motor exit points along the neural tube. For each loss-of-function mutant with a putative role in gliogenesis (*Nkx2.2*, *PDGFRab, SoxE1,* and *SoxE3*), glial distance from the neural tube measured 25.43±4.6, 28.76±5.2, 19.80±5.6, and 13.93±5.4 μm respectively (Fig. 2.5M). By contrast, the length of IF-keratin-positive glia along ventral roots in control gRNA and *n-myc* negative controls measured 164.74±8.4 and 175.9±9.9 μm respectively ($p<2e-16$ in all cases; pairwise comparisons of lengths, and statistical analyses, are provided in Table 2.4). *n-myc* was chosen as an additional negative control to confirm gliogenic specificity because it is a transcription factor that is expressed in the neural tube and is required for neural crest development, but its role is unrelated to gliogenesis (Sauka-Spengler et al., 2007). Lengths of IF-keratin-positive glia along motor roots of *n-myc* mutant embryos were not significantly different from random gRNA controls (n=16/16, p=0.65; Fig. 2.5M, O). Interestingly, radial glial cells in CRISPR mutants appear unaffected relative to controls, suggesting that observed effects are restricted to motor root glia distal to the motor exit points along the neural tube, and radial glia are likely controlled by a different regulatory program (RG in Fig. 2.5F, I, L, and N, compared with 5C and O).

To exclude the possibility that the loss of motor root glia from CRISPR mutants is related to elimination of motor axons, we examined neurofilament (NF) expression in motor roots along the trunk of mutant embryos (Fig. 2.6). Motor axons are unaffected in *PDGFRab, SoxE1* and *SoxE3* deletion mutants compared to the control group (compare

Fig. 2.6C-E with Fig.6A). Interestingly, although the ventral branch of motor axons in *Nkx2.2* mutants is also unaffected (n=16/16, Fig. 2.6B, arrow) the dorsal branch of motor axons was absent from 7 out of 16 *Nkx2.2* mutants examined (asterisk in Fig. 2.6B; compare with M_D in Fig. 2.6A). These results suggest that the effects on length of M_V motor root glia in the mutants analyzed is not related to motor axon projections, but *Nkx2.2* may play a role in formation of the M_D that has not been characterized.

In all mutants analyzed, *SoxE1* expression was retained in prechondrogenic neural crest cells of the pharyngeal arches (Fig. 2.10). This observation suggests that perturbation effects on putative gliogenic regulatory genes (*Nkx2.2*, *PDGFRab*, *SoxE3*) were not global and are unrelated to the chondrogenic role of *SoxE1* in neural crest that has been described elsewhere (Lakiza et al., 2011; McCauley and Bronner-Fraser, 2006). To this point, our results support the notion that a regulatory link may exist in lamprey between *Nkx2.2*, *PDGFRab* and *SoxE3* activity (particularly in the ventral neural tube and flanking the CNS), with regulation of glial-specific IF-keratin expression at stage T26 (Fig. 2.5E, H, K).

Since *SoxE2* (the lamprey *Sox10* ortholog) is expressed in both the ventral neural tube and in neural crest-derived DRGs flanking it (Fig. 2.2F), we next asked whether these expression domains are under different regulatory programs. Importantly, both *Nkx2.2* and *Sox9* have been shown to regulate expression of *Sox10* within OPCs during development (Stolt et al., 2003; Zhu et al., 2014). Deletion of *SoxE3* (lamprey *Sox9*) eliminated expression of *SoxE2* from the CNS, with reduced expression in DRGs and the otic vesicle (Fig. 2.7C,F) as expected from previous results (Lakiza et al., 2011). However, deletion of *Nkx2.2* eliminated only ventral neural tube expression, but *SoxE2*

expression in DRGs remained unaffected (Fig. 2.7B, E). Genotypes of *Nkx2.2* and *SoxE3* individual mutants illustrated in Fig. 2.7 are shown in Fig. 2.11. These results support a conserved genetic regulatory mechanism in lamprey involving *Sox9, Nkx2.2* and *SoxE2/Sox10* in the developing ventral neural tube that may be related to the glial-specific effects we observed in these mutant embryos.

To validate CRISPR/Cas9 deletion efficiency, five embryos were picked randomly from each injected batch at stage T26, prior to any phenotypic analysis. Genomic DNA from each group was extracted and pooled, and flanking DNA sequence encompassing the gRNA target sites was amplified (primers for genotyping are listed in Table 2.3). Twenty clones from each group of 5 injected embryos were selected at random and sequenced (Fig. 2.13). All of the embryos selected at random contained mutant alleles. *Nkx2.2*, *PDGFRab, SoxE1, SoxE2* and *SoxE3* CRISPR/Cas9 knockouts contained 95%, 100%, 90%, 90% and 100% mutant alleles respectively (Fig. 2.13), suggesting that mosaic embryos containing a wildtype allele occurred only infrequently, and that our mutagenesis protocol resulted in bi-allelic deletions at target loci (Zu et al., 2016).

DISCUSSION

Deciphering the origin of vertebrate gliogenesis is important for understanding how glial complexity has increased to support behaviors characteristic of vertebrates. To begin to understand the evolution of vertebrate gliogenesis, we investigated the expression and function of sea lamprey homologs to key regulators of gliogenesis in jawed vertebrates, focusing on genes that are known to be involved in the development of oligodendrocyte precursor cells—an evolutionary innovation of jawed vertebrates.

To compare the gene regulatory relationships that are revealed by our data for lamprey gliogenesis, with regulatory mechanisms required for oligodendrocyte development in jawed vertebrates, we assembled a core OPC gene regulatory network (GRN) module for gnathostomes from the literature (Fig. 2.8A) to highlight established *cis*-regulatory interactions (solid lines) as well as likely interactions inferred from perturbation studies (dashed lines). Signaling molecules including Sonic hedgehog (shh) and FGF may be required to initiate OPC-specific expression of transcription factors, such as *Olig2, Nkx2.2* and Sox*E* genes), with additional signaling mediated through *PDGFRa* (Hu, 2009). *Nkx2.2* controls oligodendrocyte differentiation by negatively regulating *PDGFR*a to indirectly control *Sox10,* and also regulates expression of *MBP* and myelin proteolipid protein, *PLP/DM20* through a separate pathway (Qi et al., 2001; Zhu et al., 2014). *Sox9* appears to determine glial fate in neural stem cells within the neuroepithelium, where its deletion increased the number of motor neurons at the expense of oligodendrocytes (Stolt et al., 2003). *Sox8* and *Sox10* appear to be functionally redundant during OPC development since *Sox10* directly regulates *MBP*

expression, but in its absence, *Sox8* is able to compensate (Stolt et al., 2004; Stolt et al., 2002).

During oligodendrocyte differentiation, transcriptional regulators control each other through complex feedback and feed forward regulatory interactions. For example, *Olig2* directly binds the enhancer of *Sox10* to activate its expression (Kuspert et al., 2011; Lu et al., 2002), while *Sox10* simultaneously modulates *Olig2* expression (Li et al., 2007). In the pre-myelinating stage, transcriptional regulation of myelin genes is accomplished through a series of feed-forward mechanisms. For example, following *Olig2* upregulation of *Sox10*, cooperative binding by *Sox10* and *Olig2* activates expression of *Myrf* (myelin regulatory factor) by directly binding its intron 1 enhancer (Emery, 2013; Hornig et al., 2013). Finally, *Myrf* and *Sox10* together drive expression of *MBP* and *PLP/DM20* in order to maintain the multi-layered insulating cell membrane surrounding axons (Bujalka et al., 2013; Emery, 2013).

While our analysis to this point is incomplete, our results suggest that molecular mechanisms regulating gliogenesis in lampreys contain core elements of the GRN that is required for OPC specification and differentiation in jawed vertebrates, including activity by *Nkx2.2*, *PDGFR* and SoxE genes (Fig. 2.8B). Additionally, several regulatory links among those genes appear to be conserved between lamprey and jawed vertebrates (Fig. 2.8B) despite apparent differences in the precise regulatory relationship (e.g., activation versus repression roles discussed below), as well as differences in the downstream effectors of glial differentiation. Our results suggest that the core regulatory mechanisms for gliogenesis are an ancient feature of vertebrate glial biology. Gene expression data coupled with our functional analysis of gliogenesis in

lamprey strongly suggest that ancestral vertebrates likely deployed an *Nkx2.2-PDGFR-*SoxE axis as part of a larger regulatory network that was largely responsible for global control of glial cell development *sensu lato*, and that this feature is retained among all extant vertebrates studied to date. It should be noted that our analysis includes only a subset of the genes that are likely to be required for specification and differentiation of glia in the lamprey. For instance, Sonic Hedgehog (shh) is an important ventral determinant signaling molecule expressed in the notochord and floor plate, and is important for oligodendrocyte specification (Gunhaga et al., 2000; Nery et al., 2001). *Shh* expression drives ventral *Olig2*-expressing progenitors toward an OPC fate, and blocks their differentiation into motor neurons (Hu et al., 2009). The arctic lamprey possesses three copies of hedgehog with two of them (*LjHHa* and *LjHHb*) expressed in the notochord and floor plate (Sugahara et al., 2011). This raises the possibility that hedgehog signaling may also be involved in lamprey gliogenesis in the ventral neural tube, though this remains to be tested. As regulators of OPC development, *Olig1* and *Olig2* are of strong interest for investigating the development and evolution of oligodendrocytes, but to date have not been identified in the lamprey genome. Since *Olig2* is also necessary for differentiation of motor neurons (Lu et al., 2002), and lampreys possess HB9-positive motor neurons at a comparable ventral location of the neural tube (Fig. 2.3B), it is possible that lampreys may possess an as yet undescribed Olig gene that is involved in neuro- and gliogenesis.

While we have described aspects of gliogenesis that appear to be conserved between lamprey and jawed vertebrates, important differences are also seen. For example, in jawed vertebrates, *Nkx2.2* represses *Sox10* indirectly through its repression

of *PDGFRa* to control the timing of oligodendrocyte myelination (Fig. 2.8A) (Zhu et al., 2014). However, in lamprey, which lacks myelinated axons, *Nkx2.2* appears to induce *SoxE* gene expression to drive glial-specific expression of IF-keratin (Figs. 2.5, 2.6, 2.8B). Differences in species specific GRNs such as these are likely to reflect the independent evolutionary trajectory of jawless and jawed vertebrates across half a billion years of independent evolution. The vertebrate common ancestor may have possessed a core gene module to regulate gliogenesis. Following divergence of jawed and jawless vertebrates, independent modification (rewiring) of the ancestral GRN drove the evolution and development of different glial cell types in these groups. Modifications could have involved recruitment of different co-factors, or intermediate transcription factors. Interestingly, knock-down of *Nkx2.2a* in zebrafish decreases the number of *Sox10*-positive OPCs initially, but later this perturbation results in increasing *Sox10*-positive non-myelinating OPCs that arise from different progenitors (Kucenas et al., 2008). Thus, regulatory mechanisms in oligodendrocytes are complex and involve multiple cell populations. Further studies will be required to reveal precise *cis*-regulatory relationships within the GRN required for both lamprey gliogenesis and OPC development in jawed vertebrates.

Based on observations that the major OPC populations associated with CNS neurons originate from the ventral, rather than the more proximally positioned medial neuroepithelium, Richardson and colleagues speculated that OPCs may have first evolved to accompany motor axons, facilitating responses to environmental stimuli, (e.g., escape behavior) (Pringle and Richardson, 1993; Richardson et al., 2006; Richardson et al., 2000). Our results support this scenario. The expression of *Nkx2.2* is

restricted in the CNS. However, its deletion eliminated glial specific IF-keratin, as well as *SoxE1* expression at motor roots (Fig. 2.5E, F). This result suggests that lamprey motor root glia in the periphery may be CNS-derived. Despite the deletion of dorsal branch motor axons (M_D) in *Nkx2.2* mutants, the ventral branches (M_V) remain unaffected (white arrow in Fig. 2.6B) suggesting the loss of glia along motor roots is unrelated to loss of axons. Furthermore, cell lineage tracing experiments we have conducted (data not shown) as well as recently published work from the Bronner group suggest that lamprey trunk neural crest are unlikely to migrate medially and do not contribute to glial populations along motor roots (Green et al., 2017). Such data provide additional supporting evidence that lamprey motor root glia outside the neural tube may be CNS-derived, regulated by genes required for OPC development among jawed vertebrates. CNS-derived glia migrating to the PNS have been reported in other animals. For instance, the MEP glia and perineurial glia in jawed vertebrates arise in the CNS, but become positioned along the PNS following their differentiation (Smith, 2014; Clark, 2014).

Lamprey motor root glia that express glial specific IF-keratin (Fig. 2.4E) are negative for glial fibrillary acidic protein (GFAP), even though there are GFAP positive cells elsewhere in the developing lamprey (Green et al., 2017). Nonetheless, their development appears to be controlled by regulatory interactions involving *Nkx2.2*, *PDGFR* and SoxE genes (Figs. 2.5, 2.8). This suggests deep homology where glial cell types in lamprey and jawed vertebrates utilize a conserved gene regulatory network to control the differentiation of functionally similar glial cells (Halder et al., 1995; Shubin et al., 2009). We suggest that the ancestral gliogenic GRN involving activity of an

Nkx2.2-PDGFR-SoxE axis (Fig. 2.8) has been independently rewired to accomplish the evolution of novel, but lineage-specific glial cell types, that may not be homologous among jawed and jawless vertebrates, though this remains uncertain.

Extending regulatory conservation of the "glia/OPC" GRN module back to basal jawless vertebrates supports the notion that underlying regulatory mechanisms for a novel cell type in jawed vertebrates (i.e., OPCs) can be traced to a more general, evolutionarily ancient function in cell type specification. However, this raises questions regarding how the topology of the ancestral vertebrate network for glial development broadly could have been modified to facilitate the evolution of novel glial cells types, such as OPCs. We suggest that recruitment of new downstream pre-myelinating and myelinating genes (e.g., *Myrf, MBP, PLP-DM20*), into the ancestral vertebrate glial GRN was critical for the emergence of OPCs in jawed vertebrates since this novel cell type is directly responsible for producing myelinating oligodendrocytes. Increasing the transmission speed of action potentials along axons, a key evolutionary innovation that amplified glial and CNS evolutionary complexity, likely facilitated active predation as well as rapid escape behaviors.

Taken together, our results suggest a scenario in which a pre-existing gliogenic GRN drove differentiation of glia in the ancestral vertebrate, dependent in part on an *Nkx2.2-PDGFR*-SoxE axis. Following their divergence from jawless vertebrates, jawed vertebrates elaborated on this genetic scaffold to recruit additional downstream genes, which presumably allowed for the lineage-specific evolution of novel glial cell types, such as myelinating oligodendrocytes. In sum, we have performed expression and functional analysis of genes in lamprey that are important for glial cell development,

with a focus on genes that are orthologous to those required for OPC development in jawed vertebrates. We show that the regulatory roles of lamprey SoxE, *Nkx2.2*, and *PDGFRab*, in the context of gliogenesis, highlight shared developmental relationships that are ancient features of vertebrate glial biology, and were likely rewired to facilitate the origin and evolution of novel glial cell types in early vertebrates.

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TABLE

Table 2.1. DNA sequences targeting gene-specific sites for deletion by CRISPR/Cas9

gRNA name	Target sequence PAM underlined
Nkx2.2g1	5’-GGCTCGACGGGAATCTTAGGAGG-3’
Nkx2.2g2	5’-GGAGGTTGTACTTGGTGGCGTGG-3’
Nkx2.2g3	5’-GGTAGCGCTGGTGGCGGAAGCGG-3’
PDGFRab	5’-GGAAAGCAGCCGCCCAAAGGCGG-3’
SoxE1	5’-GGGGACGCGGCGAGCCCCTGCGG-3’
SoxE2	5’-GGCGGCCGTGAGTCAAGTGCTGG-3’
SoxE3	5’-GGACATACGGGAGCTCAGTCGGG-3’
RgRNA1	5’-TAGGCGGGTAACGTCGTTAGCC-3’
RgRNA2	5’-TAGGTGGAAACCCCAACTTATT-3’

Table 2.2. Oligonucleotide sequences used to amplify lamprey *Nkx2.2* and *PDGFRab* gene sequences.

	Forward (5'-3')	Reverse (5'-3')
Nkx2.2	TCTCGAAGGCGCAGACGTTC	AGTGGAGGTTGTACTTGGTG
PDGFRab	TCTGCAATGCTCTTGAGCTC	TGGCTACAGGCAAACCACTC

Table 2.3. Oligonucleotides sequences used to amplify genomic DNA encompassing deleted sequences for *Nkx2.2, SoxE1, SoxE3,* and *PDGFRab* in CRISPR mutants.

	Forward (5'-3')	Reverse (5'-3')
Nkx2.2	CGCAGACGTTCGAGCTGGAG	GCGCACGTGTTCACTTCATC
SoxE1	TCCTGAACGAGAACGAGAAG	TCACGACACCGTAACCAATC
SoxE2	CCAGTCAGCATGTCGGATAC	GTGGATCGTCACGAGGTATC
SoxE3	CTTGCGAACAGCACTTGTCC	AAACGCTATACCGTGGGATC
PDGFRab	CTTGTTCTTCCGCAGACAAG	TTGCTCGATCACGATGGAAC

Table 2.4. Significance of difference in measurement length between experimental and control ventral branch motor root glia. Pairwise comparisons of each CRISPR mutant with either the negative control random gRNA or n-myc control. P-values determined, as described in Methods.

Pairwise comparisons	P value
Nkx2.2 – control gRNA	<2e-16
PDGFRab – control gRNA	<2e-16
SoxE1 – control gRNA	<2e-16
SoxE3 – control gRNA	<2e-16
Nkx2.2 – n-myc	<2e-16
PDGFRab – n-myc	<2e-16
SoxE1 – n-myc	<2e-16
SoxE3 – n-myc	<2e-16
n-myc – control gRNA	0.65

Table 2.5. Reference information for gene regulatory interactions illustrated in Fig. 8A.

up-stream gene	Down-stream gene	references
fgf	shh	Hu et al., 2009
shh	Sox10	Hu et al., 2009
	olig2	Hu et al., 2009
	Nkx2.2	Hu et al., 2009
Olig2	Sox10	Kuspert et al., 2011; Liu et al., 2007; Lu et al., 2002
	PDGFRa	Lu et al., 2002
	nkx2.2	Liu et al., 2007
	PLP/DM20	Lu et al., 2002
	MBP	Lu et al., 2002; Li et al., 2007
Sox9	Sox10	Stolt et al., 2003
	Sox8	Stolt et al., 2003
Sox10	Olig2	Liu et al., 2007
	Nkx2.2	Liu et al., 2007
	myrf	Hornig et al., 2013
	MBP	Li et al., 2007; Stolt et al., 2002
Sox8	PLP/DM20	Hornig et al., 2013
	MBP	Hornig et al., 2013; Stolt et al., 2004
PDGFRa	Sox10	Zhu et al., 2014
Nkx2.2	Sox10	Zhu et al., 2014
	PDGFRa	Zhu et al., 2014
	PLP/DM20	Qi et al., 2001
	MBP	Qi et al., 2001
Myrf	PLP/DM20	Bujalka et al., 2013
	MBP	Bujalka et al., 2013

FIGURE LEGENDS

Figure 2.1. Developmental origins of neural progenitor cell types and differential gene expression in ventral domains of the developing vertebrate neural tube. Glial cells and neurons in vertebrates share progenitor regions. p0 to p3 domains produce different glial and neuronal populations. The two ventral most domains, pMN and p3, where *Olig1*/2, *Nkx2.2*, *Sox8*, *Sox10*, and *PDGFR*a are expressed give rise to oligodendrocyte precursors (OPCs), as well as motor neurons and V3 interneurons (V3). AS, astrocytes; CC, central canal; FP, floor plate; GP, glial precursors; ML, mantle layer; MZ, marginal zone; NP, neuronal precursors; NVZ, neuroepithelial ventricular zone; p0-3, ventral progenitor domains (0-3); pMN, motor neuron progenitor domain; RP, roof plate; V2, V2 interneurons.

Figure 2.2. Expression of lamprey homologs of oligodendrocyte markers along the ventral neural tube. (A) Whole mount lateral view of *SoxE1* mRNA expression at stage 24. (B) Transverse section through embryo in A at level B', showing that *SoxE1* is expressed on both sides (arrows) of the ventral neural tube CNS-PNS interface. (C) Lateral view of *SoxE1* expression in dorsoventral stripes positioned along the anterior-posterior axis of the trunk in a T26 embryo. (D) Transverse section through (C): *SoxE1* expressing cells flank the neural tube and notochord (arrows). (E) Whole mount lateral view of *SoxE2* mRNA expression at stage 24. *SoxE2* is expressed in the otic vesicle (black arrow), cranial neural crest in the epibranchial region (arrowheads), and in dorsal root ganglia along the trunk (red arrows). (F) Transverse section through (E); similar to *SoxE1* (B), *SoxE2* transcripts are also localized on both sides of the CNS-PNS interface

(large and small double black arrows), in DRGs as well as within the dorsal neural tube adjacent to DRGs (red arrow). (G) *SoxE2* transcripts localized to DRGs along the trunk at T25. (H) Transverse section through (G) indicates *SoxE2* expression at a DRG, but lacking from the ventral CNS-PNS interface at T25 (asterisks). (I-L) *SoxE3* expression: *SoxE3* is expressed along the length of the neural tube at stages T23 and T24 (arrows in I and K). Transverse sections reveal *SoxE3* transcripts in the ventral ventricular zone at T23 (arrow in J), with expanded expression dorsally by stage 24 (arrow in L). Arrowhead in J indicates migrating neural crest. (M-R) *Nkx2.2* mRNA expression: At stage 22, *Nkx2.2* transcripts are observed internally in the cranial region (arrows in M), that in transverse section (N) reveals transcripts localized to the ventral neural tube. (O and P) By stage T23, *Nkx2.2* is expressed along the ventral neural tube and extending into the trunk region (arrows in O) but in transverse section reveals *Nkx2.2* transcripts are absent from the floor plate (dashed line in P). (Q and R) At T25, *Nkx2.2* transcripts are localized to the ventral neural tube along the anterior-posterior axis (R), with prominent expression in the cranial region extending into the forebrain (arrows in Q). *PDGFRab* expression is observed as dorsoventral stripes along the trunk (arrowhead in S). (T) A transverse section of the embryo shown in S indicates *PDGFRab* transcripts flanking the neural tube and notochord (arrows in T), as well as in dermatome beneath the ectoderm (arrowhead in T). (U, V) Summary diagrams illustrating spatiotemporal expression of homologs in lamprey (U) required for oligodendrocyte development in jawed vertebrates (e.g., mouse) (V). Colored shapes illustrate different gene expression domains at separate stages as shown. Dashed circle in B represents the notochord which is missing as a result of a sectioning artifact. FP, floor plate; MHB, mid-hindbrain

boundary; ML, mantle layer; MZ, marginal zone; N, notochord; NT, neural tube; NVZ, neuroepithelial ventricular zone; OV, otic vesicle; RP, roof plate; “E” in U and V indicates embryonic day of mouse development. Scale bar =50μm.

Figure 2.3. Non-neuronal expression of *SoxE1*. (A) Ventral view of a neural tube dissected from T24 stage embryo. *SoxE1* mRNA expression is visible in punctate domains along the ventral surface of the trunk neural tube (arrows). (B) Co-expression of *SoxE1* and the motor neuron marker, HB9, at stage T24: *SoxE1*-expressing cells in the ventral neural tube (black arrow) do not overlap with ventral motor neurons (red arrowheads). (C-E) Co-labeling of *SoxE1* (black arrow in C and E) and the pan-neuronal marker (HuC/D) (white arrows in D and E) in the ventral neural tube, shown as a single merged image in E. Note the lack of overlap between these markers (E). N, notochord; NT, neural tube. Scale bar =50μm.

Figure 2.4. Glial-specific expression of intermediate filament (IF) keratin in both CNS and PNS glia during embryonic stages. Embryos were processed for imaging using either the chromogenic reaction of an HRP or AP-conjugated secondary antibody with transmitted light, or a secondary antibody conjugated to Alexafluor 546 and processed using fluorescence microscopy. (A) Transverse section through trunk at stage T24. IF-keratin expression in motor glia projecting from the ventral neural tube (arrowhead) and in radial glia near the periphery of the CNS. (B) IF-keratin expression at stage 26 in radial glia (RG) of the CNS, and cells that accompany trigeminal neurons (V) in the hindbrain. (C) Whole mount lateral view of IF-keratin expression in cranial

glia at stage 26. (D) IF-keratin expression in lateral view of a whole mount stage 26 embryo illustrates motor (white arrowhead) and sensory (black arrowhead) glia. D illustrates IF-keratin expression as a composite Z-stack image of 2 different focal planes illustrating anteroposterior differences in the location of sensory and motor roots, that are also found at different mediolateral positions as illustrated in E and F. (E) dorsal (M_D) and ventral (M_V) motor root glia are positioned immediately adjacent to the neural tube (M_D) and notochord (M_V). IF-keratin is also expressed in radial glia (RG) within the CNS and along the lateral line (PLLN in C, E, F). (F) IF-keratin expression in glia associated with DRGs. (G-I) Double IHC depicting neurofilament in axons and IF-keratin in glia of the motor root. (G) Bright field IHC using alkaline phosphatase-conjugated secondary antibody to show NF expression in motor axons peripherally, and in CNS axons within the neural tube. (H) Two-channel image of the section shown in G, showing fluorescent labeling of IF-keratin, illustrating keratin expression on motor roots and in the neural tube. (I) Inset in H illustrates lack of overlap between NF-positive motor axons (black arrow) and IF-keratin-positive glia (white arrowhead). (J) Neurofilament staining of motor root axons (M_D, M_V) at stage 26. Alexafluor-546 fluorescence (pseudocolored green) was saturated in the NT marginal zone to reveal single axons in the motor root. (K) Co-expression of *SoxE1* (black arrow) and IF-keratin (red arrowhead) at stage 24: *SoxE1* and IF-keratin span both sides of the neural tube motor exit point. (L) Overlapping IF-keratin and *SoxE1* expression flanking the neural tube and notochord in the stage T26 embryo (black arrows). DRG, dorsal root ganglia; ETX, epibranchial tract of the vagal nerve; M_D, dorsal branch of motor root; M_V, ventral branch of motor root; MZ, marginal zone; N, notochord; NT, neural tube; OV,

otic vesicle; PLLN, posterior lateral line nerves; RG, radial glia; V, trigeminal nerves. Scale bar: 50µm. (D) Scale bars: 50µm.

Figure 2.5. *SoxE1* and IF-keratin expression in CRISPR/Cas mutants. *SoxE1* mRNA expression (bluish-purple) in control random gRNA injected embryos at stage 24 (A) and 26 (B) showing transcripts on both sides of the CNS-PNS interface (A) and adjacent to the neural tube and notochord (B) as seen in wild type embryos (Fig. 2.2B, D). In *Nkx2.2* deletion mutants (Δ*Nkx2.2*), *SoxE1* expression remains in the ventral neural tube (D) but has been lost outside the neural tube across the motor exit point at stage 24 (asterisks). In *PDGFRab* mutants, *SoxE1* expression is unaffected at stage 24 (G), while its expression is lost in Δ*SoxE3* deletion mutants from the same stage (J). In stage T26 embryos, *SoxE1* expression flanking the neural tube and notochord is eliminated in all mutants examined (compare *SoxE1* expression in B with E, H, and K). IF-keratin expression is lost from the dorsal branch of motor glia in all mutants examined, except for the Δ*n-myc* control mutant (red asterisks in F, I, L, N), while the ventral branches are reduced in length compared with the control shown in (C) (red arrows in C, F, I, L and N). (O) IF-keratin expression in both M_D and M_V motor roots was unaffected in *Δn-myc* control mutants. IF-keratin-positive glia accompanying the M_V were measured in wildtype embryos and compared with deletion mutants to quantify effects on peripheral glia. M_V median length ± SEM is shown above each box and whisker plot. p<2e-16 for *Nkx2.2, PDGFRab, SoxE1,* and *SoxE3* mutants, compared to the median M_V length in the random gRNA and *Δn-myc* negative control

groups (n=30 for each mutant). N, notochord; NT, neural tube; WT, wildtype. Scale bar: 50µm.

Figure 2.6. Neurofilament expression in CRISPR/Cas mutants. (A) Neurofilament (NF) expression in the negative control. (B) NF expression in an Δ*Nkx2.2* mutant; note the absence in (B) of a dorsal motor branch (M_D) projection, whereas the ventral branch (M_V) (white arrow) remains unaffected. In Δ*PDGFRab* (C), Δ*SoxE1* (D),and Δ*SoxE3* (E) mutant embryos both dorsal and ventral motor branches (white arrowheads and arrows respectively in C-E) appear unaffected (compare M_D and M_V in C-E with A). MR, motor roots; MZ, marginal zone; N, notochord; NT, neural tube; PLLN, posterior lateral line nerves. Scale bar: 50µm, same magnification in all images.

Figure 2.7. *SoxE2* expression in *Nkx2.2* and *SoxE3* CRISPR/Cas mutants. (A) *SoxE2* expression in a T24 negative control whole mount embryo, sectioned in transverse at D', as shown in D. (B) In the Δ*Nkx2.2* mutant, *SoxE2* is expressed in the OV and neural crest (arrows), similar to the negative control (A, D). (E) Transverse section through the trunk (E' in B) reveals *SoxE2* expression retained in dorsal root ganglia (DRG), but has been lost from the ventral neural tube (compare arrowheads in D and E). (C) In *SoxE3* deletion mutants (Δ*SoxE3*), expression of *SoxE2* in the OV is reduced and diffuse, compared to the control and neural crest expression is not detected. (F) Transverse section at F' in C; *SoxE2* expression lost from both the ventral neural tube and MEP, as well as from DRGs (compare D and F). N, notochord; NT, neural tube; OV, otic vesicle. Scale bar: 50µm.

Figure 2.8. Gene regulatory interactions controlling OPC development and lamprey gliogenesis. (A) Extracellular signals (fibroblast growth factor, FGF, and sonic hedgehog, shh) mediate the specification of oligodendrocyte precursors in jawed vertebrates. Key transcriptional regulators that are necessary for the OPC differentiation include *Nkx2.2*, and SoxE genes, with signals also mediated through the *PDGFR* receptor tyrosine kinase. During the pre-myelinating stage, myelin regulatory factor (Myrf) is activated by *Olig2* and *Sox10*, and cooperates with *Sox10* to induce *MBP* and *PLP/DM20* expression (See Table 2.5 for references information). (B) Lamprey gene regulatory interactions for gliogenesis include genes required for OPC development. *Nkx2.2, PDGFRab* and SoxE genes are required for glial specific IF-keratin expression. The HH signaling pathway may participate in gliogenesis in the lamprey ventral neural tube (HHa/HHb ?), but it is unclear whether lamprey possesses Olig genes (not shown) that could be involved in glial development within the CNS. Solid lines represent established *cis*-regulatory interactions, while dashed lines indicate likely interactions based on functional perturbation studies. Pointed and flat arrowheads represent activating and repressing regulatory interactions respectively.

Figure 2.9. Maximum likelihood analysis of Nkx2.2 (A) and PDGFR (B). Sea lamprey (*P. marinus*) *PmNkx2.2* is clustered in the vertebrate Nkx2.2 clade in (A). Sea lamprey *PmPDGFRab* clusters with other vertebrate PDGFR genes, but is positioned basal to both PDGFRa or PDGFRb clades (B). Accession Nos: *Homo sapiens* Nkx2.2 (HsNkx2.2: NP_002500), *Mus musculus* Nkx2.2 (MmNkx2.2: AAI38160), *Gallus gallus* Nkx2.2 (GgNkx2.2: NP_001264647), *Xenopus tropicalis* Nkx2.2 (XtNkx2.2:

XP_002939477), *Danio rerio* Nkx2.2a (DrNkx2.2a: NP_001295569), *Callorhinchus milii* Nkx2.2 (CmNkx2.2: XP_007896539), *Xenopus tropicalis* Nkx2.1(XtNkx2.1: XP_002935383), *Mus musculus* Nkx2.1 (MmNkx2.1: NP_033411) , *Callorhinchus milii* Nkx2.3 (CmNkx2.3: XP_007907293), *Danio rerio* Nkx2.3 (DrNkx2.3: AAC05228), *Callorhinchus milii* Nkx2.5 (CmNkx2.5: XP_007904842), *Danio rerio* Nkx2.5 (DrNkx2.5: AAC05229), *Mus musculus* PDGFRa (MmPDGFRa: AAH53036), *Homo sapiens* PDGFRa (HsPDGFRa: NP_006197), *Gallus gallus* PDGFRa (GgPDGFRa: NP_990080), *Xenopus laevis* PDGFRa (XlPDGFRa:AAH43948), *Danio rerio* PDGFRa (DrPDGFRa: NP_571534), *Homo sapiens* PDGFRb (HsPDGFRa: NP_002600), *Danio rerio* PDGFRb (DrPDGFRb: NP_001177862), *Xenopus Tropicalis* PDGFRb (XlPDGFRb: XP_012815128), *Gallus gallus* PDGFRb (GgPDGFRb), *Homo sapiens* Kit (HsKit: NP_000213), *Xenopus tropicalis* FLT3 (XtFLT3: XP_012813386), *Homo sapiens* FLT3 (HsFLT3: CAA81393).

Figure 2.10. SoxE1 expression in pharyngeal arches (red arrowheads) was not affected in *ΔNkx2.2, ΔSoxE3* and *ΔPDGFRab* deletion mutants, suggesting that observed knockout effects related to changes in SoxE1 expression are unrelated to its expression in chondrogeneic neural crest within pharyngeal arches.

Figure 2.11. Individual allelic sequences of mutant lamprey embryos from Figs. 5 and 7. The control sequences random gRNA) are listed at the first line of each panel, with the target site in red and PAM site in blue. Three clones from each individual embryo were sequenced to confirm the presence of mutations. The numbers of

nucleotide deletions are listed to the right of each sequence. For some embryos, different clones represent sequences of the same allele obtained from multiple clones of the same individual (e.g. clones 1, 2, 3 in panel B; clones 2, 3 in panel C), and most embryos sequenced contained 2 different alleles. Only one embryo (Panel A, representing the Nkx2.2 mutant shown in Figure 5D), contained 3 different alleles. The Nkx2.2 gRNA sequence shown above (Nkx2.2 gRNA1) generated mostly in-frame deletions (Fig. 2.11 A,B and H; see also Figure S5) yet yielded strong loss of function phenotypes (Fig. 5D-F). Two other gRNAs, Nkx2.2 gRNA2 and Nkx2.2 gRNA3 (Table 2.1) respectively showed either low efficiency (20%), or were lethal (data no shown). Notably, the *Nkx2.2* gRNA1 deletion site eliminated a leucine from the Nk2-specific domain (Nk2-SD) of *Nkx2.2* (boxed codon in Fig. 2.11A, B and H), that is conserved across species (see Fig. 2.12 below), suggesting this position may play a critical role in Nkx2.2 gene activity.

Figure 2.12. Amino acid sequence alignment of vertebrate Nkx2 proteins. The *PmNkx2.2* guide RNA targets a genomic DNA sequence that corresponds to the translated amino acid sequence highlighted in red. The deleted sequence includes a codon encoding a leucine residue that is conserved among all Nkx2.2 proteins, but is not present in any other Nkx2 proteins. Nkx2 class amino acid sequences are translated from coding sequences shown in Fig.9.

Figure 2.13. Sequencing results from five randomly pooled CRISPR/Cas9 and gRNA injected T26 embryos. Lamprey Nkx2.2 (A), PDGFRab (B), SoxE1 (C), SoxE2

(D) and SoxE3 (E) wildtype sequences are listed at the top of each panel, with the gRNA target site shown in red and the PAM site in blue. Twenty clones were sequenced from total genomic DNA isolated from 5 pooled embryos, and sequences at each target are listed below the wildtype sequence of each corresponding gene. Numbers of nucleotide base pair deletions (-), insertions (+) or unchanged (Green et al.) are shown at the 3' site of each sequence. Insertions and substitutions are shown in green. Mutagenesis efficiencies calculated from randomly selected and sequenced clones from the 5 pooled embryos in each group are 95%, 100%, 90%, 90% and 100% for Nkx2.2, PDGFRab, SoxE1, SoxE2 and SoxE3 respectively, confirming the high efficiency of CRISPR/Cas9 mutagenesis in lampreys that has been noted elsewhere (Square et al., 2015; Zu et al., 2016).

FIGURES

Figure 2.1. Developmental origins of neural progenitor cell types and differential gene genes expression in ventral domains of the developing vertebrate neural tube.

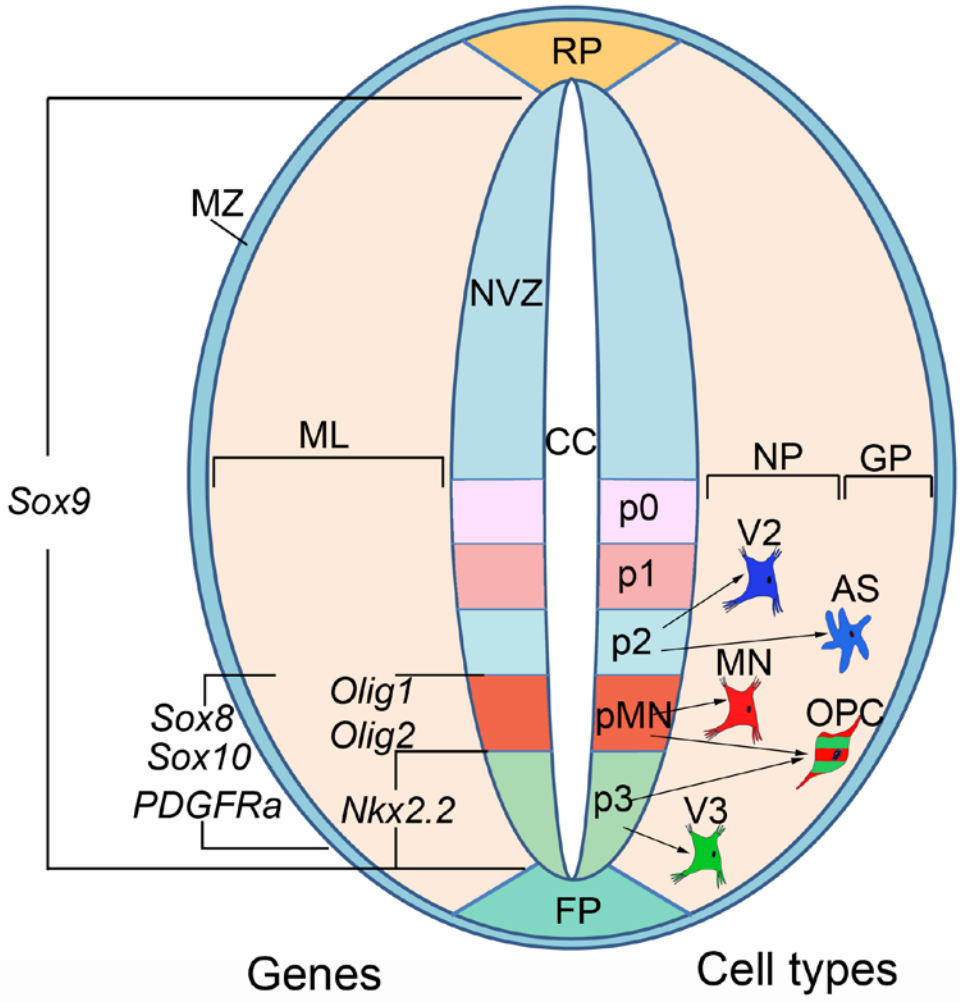


Figure 2.2. Expression of lamprey homologs of oligodendrocyte markers along the ventral neural tube.

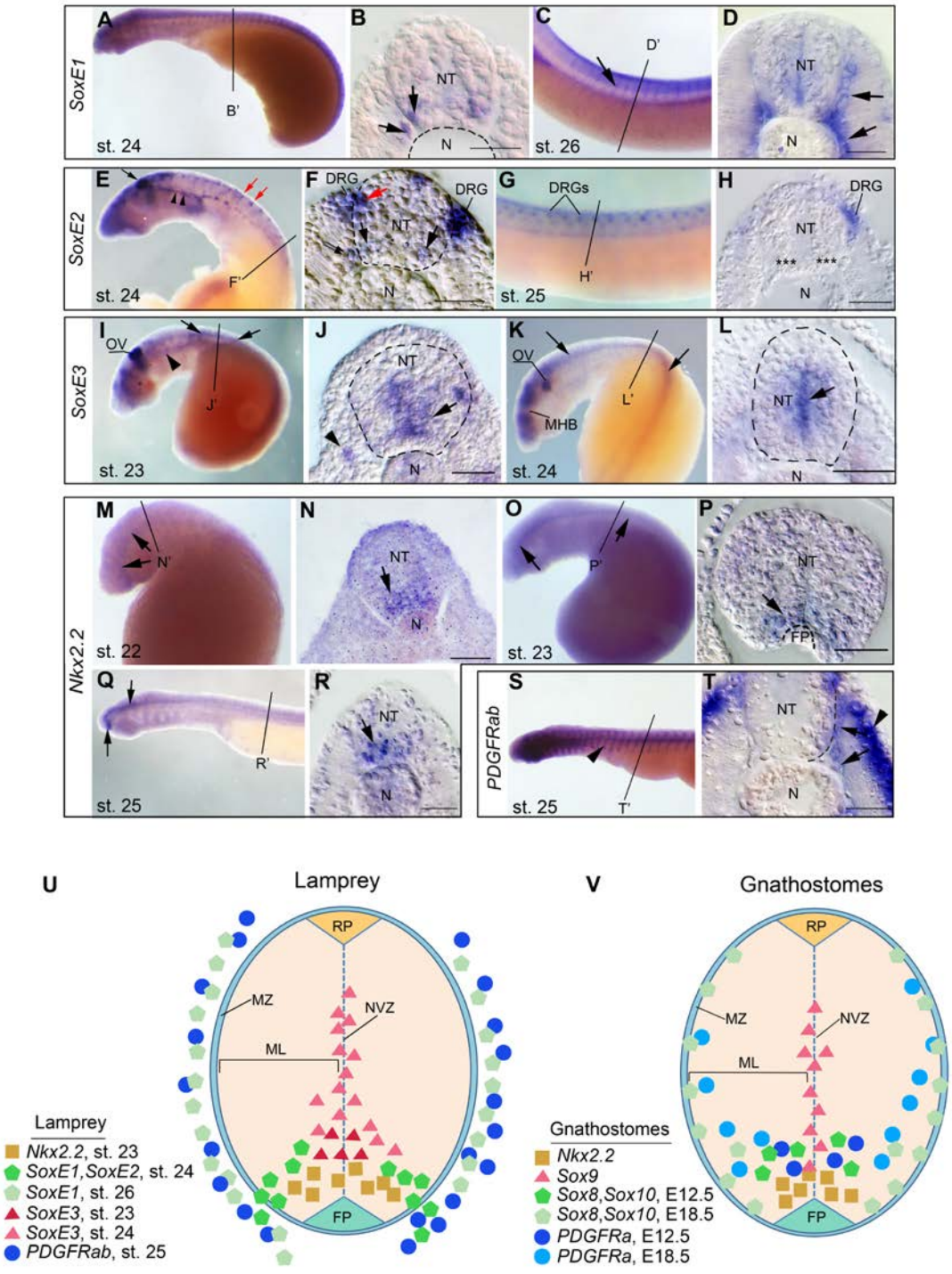


Figure 2.3. Non-neuronal expression of *SoxE1*.

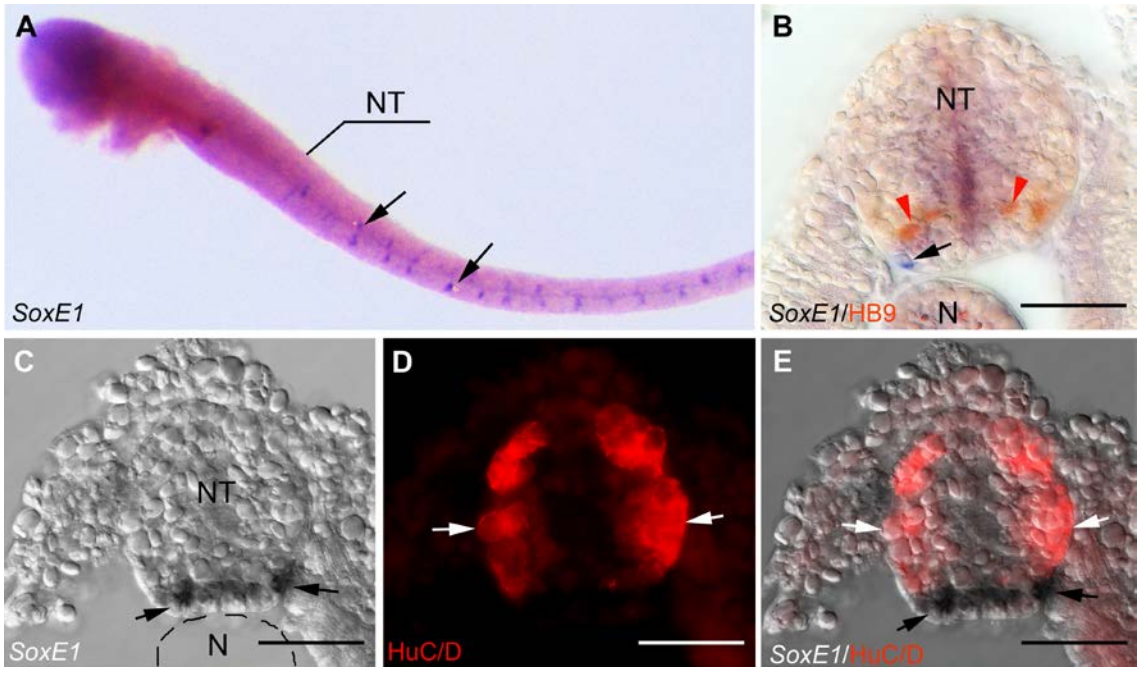


Figure 2.4. Glial-specific expression of intermediate filament (IF) keratin in both CNS and PNS glia during embryonic stages.

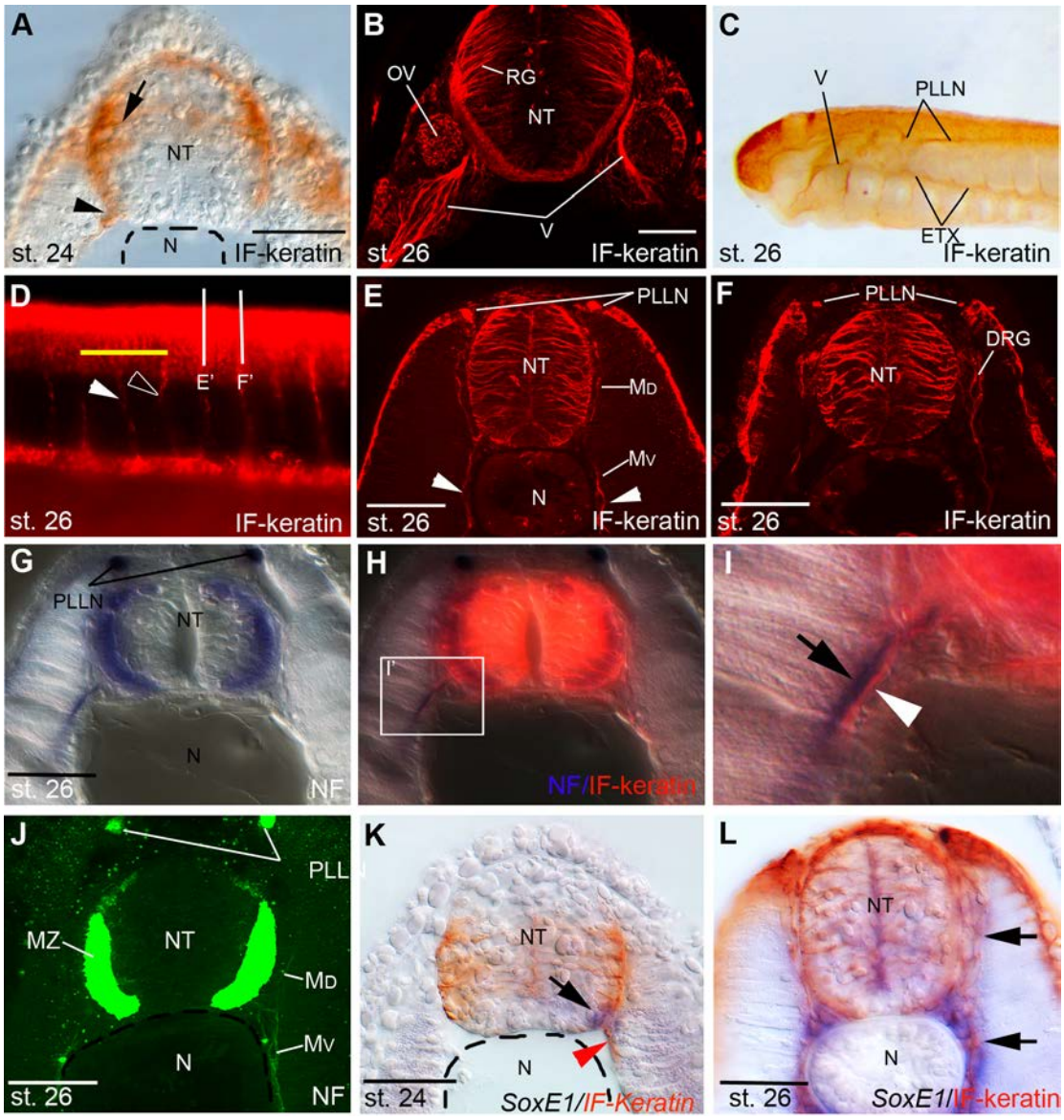


Figure 2.5. *SoxE1* and IF-keratin expression in CRISPR/Cas mutants.

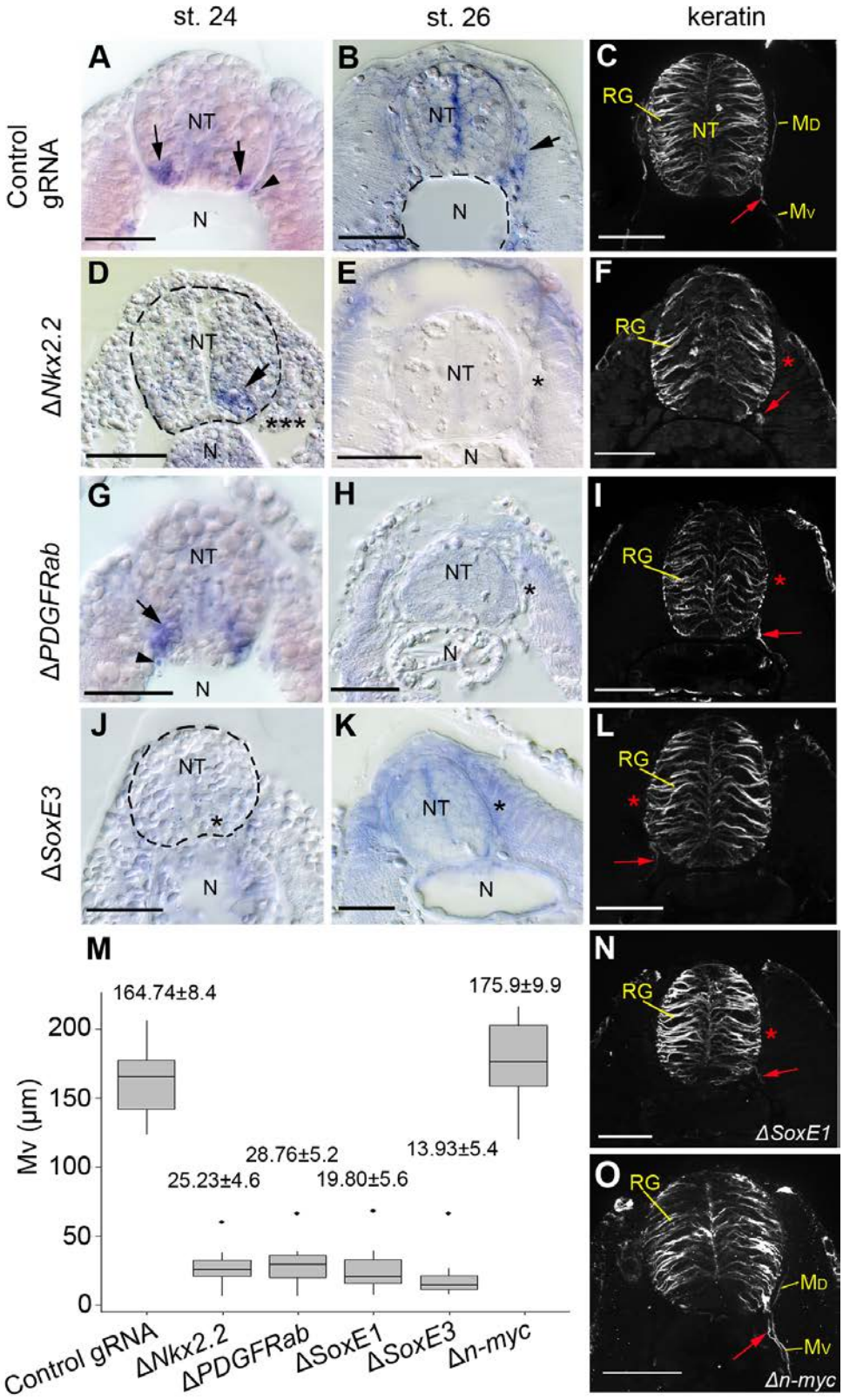


Figure 2.6. Neurofilament expression in CRISPR/Cas mutants.

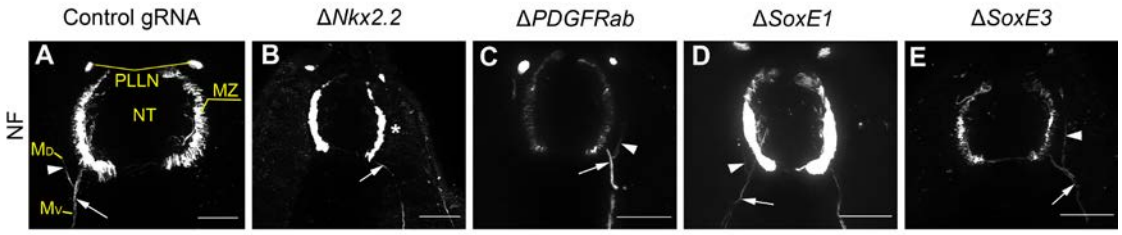


Figure 2.7. *SoxE2* expression in *Nkx2.2* and *SoxE3* CRISPR/Cas mutants.

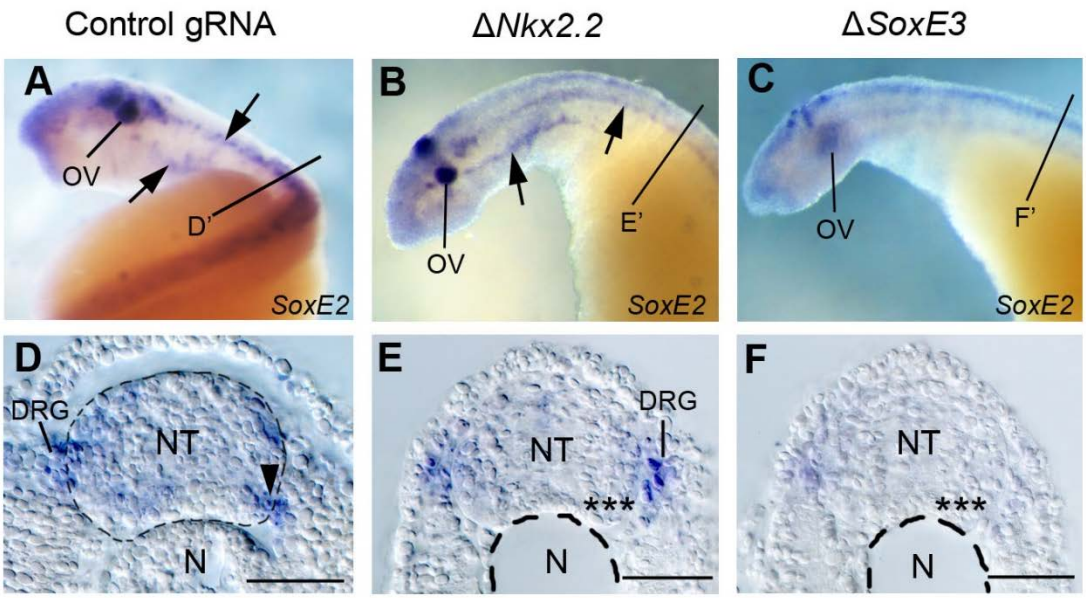


Figure 2.8. Gene regulatory interactions controlling OPC development and lamprey gliogenesis.

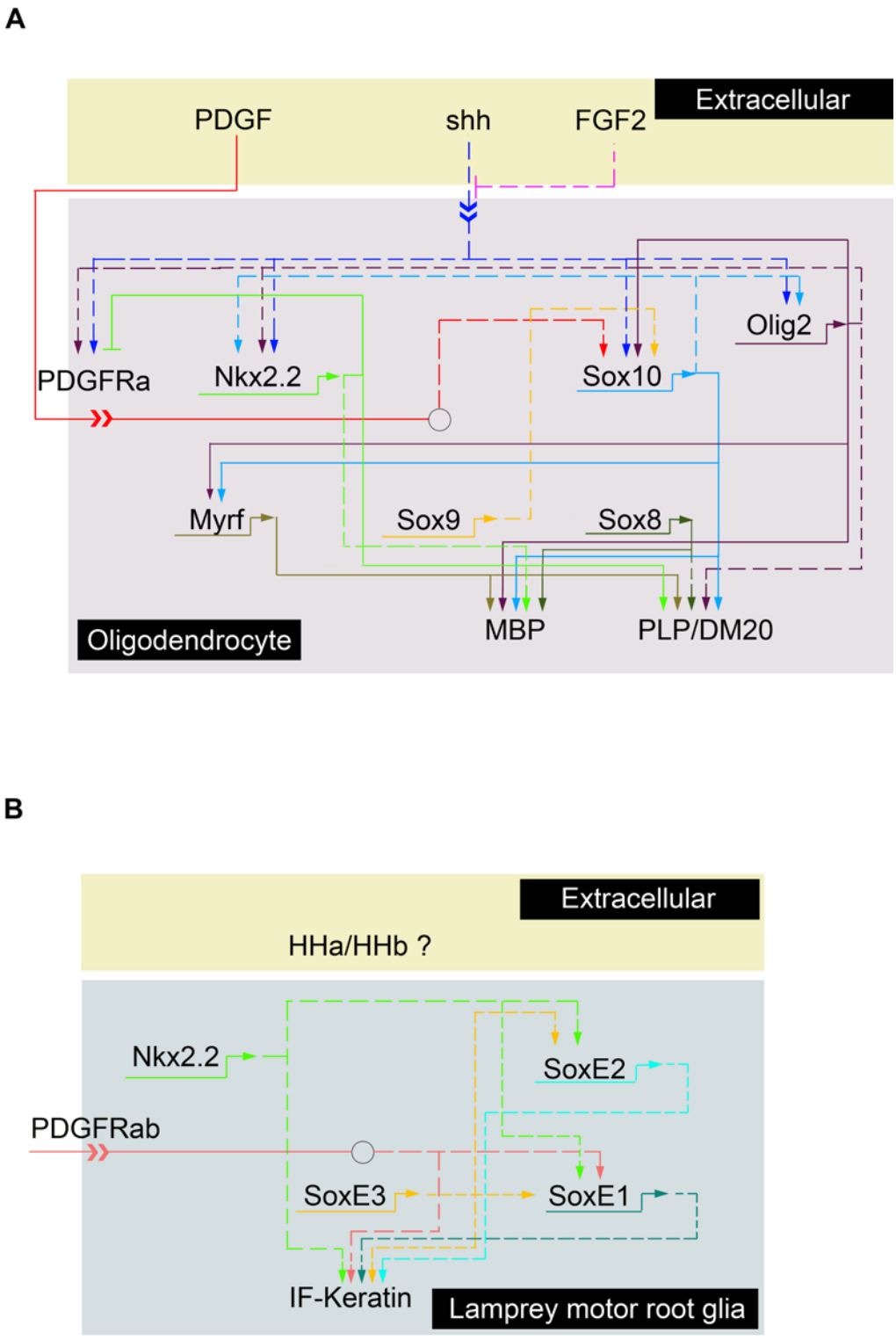


Figure 2.9. Maximum likelihood analysis of Nkx2.2 (A) and PDGFR (B).

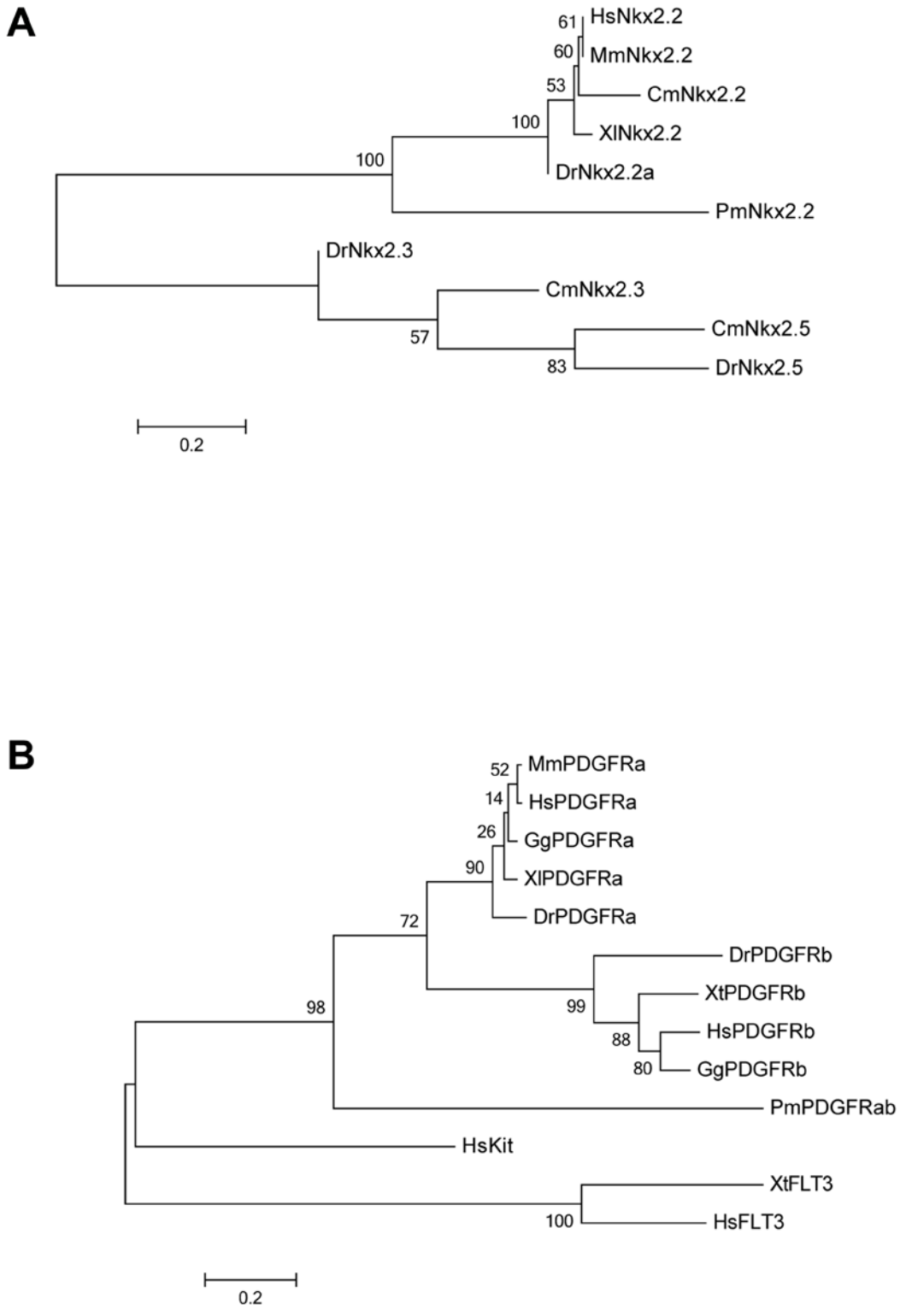


Figure 2.10.

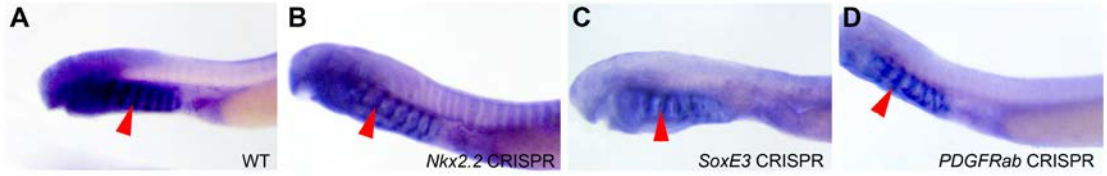


Figure 2.11. Individual allelic sequences of mutant lamprey embryos from Figs. 2.5 and 2.7.

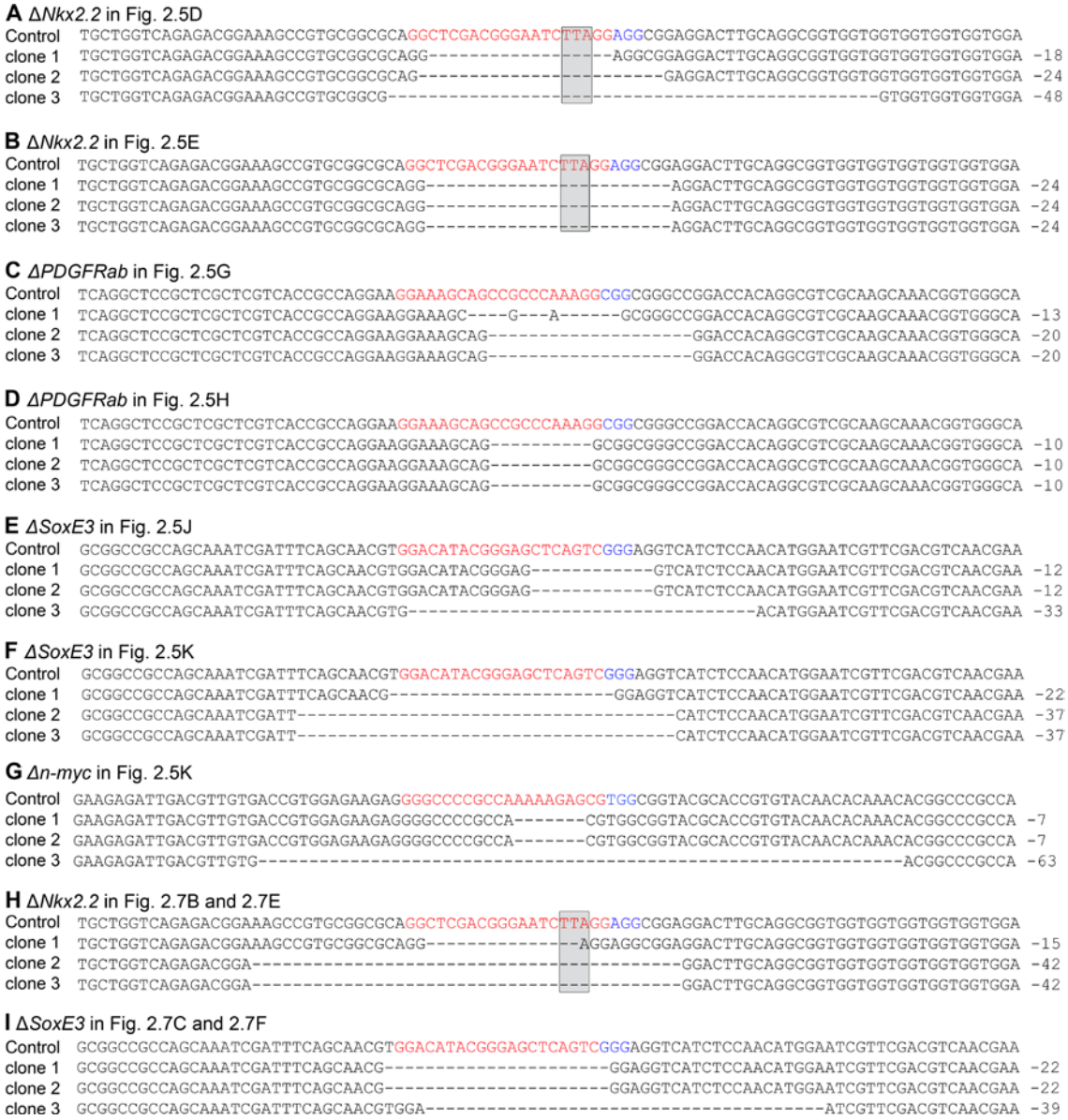


Figure 2.12. Amino acid sequence alignment of vertebrate Nkx2 proteins.

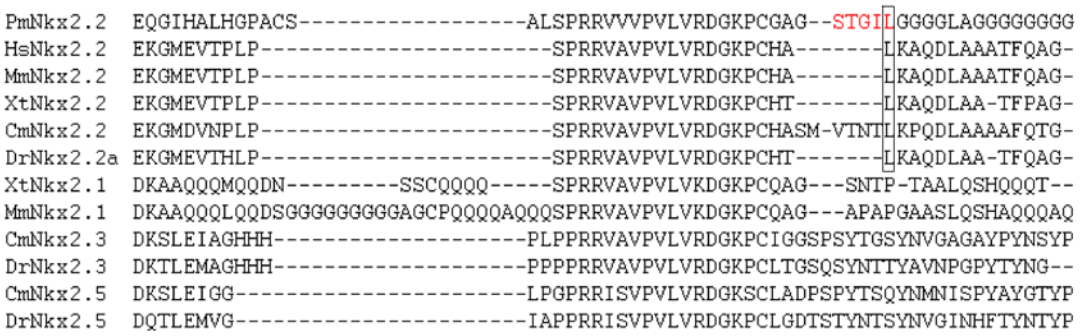
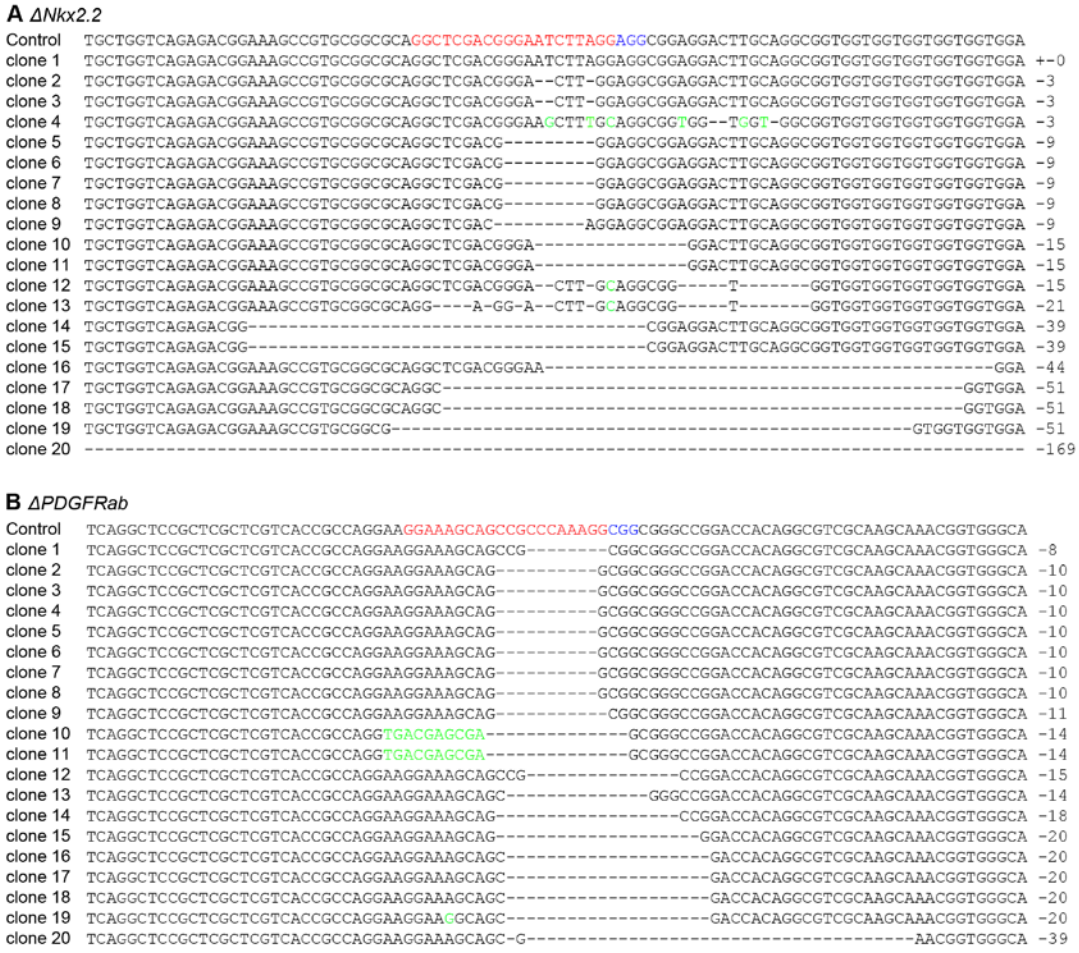


Figure 2.13. Sequencing results from five randomly pooled CRISPR/Cas9 and gRNA injected T26 embryos.



C *ΔSoxE1*

```
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clone 1   GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAGCCCCTGCGGGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG WT
clone 2   GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAGCCCCTGCGGGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG WT
clone 3   GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCG----------GGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -10
clone 4   GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCG----------GGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -10
clone 5   GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAG-----------CGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -11
clone 6   GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAG-----------CGGACCCC-ACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -12
clone 7   GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAG-----------CGGACCCC-ACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -12
clone 8   GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCG-------------GGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -13
clone 9   GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCG-------------GGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCGTGGCGAG -13
clone 10  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCG-------------GGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -13
clone 11  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGC--------------GGGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -13
clone 12  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCG----------------GACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -15
clone 13  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGC-------------------GGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -18
clone 14  GGAAATCGGTCAAGGGCTCCGGCGACGGGGA-----------------------CCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -24
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clone 16  GGAAATCGGTCAAGGGCTCCGGCGACGGGG-------------------------ACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -30
clone 17  GG-------------------------------------------------ACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -52
clone 18  GG-------------------------------------------------ACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -52
clone 19  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAG------------------------------------------------- -96
clone 20  ------------------------------------------------------------------------GGGGGTCCATGGCGAG -124
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D *ΔSoxE2*

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Control   GCGCGGACGACCCCTTCTCGGAGAGCATCCAGGCGGCCGTGAGTCAAGTGCTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC
clone 1   CGGAGAGCATCCAGGCGGCCGTGAGTCAAGAGCATCCAGGCGGCCGTGAGTCAGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC +18
clone 2   CCTTCTCGGAGAGCATCCAGGCGGCCGTGAGTCAAGGTCGTCCGCGCCTACTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC +12
clone 3   GCGCGGACGACCCCTTCTCGGAGAGCATCCAGGCGGCCGTGAGTCAAGTGCTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC WT
clone 4   GCGCGGACGACCCCTTCTCGGAGAGCATCCAGGCGGCCGTGAGTCAAGTGCTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC WT
clone 5   GCGCGGACGACCCCTTCTCGGAGAGCATCCAGGCGG---------ATGCTCTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -9
clone 6   GCGCGGACGACCCCTTCTCGGAGAGCATCCAGGCGGCCGTGAGTCA---------CGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -9
clone 7   GCGCGGACGACCCCTTCTCGGAGAGCATCCAGGCGG--ATGCT-C------TGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -9
clone 8   GCGCGGACGACCCCTTCTCGGAGAGCATCC---------------GAGAAGGGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -15
clone 9   GCGCGGACGACCCCTTCTCGGAGAGCATCCAGG--------------------ACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -21
clone 10  ------------------------------------------------GCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -195
clone 11  ----------------------------------------------------CGACTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -257
clone 12  ---------------------------------------------CGACAGCTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -204
clone 13  ---------------------------------------------CGACATCTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -204
clone 14  ---------------------------------------------CGACATCTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -204
clone 15  ----------------------------------------------------CGACTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -257
clone 16  ----------------------------------------------------CGACTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -257
clone 17  ----------------------------------------------------CGACTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -257
clone 18  ----------------------------------------------------CGACTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -257
clone 19  ----------------------------------------------------CGACTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -257
clone 20  ----------------------------------------------------CGACTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC -257
```

E *ΔSoxE3*

```
Control   GCGGCCGCCAGCAAATCGATTTCAGCAACGTGGACATACGGGAGCTCAGTCGGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA
clone 1   GCGGCCGCCAGCAAATCGATTTCAGCAACGTGGACATACGGGAGCTC----GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -4
clone 2   GCGGCCGCCAGCAAATCGATTTCAGCAACGTGGACATAC-----------GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -12
clone 3   GCGGCCGCCAGCAAATCGATTTCAGCAACGTGGACATAC-----------GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -12
clone 4   GCGGCCGCCAGCAAATCGATTTCAGCAACGTGGACATAC-----------GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -12
clone 5   GCGGCCGCCAGCAAATCGATTTCAGCAACGTGGACATAC------------GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -12
clone 6   GCGGCCGCCAGCAAATCGATTTCAGCAACGTGGACATAC-----------GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -12
clone 7   GCGGCCGCCAGCAAATCGATTTCAGCAAC----------------------GGGAGGTCATCCCCAACATGGAATCGTTCGACGTCAACGAA -22
clone 8   GCGGCCGCCAGCAAATCGATTTCAGCAAC----------------------GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -22
clone 9   GCGGCCGCCAGCAAATCGATTTCAGCAAC----------------------GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -22
clone 10  GCGGCCGCCAGCAAATCGATTTC---------------------------GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -28
clone 11  GCGGCCGCCAGCAAATCGATTTC---------------------------GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -28
clone 12  GCGGCCGCCAGCAAATCGATTTC---------------------------GGGAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -28
clone 13  GCGGCCGCCAGCAAATCGATTTCAGCA----------------------------GGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -28
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clone 16  GCGGCCGCCAGCAAATCGATTTCAGCAACGTGGACAT-----------------------------GGAATCGTTCGACGTCAACGAA -33
clone 17  GCGGCCGCCAGCAAATCGATT-----------------------------------CATCTCCAACATGGAATCGTTCGACGTCAACGAA -37
clone 18  GCGGCCGCCAGCAAATCGATTTCAG-------------------------------CAACGTGGAATCGTTCGACGTCAACGAA -39
clone 19  GCGG-------------------------------------------GAGGTCATCTCCAACATGGAATCGTTCGACGTCAACGAA -49
clone 20  GC---------------------------------------------------------------------------------- -90
```

CHAPTER 3: NEURAL CREST AND EPIDERMAL PLACODE CONTRIBUTIONS IN THE EVOLUTION AND DEVELOPMENT OF CRANIAL SENSORY GANGLIA

Tian Yuan, Joshua R. York, and David W. McCauley

Keywords:

Placode, Cranial sensory neurons, CRISPR/Cas9, Lamprey, *DlxB*, *FoxD-A*, *SoxE2*, *TwistA*

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ABSTRACT

Vertebrates possess paired cranial sensory ganglia that are derived from two different embryonic cell populations, neural crest cells and placodes. Cranial sensory ganglia arose prior to the divergence of jawless and jawed vertebrates, but mechanisms that facilitated their evolution are unknown. Here, we provide evidence that in the basal jawless vertebrate sea lamprey, sensory neurons in cranial ganglia form exclusively from cranial epidemal placodes without a requirement for neural crest, which appear only to make non-neuronal contributions to ganglia. Functional analyses suggest that neural crest cells are instead required for proper patterning of sensory neurons within ganglia. Our results, together with observations that proto-placode cells form individual sensory neurons in invertebrate chordates, suggest a scenario in which cranial sensory ganglionic neurons in the first vertebrates were likely derived exclusively from ectodermal placodes, with the neural crest functioning primarily in patterning ganglionic neurons, as well as contributing presumptive glial cells. This resulted in clustered sensory neurons that form cranial ganglia characteristic of all vertebrates. After the divergence of jawless and jawed vertebrates, neural crest cells were subsequently recruited to contribute sensory neurons located in the proximal region of cranial sensory ganglia.

INTRODUCTION

The origin of the vertebrates has remained one of the most fascinating and puzzling issues in evolutionary biology for over 150 years (Gee, 1996; Gegenbaur, 1878; Geoffroy Saint-Hilaire, 1830; Romer, 1950). According to the “new head” hypothesis, and evidence from the fossil record, early vertebrates distinguished themselves morphologically from invertebrate chordates by evolving a complex head region equipped with novel morphological features, including a cellular head skeleton to support and protect an enlarged tripartite brain, and a muscularized pumping pharynx that facilitated an active predatory lifestyle (Gans and Northcutt, 1983; Northcutt and Gans, 1983). One striking novelty in the new vertebrate head was the organization of large groups of peripheral sensory neurons into compact clusters known as cranial sensory ganglia (Gans, 1989; Gans and Northcutt, 1983; Northcutt and Gans, 1983). The evolution of specialized sensory ganglia may have allowed early vertebrates to engage in behaviors dependent on specific control of different craniofacial structures that would have included eyes, ears, and the olfactory epithelium among others (Baker and Schlosser, 2005; Gans and Northcutt, 1983; Kardong, 2002; Manzanares and Nieto, 2003; Northcutt and Gans, 1983). Despite the evolutionary significance of cranial ganglia during early vertebrate diversification, their evolutionary origins are not clear. Vertebrate cranial ganglia are derived from two separate embryonic cell populations that are both innovations of the vertebrate clade, ectodermal neurogenic placodes and neural crest cells (Barlow, 2002; D'Amico-Martel and Noden, 1983; Harlow and Barlow, 2007; Steventon et al., 2014). The neural crest is a stem cell-like population that arises along the neural plate border, an embryonic territory located between the

medially positioned neural plate and lateral epidermal ectoderm (Gammill and Bronner-Fraser, 2003; Le Douarin, 1999; Sauka-Spengler and Bronner-Fraser, 2008; Trainor, 2013). During neurulation, cells within the neural border region are brought dorsally toward the closing neural tube, where they become specified into bona fide neural crest through the activity of a complex gene regulatory network (GRN) involving cell-cell signaling molecules and numerous transcription factors (Betancur et al., 2010; Sauka-Spengler and Bronner-Fraser, 2008; Simoes-Costa and Bronner, 2015). As anterior neural folds fuse, newly specified cranial neural crest cells detach from the neural tube and migrate in streams throughout the embryonic head (Bronner, 2012; Cordero et al., 2011). These cells subsequently undergo terminal differentiation into a diverse array of derivatives, including craniofacial cartilage and bone, smooth muscle and pigment (Baker, 2008; Green et al., 2015; Hall, 2008). In addition to these differentiated cell types, neural crest cells in jawed vertebrates are also a key source of glial cells as well as sensory neurons that comprise vertebrate cranial sensory ganglia, including the trigeminal, facial, vestibulocochlear and glossopharyngeal ganglia (Barlow, 2002; Sestak et al., 2013).

In contrast to neural crest cells, ectodermal neurogenic placodes arise embryonically in the pre-placodal ectoderm, a region located immediately lateral to the neural plate border (Graham and Shimeld, 2013; Patthey et al., 2014; Schlosser, 2014). Similar to neural crest cell development, embryonic development of placodes is orchestrated by a placode-specific GRN involving the activity of transcription factors including Six1,2,4, Eya1, Pax8 and Dlx3, as well as some of the same transcriptional regulators that control neural crest fate, including Tfap2α and Dlx5 among them (Baker

and Bronner-Fraser, 2001; Maharana et al., 2017; Moody and LaMantia, 2015; Schlosser, 2006; Streit, 2008). Following specification as pre-placodal ectoderm, each placode acquires a characteristic shape of thickened epithelium from which migratory cells soon emerge, being followed closely behind by migratory neural crest (Theveneau et al., 2013). Migratory placode cells then colonize targeted regions of the head to form the cranial ganglia primordia and, together with neural crest cells, differentiate into sensory neurons within each respective ganglion (Baker and Bronner-Fraser, 2001; Schlosser, 2014; Singh and Groves, 2016).

Although bona fide cranial neural crest and placodes and the cranial ganglia that they form emerged as evolutionary novelties in the vertebrate lineage, we know almost nothing regarding the ancestral basis for the development of cranial sensory ganglia in vertebrates. Recent work in invertebrate chordates has attempted to resolve this issue by demonstrating that a “proto-placode” domain and “neural crest-like cells” predates vertebrate origins. In the tunicate, *Ciona*, for example, proto-placode-like sensory neurons and neural crest-like pigment cells form in the anterior larval head (Abitua et al., 2015; Abitua et al., 2012; Mazet et al., 2005). Importantly however, sensory neurons in *Ciona* and other invertebrate chordates are unable to cluster to form vertebrate-like ganglia (Abitua et al., 2015; Manni et al., 2004; Meulemans and Bronner-Fraser, 2007). These observations suggest that, although genetic signatures of neural crest and placodes predate the vertebrates, the ability to organize groups of sensory neurons into clustered cranial sensory ganglia is a uniquely vertebrate feature. To date, the origins of the developmental-genetic mechanisms that regulated this process in early vertebrates remain unclear.

To address this knowledge gap, we used the sea lamprey (*Petromyzon marinus*) as a comparative model to investigate molecular genetic and cellular mechanisms coordinating development and evolution of vertebrate cranial sensory ganglia. Lampreys are a group of basal, primitively jawless vertebrates that constitute the sister group to all other vertebrates, and are therefore ideal comparative models for probing the evolution of developmental mechanisms in early vertebrates (Green and Bronner, 2014; McCauley et al., 2015). Similar to their jawed vertebrate sister taxa, lampreys have bona fide neural crest cells and placodes, and possess many of the same differentiated cell types as jawed vertebrates, including cranial sensory ganglia (Kuratani et al., 1997; McCauley and Bronner-Fraser, 2003; Modrell et al., 2014). Using a combination of gene expression analysis, CRISPR/Cas9-mediated genome editing, and cell lineage tracing of both placodes and neural crest, we probed developmental-genetic mechanisms responsible for specifying and patterning cranial ganglia in lamprey. By documenting relative contributions and functional roles of both neural crest and placodes for early head development, we show that lamprey cranial neural crest cells migrate into ganglia as has been reported (Modrell et al., 2014), but they do not appear to form sensory neurons within cranial ganglia, and instead may function to guide and pattern the formation of these placode-derived ganglionic sensory neurons. We discuss our findings in the context of a possible scenario for evolution of ancestral vertebrate cranial ganglia in which two cell populations, neurogenic placodes and neural crest originally acquired disparate roles in cranial ganglion neurogenesis and patterning respectively.

RESULTS

Neural crest and placode contributions to cranial sensory ganglia

To investigate the contributions of neural crest and placode cells to developing cranial sensory ganglia, we labeled both populations contemporaneously by focally injecting a vital dye (DiO) to label neural crest in stage T21 embryos (embryonic day 6; E6) that had first been labeled pan-ectodermally by immersion in DiI (Fig. 3.1A). We examined the contribution of both cell types to trigeminal and vestibuloacoustic ganglia in the context of differentiated cranial sensory neurons at stage T26 (E15) (Fig. 3.1B-M) (Juarez et al., 2013; Nikitina et al., 2009). At T26, DiI-labeled placodal cells are observed in trigeminal ganglia (arrows in Fig. 3.1B, D), while neural crest cells had migrated from the site of injection toward cranial ganglia (arrowheads in Fig. 3.1C, D). Sectioning revealed that DiO-labeled neural crest cells (pseudocolored green) surrounded, but were absent from within the DiI-labeled (placode-derived) cells (pseudocolored red) residing at the core of the ophthalmic trigeminal ganglion (opV; Fig. 3.1E and F) (Modrell et al., 2014). A separate set of DiO-labeled T21 embryos was allowed to develop to T26 (Fig. 3.1G), then immunolabeled using a pan-neuronal marker, HuC/D, to identify cranial sensory ganglia (Fig. 3.1H-M). DiO-labeled neural crest cells extended ventrally from the dorsal neural tube, and became positioned medial to and surrounding Hu-positive neuronal clusters at the core of the maxillomandibular branch of the trigeminal ganglion (arrowheads in Fig. 3.1H, I). Interestingly, we observed a centrally located neuron-free zone within the mmV ganglion (region inside dashed line oval in Fig. 3.1I), that contained DiO-labeled neural crest (arrowheads at center of mmV in Fig. 3.1I,J). In the region immediately caudal to mmV (Fig. 3.1G,

plane of section K' shown in K), neural crest cells surrounded and were also seen to integrate with cells of the vestibuloacoustic ganglion (va) (Fig. 3.1K-M). Note the absence of HuC/D expression from labeled neural crest (compare Fig. 3.1L and M). This observation confirms results from a previous study that neural crest cells contribute to cranial sensory ganglia (Modrell et al., 2014), but despite their location among HuC/D positive sensory neurons of the vestibular ganglion, and a central neuron-free region of the mmV, neural crest cells do not appear to express HuC/D protein, suggesting a non-neuronal identity of these cells (Fig. 3.1I, J, L, M). In addition to their location surrounding and within cranial ganglia, DiO-labeled cells were observed within the pharyngeal prechondrocytes, and in perichondrial cells adjacent to the forming cartilage bar (Fig. 3.3N, O merged in P; see arrowheads in Fig. 3O, P) previously shown to be derived from neural crest (Martin et al., 2009; McCauley and Bronner-Fraser, 2006), and providing additional confirmation that our DiO injection technique labeled neural crest. These results support the notion that neural crest cells contribute to cranial ganglia in lamprey, but may not form cranial sensory neurons within trigeminal or vestibuloacoustic ganglia that we examined.

To investigate the role of neural crest in cranial ganglia development within lamprey, we next examined expression of several neural crest markers that are known to contribute to development of the cranial peripheral nervous system. Sox10, a transcription factor critical to the development of the neural crest-derived peripheral nervous system in a number of vertebrates (Carney et al., 2006; Dutton et al., 2001b; Lakiza et al., 2011; Lee et al., 2016; McCauley and Bronner-Fraser, 2006), is expressed strongly in the cranial ganglion in the mouse and chick (Giovannone et al., 2015;

Paudyal et al., 2010). Similarly, another transcription factor, *TwistA*, is expressed in post-migratory neural crest in lamprey, including components of the peripheral nervous system (Sauka-Spengler et al., 2007).These reports suggest a possible role for *SoxE2* (the lamprey Sox10 ortholog) and *TwistA* in development of the cranial ganglia in lamprey (Lakiza et al., 2011; McCauley and Bronner-Fraser, 2003; Sauka-Spengler et al., 2007). In order to test this possibility, we revisited their expression patterns in the context of peripheral cranial neurogenesis.

Examination of *SoxE2* and *TwistA* expression in the head reveals that, rather than being expressed largely within the core of the cranial ganglia, these genes appear to be expressed primarily in cells surrounding cranial ganglionic clusters, at Tahara stage 26 (T26) (Fig. 3.2A, F) (McCauley and Bronner-Fraser, 2003; Sauka-Spengler et al., 2007) , with scattered expression of SoxE2 located within the maxillomandibular branch (mmV) of the trigeminal ganglion (Figs. 3.2K and 3.3B). To register their precise expression domains with cranial ganglia, we also examined *SoxE2* and *TwistA* expression in the context of the pan-neuronal marker, HuC/D to determine if there is overlap between *SoxE2* and *TwistA* positive neural crest and Hu positive cranial sensory neurons. Cells expressing *SoxE2* and *TwistA* appear to surround clusters of Hu-positive cells in each of the cranial ganglia (arrows in Fig. 3.2B, G). Horizontal sections through cranial ganglia confirmed the lack of overlap in expression of *TwistA* with Hu-positive neurons in the core of ganglia (Fig. 3.2H and I, merged in J), suggesting that *TwistA*-positive cells associated with lamprey cranial ganglia are unlikely to be sensory neurons.

Since these cells do not appear to be neuronal, we next asked if the cells expressing *SoxE2* and *TwistA* surrounding the cranial ganglia were glial in nature. To test this possibility, we examined the expression of a lamprey glial-specific marker, LCM29, in embryos co-labeled for expression of *SoxE2* or *TwistA*. LCM29 is specific for intermediate filament (IF) cytokeratin expressed in glial cells in the lamprey (Merrick et al., 1995). *TwistA* and *SoxE2* expression domains surrounding ganglia do not overlap with LCM29 positive glia at stage T26 (Fig. 3.2K, L, inset M, and N, O, inset P; see arrows in Fig. 3.2K-P), nor with other glial cells outside the ganglia (arrowhead in Fig. 3.2O). Interestingly, a small number of SoxE2 expressing cells within the mmV appear to overlap with glial-specific IF-keratin expression (arrowheads in Fig. 3.2L,M), indicating the glial identity of those SoxE2-positive cells within the mmV. However, these results do not rule out the possibility that cells expressing *TwistA* and *SoxE2* surrounding the cranial ganglia may be different glial cell types that do not express the IF-keratin protein recognized by the LCM29 antibody.

Although *Sox10* and *Twist* are reliable markers of neural crest development in vertebrates, it is possible that the *SoxE2*- and *TwistA*-positive cells surrounding cranial ganglia in lamprey are not of neural crest origin. To verify the neural crest identity of these cells, we used CRISPR/Cas9 genome editing to delete coding sequences from the *FoxD-A* and *SoxE1* genes, since these genes are essential for neural crest development (Sauka-Spengler et al., 2007) and both are expressed in premigratory neural crest of lamprey but are not co-expressed with *SoxE2* and *TwistA* later in the cranial nervous system (Sauka-Spengler et al., 2007). gRNAs for CRISPR/Cas9-mediated genome editing were designed to minimize potential off-targets, as described in Materials and

Methods. We examined expression of *SoxE2* and *TwistA* around cranial sensory ganglia in CRISPR mutants. Deletion of *FoxD-A* sequence resulted in loss of expression of both *SoxE2* (n=18/20) and *TwistA* (n=16/21) from the region surrounding cranial ganglia in T26 embryos respectively, as shown both in whole mounts (Fig. 3.3E, G) and horizontal sections (Fig. 3.3F, H). Comparison of arrows in Fig. 3.2B, D with the same region in Fig. 3.3F, H (highlighted with asterisks), indicates that both *SoxE2* and *TwistA* expression have been lost from the region surrounding mmV. Similarly, elimination of *SoxE1* resulted in loss of expression of both these genes surrounding cranial ganglia (Fig. 3.3I-L, compare arrows in B, D with the same region shown in J and L highlighted with asterisks; *SoxE2*, n=16/17; *TwistA*, n=18/20). Further, SoxE2 expression was also absent from the mmV in *FoxD-A* and *SoxE1* mutants, supporting the neural crest identity of these cells within the trigeminal ganglion (Fig. 3.3B, F, J). Importantly, non-neural crest expression of *SoxE2* in the otic vesicle (OV) was unaffected in either *FoxD-A* (n=20/20) or *SoxE1* (17/17) mutants (Fig. 3.3E, F, I, J) compared to control embryos (Fig. 3.3A, B), suggesting *SoxE2* expression surrounding cranial ganglia, and within mmV, but not in the otic vesicle, is dependent on neural crest specification. In summary, these findings support the neural crest identity of *SoxE2*- and *TwistA*-expressing cells in and around lamprey cranial sensory ganglia. Genotypes of putatively mutant embryos were verified by sequencing of target loci, and were validated for injection efficiency, indicating *FoxD-A* (20/20,100%) and *SoxE1* (36/40, 90%) contained biallelic deletions as detailed in supplementary information (Fig. 3.8).

Lamprey neural crest cells pattern placode-derived cranial sensory neurons

Since neural crest cells do not appear to contribute sensory neurons, or IF-keratin positive glia to cranial ganglia, we next investigated possible functions of neural crest cells during cranial ganglion development, reasoning that they may play a role in pattern formation of sensory neurons within ganglia. To test this notion, we eliminated the migratory neural crest transcription factors *SoxE2* and *FoxD-A*, and first examined effects on placode formation, and subsequently examined patterning of ganglionic sensory neurons during development. To determine effects on placode formation, we examined expression of the canonical placode marker genes, *Six1/2* and *Pax3/7* following deletion of *SoxE2* and *FoxD-A* gene sequences (Baker and Bronner-Fraser, 2001; Streit, 2004). We isolated a full-length putative lamprey Six gene coding sequence, based on a previously identified partial sequence, AF108812, (Seo et al., 1999), that our phylogenetic analysis revealed to be orthologous to gnathostome *Six1/2* (Fig. 3.7). Lamprey *Six1/2* is expressed around the otic vesicle, and in the petrosal and posterior lateral line ganglia at stage T25 (Fig. 3.4A,B). Among *SoxE2* and *FoxD-A* mutant embryos at stage T25, *Six1/2* expression domains appear unaffected (compare Fig. 3.4D, E, G, H with A, B; *ΔSoxE2*, n=21/21; *ΔFoxD-A*, n=19/19). At T24, lamprey *Pax3/7*, is expressed in the neural tube and ophthalmic branch of the trigeminal ganglion (opV) (Fig. 3.4C) (Kusakabe et al., 2011; McCauley and Bronner-Fraser, 2002; Modrell et al., 2014). Compared to control embryos, *Pax3/7* expression in *SoxE2* and *FoxD-A* CRISPR mutants appears unaffected at T24 (Fig. 3.4F, I; *ΔSoxE2*, n=17/17; *ΔFoxD-A*, n=20/20). In contrast, deletion of *DlxB*, a member of Dlx genes known to regulate placode induction and specification (Neidert et al., 2001; Schlosser and Ahrens, 2004), affected expression of placode markers. In early neurulae, *DlxB* is

expressed in non-neural ectoderm, but is excluded from the neural plate border, suggesting its role in placode specification within lampreys (Sauka-Spengler et al., 2007). As expected, *Six1/2* and *Pax3/7* expression are abolished from *DlxB* CRISPR mutants, confirming the placodal identity of *Six1/2* and *Pax3/7*-positive cells in cranial ganglia (Fig. 3.4J-L; *Six1/2*, n=19/19; *Pax3/7*, n=16/21). These results suggest that genetic perturbations of key migratory neural crest transcriptional regulators in lamprey does not affect the specification, or migration of cranial neurogenic placode-derived cells in lamprey. Genotypes of putatively mutant *SoxE2* and *DlxB* embryos were verified by sequencing of target loci from five randomly selected injected embryos. Mutant loci were PCR-amplified from the pooled genomic DNA and subcloned into the pGEM T-easy vector using primer sets indicated in Supplementary Table 3.2. Injection efficiency for each mutant was determined by sequencing either 40 clones (*SoxE2*, 35/40, 87.5%) or 20 clones (*DlxB* 20/20, 100%), selected at random (Fig. 3.8).

We then examined the morphology of ganglia in *SoxE2* and *FoxD-A* mutants to determine a possible role for neural crest in patterning cranial ganglionic neurons (Fig. 3.5). In marked contrast to early embryonic stages, we found that CRISPR/Cas9-mediated mutagenesis of both *SoxE2* (18/19) and *FoxD-A* (16/19) results in patterning defects associated with differentiated Hu-positive cranial sensory ganglia (Fig. 3.5D,G). For example, in the *SoxE2* mutant embryo shown in Figure 5D, the opV trigeminal appears to be elongated and projects in the rostro-ventral direction (Fig. 3.5E), and epibranchial ganglia appear to be fused to each other (arrows in Fig. 3.5D). Indeterminate Hu-positive sensory neurons appeared adjacent to the posterior lateral line but did not appear to be associated with any other ganglia (arrows in Fig. 3.5F). In

the *FoxD-A* mutant embryo shown in Figure 5G, mmV protrudes toward the posterior (asterisk in Fig 5H) and the geniculate/anterior lateral line ganglia (g/all) appear elongated and located in a more dorsal position (compare Fig. 3.5B and H). Indeterminate Hu-positive neurons are observed adjacent to the petrosal ganglion (arrow in Fig. 3.5I). Importantly, despite these consistent patterning defects, we never observed loss of ganglia in any of our neural crest mutants. In contrast to mutants with perturbed neural crest development (i.e., *SoxE2*, *FoxD-A*), loss of *DlxB* eliminated formation of organized cranial sensory neurons in the region where the trigeminal, geniculate, and anterior/posterior lateral line ganglia would form (Fig. 3.5J, K; n=13/19). Epibranchial ganglia (n1-n6) are completely absent, though a few scattered Hu positive cells are present in the epibranchial region (Fig. 3.5L), while Hu expression remains in the CNS (NT in Fig. 3.5J). These results, together with the observations of unaffected placodal gene expression in neural crest genetic mutants at early stages (Fig. 3.4), suggest that lamprey neural crest cells may not be required for specification or migration of placode-derived cranial sensory neurons, but instead may pattern sensory neurons into correctly positioned ganglionic clusters as they condense.

In summary, our results show that neural crest cells surround and integrate into cranial ganglia, but may not form sensory neurons. We also show that loss of migratory neural crest does not prevent the differentiation of sensory neurons, but disrupts the organization of these neurons in cranial sensory ganglia. In contrast, the loss of placode-specific gene expression eliminates formation of cranial sensory ganglia. Our data suggest that in the lamprey, sensory neurons within cranial ganglia may be derived

solely from neurogenic placodes, while neural crest cells play a patterning role but are not required for formation of cranial sensory ganglia.

DISCUSSION

The peripheral sensory nervous system in the vertebrate head consists of a series of paired ganglia that process a wide range of environmental stimuli received by sensory organs. The emergence of these paired sensory organs is thought to have been one of the key evolutionary innovations that facilitated the transition from passive filter feeding to active predation in ancestral vertebrates, yet the evolutionary-developmental mechanisms that were required for the origin of vertebrate cranial ganglia has remained obscure. Our labeling and immunohistochemistry, coupled with functional analyses in lamprey, lead us to suggest that cranial sensory neurons within some ganglia (e.g., mmV, va) within this animal may be entirely placodal in origin and that the ability to organize large groups of cranial sensory neurons into iterated cranial sensory ganglia may have been linked in part to novel patterning activities mediated by cranial neural crest cells. This may have been a key step required for the organization of cranial sensory ganglia in ancestral vertebrates since only individual proto-placodal ectoderm (PPE)-derived sensory neurons are present in invertebrate chordates (Fig. 3.6). Early in vertebrate evolution, neural crest cells likely acquired a patterning role in formation of cranial sensory ganglia. Following the divergence of jawless and jawed vertebrates, neural crest cells were subsequently recruited to contribute sensory neurons in the proximal portion of cranial ganglia (Fig. 3.6).

Phylogenomic analyses have revealed that the tunicates, rather than the cephalochordates (i.e., amphioxus), constitute the closest extant relatives of the vertebrates (Delsuc et al., 2006). Interestingly, recent studies have suggested that tunicates have a small group of sensory neurons that are derived from a “proto-

neurogenic placode" region (Abitua et al., 2015), which expresses several homologues of genes that are known to regulate cranial placode and sensory ganglion development in vertebrates. However, unlike the condition in vertebrates, the sensory neurons of tunicates are scattered as individual cells in the larval head and never take on a vertebrate-like clustered ganglionic structure (Abitua et al., 2015). Moreover, tunicates have neural crest-like cells that have a regulatory network similar to the GRN controlling vertebrate neural crest, but these cells never migrate endogenously and are developmentally independent from the proto-placode region (Abitua et al., 2012). We observed that in the basal jawless vertebrate sea lamprey, a population of neural crest cells surrounds the cranial ganglia, with a few NC cells contributing to non-neuronal cells within the va and mmV ganglia (Fig. 3.1). Gene expression and functional analyses confirm that neural crest cells expressing *Twist-A* and *SoxE2* mainly surround ganglia and do not contribute to formation of sensory neurons, while only a few *SoxE2*-positive cells locate in the mmV ganglion appearing to overlap with glial marker (Figs. 2, 3). This expression pattern is different from the observations in mouse and chick, in which *Sox10* is expressed throughout the cranial ganglia (Giovannone et al., 2015; Paudyal et al., 2010). These results, together with the previous observation that lamprey neural crest cells contribute to cranial ganglia as presumptive glial cells (Modrell et al., 2014), suggest that lamprey cranial neural crest do not contribute to neurons in cranial ganglia. Based on our findings here in lamprey, and comparisons with invertebrate chordates (Abitua et al., 2015), we hypothesize that ancestral organization of sensory neurons into clusters of cranial sensory ganglia may have been dependent on novel developmental cooperation of two cell populations: placode-derived cells for

specification of cranial neurons, and neural crest cells to pattern and organize these neurons into clustered cranial ganglia (Fig. 3.6). This hypothesis is supported by data from our functional analysis, that deletion of neural crest affects only the organization of cranial ganglia, while elimination of placodes results in loss of sensory neurons that form them (Fig. 3.5). Interestingly, deletion of neural crest did not affect the clustering of sensory neurons, although the patterning of these neurons is abnormal. Thus, the evolution of clustered sensory neurons into paired cranial ganglia may be driven by other mechanisms, such as cell-cell adhesion. Interestingly, a previous study in the chick showed that N-cadherin knockdown resulted in scattered trigeminal ganglionic neurons (Shiau and Bronner-Fraser, 2009). Thus, it is possible that the vertebrate ancestor recruited neural crest to pattern and organize clustered neurons into properly shaped structures to convey sensory information.

The lack of an obvious neural crest contribution to cranial sensory neurons was originally reported by McCauley and Bronner-Fraser (McCauley and Bronner-Fraser, 2003). Using immunohistochemistry, McCauley and Bronner-Fraser found that Sox10 was localized around cranial ganglia, a pattern that we confirm here (Fig. 3.2). However, lamprey cranial sensory ganglia were devoid of Sox10 protein, a key genetic marker used for tracking neural crest-derived cranial sensory neuron development in jawed vertebrates (Aoki et al., 2003; Betancur et al., 2009; Carney et al., 2006; Dutton et al., 2001a). Results from the current study support the conclusion by Modrell et al. that neural crest do in fact contribute to cranial sensory ganglia (Modrell et al., 2014), but are excluded from the population of sensory neurons within ganglia. As Modrell et al point out, temporal differences in labeling may have contributed to these

contradictory results. In addition, we note that the earlier study suggesting the lack of a neural crest contribution was dependent on Sox10 immunohistochemistry, whereas our results in the present study are based in part on mRNA expression of the Sox10 ortholog in lamprey, *SoxE2* (Figs. 3.2, 3.3). Results from McCauley and Bronner-Fraser (2003) and the current study are consistent with the possibility that neural crest cells migrating into ganglia still express the *SoxE2* gene (Fig. 3.1). Subsequently, as these cells integrate into cranial ganglia they downregulate *SoxE2* expression such that the SoxE2 protein is absent from the core of ganglia, but is translated in the cells that surround them (McCauley and Bronner-Fraser, 2003).

Developmental interactions between placodes and neural crest cells that facilitated the evolution of cranial ganglia may have been driven in part by the deployment of novel intercellular guidance cues. For example, new expression of an intercellular receptor in one cell type (neural crest) can enable a response to secreted ligands from neighboring cells (placodes) into the extracellular environment. One such candidate signaling pathway involves the secreted chemokine Stromal-derived factor 1 (Sdf1) and its receptor CXCR4 (Steventon et al., 2014; Theveneau et al., 2010; Theveneau et al., 2013). In jawed vertebrates, CXCR4 is expressed on premigratory and early migrating neural crest and binds the Sdf1 secreted factor from early migrating placode cells (Theveneau and Mayor, 2012). This signaling pathway establishes continued intercellular communication and developmental coupling of neural crest and placodes that ultimately results in their dual contribution to cranial ganglion primordia in the head (Theveneau et al., 2013). Compared to jawed vertebrates, the role of intercellular guidance cues and patterning mechanisms of neural crest and placodes in

lamprey, as well as “neural crest-like cells” and “proto-placode cells” in tunicates, remain largely unknown. However, our results showing neural crest cells surrounding and integrating into cranial ganglia (Fig. 3.1) coupled with results indicating a neural crest function to pattern placode-derived sensory neurons in cranial ganglia (Fig. 3.5) suggests possible communication between these two cell populations. Going forward, it may be important to focus on the evolutionary significance of intercellular communication between placode and neural crest in basal vertebrates and invertebrate chordates to better understand the developmental mechanisms responsible for integrating these two cell populations.

The notion of pure placode-derived sensory neurons in lamprey cranial ganglia is similar to that observed in the tunicate, *Ciona*, in which the proto-neural crest domain and proto-placode domain are developmentally separate, and the latter gives rise to sensory neurons. It is likely that early jawless vertebrates retained separate developmental contributions from neural crest cells and placodes to form their cranial ganglia (Abitua et al., 2015; Abitua et al., 2012). Taken together, these results suggest that cranial sensory ganglion development in early vertebrates may have consisted initially of an exclusively placode-derived sensory neuron contribution. Early in the ancestry of jawed vertebrates, neural crest cells would have acquired the ability to contribute sensory neurons to cranial sensory ganglia that formed from placodes, resulting in the mosaic contribution of both neural crest and placodes that is characteristic of cranial ganglia development across the jawed vertebrate clade. In extant jawed vertebrates, the neural crest contribution to cranial sensory neurons mainly involves forming the proximal portion of cranial ganglia responsible for communicating

sensory information back to the CNS (Fig. 3.6). By contrast, in *Ciona* proto-placode-derived sensory neurons in the head are responsible for directly sending axons to the CNS (Abitua et al., 2015). The putative placode-specific origin of cranial sensory neurons in lamprey (Fig. 3.1) suggests that early jawless vertebrates retained this exclusively placode-based connection between peripheral sensory organs and the CNS. After the divergence of jawless and jawed vertebrates, neural crest cells in the jawed vertebrate lineage gradually replaced the placode-derived proximally positioned sensory neurons, leaving placode-derived sensory neurons to comprise the distal portion of each ganglion (Fig. 3.6). In anamniotes, the proximal (neural crest-derived) and distal (placode-derived) sensory neurons are intermixed, whereas early in amniote evolution, the proximal and distal elements of each sensory ganglion became completely separated. Thus, in a comparative framework, these results highlight for the first time a plausible stepwise scenario for the origin and evolution of vertebrate cranial sensory ganglia from one of initially placode-derived cranial ganglionic neurons to one that gradually came to be formed by the developmental integration of placodes and neural crest cells.

MATERIALS AND METHODS

Embryo collection and fixation

Adult sea lamprey, *Petromyzon marinus*, were collected near the Hammond Bay Biological Station, Millersburg, MI, and shipped to the University of Oklahoma. Animals were kept at 14°C within a recirculating water system. Eggs and sperm from individual females and males were manually collected and eggs fertilized *in vitro*, then reared in 0.05X Marc's Modified Ringer solution (MMR) at constant temperature (18°C). For *in situ* hybridization and immunohistochemistry, embryos at selected stages were fixed in a MEMFA solution (0.1 M MOPS pH7.4, 2mM EGTA, 1mM MgSO4 and 4% Paraformaldehyde) overnight at 4°C,then dehydrated through a methanol/PBST series (25%-50%-75%-100%) and stored at -20°C. All experiments were conducted according to the University of Oklahoma, Institutional Animal Care and Use Committee, protocol R15-027.

Gene cloning and phylogenetic analysis

A fragment of a putative sea lamprey *Six1/2* gene sequence was identified from the 2007 version of the sea lamprey genome assembly from a BLAT search of the Japanese lamprey, *Lethenteron japonicum*, *Six1/2* gene (NCBI, GenBank: AF108812) (Guerin et al., 2009). The predicted cDNA sequence for *P. marinus Six1/2* was obtained from genomic DNA sequence via the GENSCAN Web Server at MIT. The following primers (Forward: 5'-TCCACAAGAACGAGAGCGTG-3'; Reverse: 5'-TGCTGAGACATGTGGCTCTG-3') were used to amplify a 706bp cDNA fragment that encompassed the full-length open reading frame. The predicted amino acid sequence was aligned with untrimmed Six1, Six2, Six3, Six4 Six5 and Six6 amino acid

sequences from other vertebrates using ClustalW. Sequence orthology was determined using a neighbor joining phylogenetic analysis in MEGA 7 with results obtained after 500 parametric bootstrap replicates (Tamura et al., 2013).

CRISPR/Cas9 and microinjection

Guide RNAs (gRNAs) for CRISPR/Cas9-mediated genome editing were designed and synthesized following previously described protocols (Hwang et al., 2013a; Hwang et al., 2013b; Square et al., 2015; Zu et al., 2016). Genomic gRNA target sites for each gene are provided in Table 3.1. Potential off targets were identified by BLAST search against the sea lamprey genome. Only gRNAs with no absolute mismatch in the 10 nucleotides proximal to PAM site were chosen (Cong et al., 2013). The target sites were also chosen to be close to the 5' end of the gene sequence. Two random gRNA sequences that do not target the lamprey genome were designed as control for toxicity effects (RgRNA1 F: TAGGCGGGTAACGTCGTTAGCC; RgRNA1 R: AAACGGCTAACGACGTTACCCG; RgRNA2 F: TAGGTGGAAACCCCAACTTATT; RgRNA3: AAACAATAAGTTGGGGTTTCCA). A cocktail containing gRNA (~800pg), Cas9 protein (1000pg) (PNA Bio) and 10% fluorescein dextran was injected into lamprey zygotes (approx. 3 nl per embryo). Injected embryos were screened after 24 hr to confirm CRISPR/Cas9 injection and only embryos with fluorescence were raised for subsequent analysis. Genotypes of putatively mutant embryos were determined by sequencing the targeted locus from five randomly selected embryos pooled from each set of embryos injected with each gene-specific gRNA-CRISPR/Cas9 cocktail. Pooled genomic DNA was isolated and mutant loci were PCR-amplified, then subcloned into the pGEM T-easy vector, transformed into DH5α

cells and plated on nutrient agar media. Twenty or 40 clones from each putative mutant group were miniprepped and sequenced to determine % efficiencies of CRISPR-mediated deletions (York et al., 2017). Flanking primer sets used to amplify each genomic locus encompassing the target loci are listed in Table 3.2.

In situ hybridization and Immunohistochemistry

Analysis of mRNA expression by *in situ* hybridization was performed as previously described (Nikitina, 2009). For immunohistochemistry (LCM29, HuC/D), fixed embryos were rehydrated into TBST (12 mM Tris pH 7.5, 150 mM NaCl, 0.1% tween-20) through a methanol/TBST series (25%-50%-75%-100% TBST) and bleached in 9% H_2O_2. For LCM29 immunohistochemistry (gift from M. Selzer, Temple University), embryos were treated with 20µg/ml proteinase K for 10 mins and post fixed (0.2% glutaraldehyde; 4% paraformaldehyde in H_2O) for 20 mins. After 3X30 min washes in TBST, embryos were blocked in a blocking buffer (TBS supplemented with 2mg ml^{-1} BSA, 5% heat inactivated normal goat serum, and 0.1% Triton x-100) for one hour at room temperature (RT). Embryos were incubated in primary antibody (LCM29, 1:100; anti-HuC/D, 1:300, Invitrogen) overnight at 4°C, then washed in TBST 3X60 mins at RT, followed by overnight incubation (4°C) in secondary antibodies conjugated with Alexa fluor 546 diluted in blocking solution (1:500). After secondary incubation, embryos were washed 3X, 1 hour each in TBST at RT and mounted whole on a slide or were sectioned using a Vibratome (20µm) and imaged as described below.

Cell lineage tracing

Tahara stage 21 (T21) embryos (Tahara, 1988) were manually dechorionated in 0.05X MMR. To label placodes, live embryos were immersed in 50μM CM-DiI Vybrant cell-labeling solution (Invitrogen) diluted in 0.05X MMR for 20 sec to label the ectoderm, and then washed in 0.05X MMR. DiO (1mM) was focally injected into the dorsal neural tube of head regions as described (McCauley and Bronner-Fraser, 2003) to label disparate cranial neural crest populations. Labeled embryos were held in the dark (18°C, 0.05XMMR) and allowed to develop to selected stages, then fixed in MEMFA as described above. Fixed dye-labeled embryos were washed 3X10mins in PBST and then stored in the dark in PBST containing 0.02% sodium azide (w/v) at 4°C to retard fungal growth.

Imaging

Whole mount embryos were embedded in 75% glycerol and viewed on a Zeiss Discovery V12 stereomicroscope. Alternatively, embryos were mounted in 5% agarose and sectioned using a Vibratome (20μm), then mounted in glycerol and imaged using a Zeiss Axioimager Z1 compound microscope equipped with an Apotome structured illumination module, and Axiocam MRm camera. Image channels were merged using Axiovision (v4.8.2) and figures assembled using Adobe Photoshop (CS4). For fluorescence imaging, Zeiss no. 43 (DiI) and no.38 (DiO) filters sets were used.

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TABLES

Table 3.1. DNA sequences targeting sites for CRISPR/Cas9 mutants

gRNA name	Target sequence with PAM underlined
DlxB	5’-GGATGCGCCCTATGACGCCAAGG-3’
FoxD-A	5’-CCTTCATGATGCACGGGTTGACC-3’
SoxE1	5’-GGGGACGCGGCGAGCCCCTGCGG-3’
SoxE2	5’-GGCGGCCGTGAGTCAAGTGCTGG-3’
RgRNA1	5’-TAGGCGGGTAACGTCGTTAGCC-3’
RgRNA2	5’-TAGGTGGAAACCCCAACTTATT-3’

Table 3.2. Oligonucleotides sequences used to amplify genomic DNA encompassing deleted sequences for *DlxB, FoxD-A, SoxE1,* and *SoxE2* in CRISPR mutants.

gRNA	Forward (5'-3')	Reverse (5'-3')
DlxB	GGGAGCTACTTAAACCATAG	GTTGTTGACACTACCACAAG
FoxD-A	GAGTGTTGCTGTTCTGCGTG	CATCATCATCGTCGTCAATC
SoxE1	TCCTGAACGAGAACGAGAAG	TCACGACACCGTAACCAATC
SoxE2	AACCGCACCGAACGTGATAC	CTGAGCTCGGCGTTGTGAAG

FIGURE LEGENDS

Figure 3.1. Cell lineage tracing to determine neural crest and placodal contributions to cranial sensory ganglia.

(A) T21 stage embryos were immersed in DiI (red) to label ectoderm, including placodal cells, and then immediately focally injected in the neural tube with DiO (green) to label neural crest. (B-E) T26 embryo with placodal (DiI) and neural crest (DiO) cells, labeled at T21 as in A. Labeled placode (B) and neural crest (C) merged in D. The arrow in B and D indicates DiI-labeled cells localized to the trigeminal ganglion, while the arrowhead (C, D) indicates DiO-labeled neural crest. (E) Transverse section through embryo in D (at plane of section E') shows DiO-labeled neural crest outside the neural tube, and the DiI-labeled opV ganglion. (F) Inset in E (F'), enlarged to show DiO-labeled neural crest cells (green) surround but do not overlap DiI-labeled opV cells (red). (G) T26 embryo with DiO (green) labeled neural tube and cranial neural crest. (H) Transverse section through (G) at axial level H' highlights HuC/D labeled neurons (magenta) in both the neural tube (NT) and in cranial ganglia (mmV). (I,J) Inset in H; Neural crest cells (green surround a Hu-positive core of trigeminal (mmV) neurons. A Hu-negative central region of the mmV in I (inside dashed line) contains neural crest (green), shown as a single channel image in J (arrowhead). (K) Transverse section through axial level K' in G. Enlarged inset shown in L and M Hu-positive sensory neurons are present in the core of the vestibuloacoustic ganglion (va), surrounded by DiO-labeled neural crest (green). Neural crest cells are also found in the va ganglion but do not overlap with HuC/D-positive neurons (arrowheads in L, M). M is shown as a single channel image highlighting the non-overlapping of neural crest cells and va

neurons. (N-P) Transverse section through axial level O‘ (pharynx) in G. The dashed line outlines DiO-labeled neural crest-derived pharyngeal prechondrocytes (C) and adjacent perichondrial cells (PC), supporting the neural crest origin of DiO-labeled cells that exited the neural tube. Abbreviations: mmV, maxillomandibular trigeminal ganglion; opV, ophthalmic trigeminal ganglion; p, petrosal ganglion; PC, perichondrial cells; pll, posterior lateral line ganglion; C, pharyngeal prechondrocytes; va, vestibuloacoudtic ganglion. Scale bars in E, H, K: 50μm; scale bars in F, I, J, L, M: 20μm.

Figure 3.2. The transcription factors *SoxE2* and *TwistA*, are expressed in cells surrounding lamprey cranial ganglia.

(A) *SoxE2* is expressed in the presumptive cranial nervous system, otic vesicle and pharyngeal arches at stage T25 (arrows). (B) *SoxE2* co-labeled with HuC/D. (C-E) Horizontal section through B confirms expression of *SoxE2* surrounding cranial ganglia. (F) Twist expression in the presumptive cranial nervous system at T26. (G) Twist co-labeled with HuC/D (H-J) Horizontal section through G confirms Twist expression surrounding the cranial ganglia, similar to *SoxE2*-HuC/D coexpression results (B-E). (K and L) Horizontal section through A (K’), co-labeled with a lamprey glial-specific keratin marker, LCM29 (merged in L). SoxE2 expression surrounds ophthalmic (opV) and maxillomandibular (mmV) ganglia. (M) Enlarged mmV region in L, showing a population of SoxE2-positive cells that surround but do no overlap with the expression of glial –specific keratin (arrows). Individual SoxE2-positive cell is also observed within mmV ganglion and appeared to overlap with keratin (arrowhead). (N-P) Plane of

section N' in F. (N) *TwistA* expression surrounds opV, mmV and petrosal (p) ganglia. Glial-specific keratin expression merged with *TwistA* (O) indicates non-overlapping *TwistA* and glial marker expression in opV, mmV and p. Abbreviations: all, anterior lateral line ganglion; g, geniculate ganglion; mmV, maxillomandibular trigeminal ganglion; opV, ophthalmic trigeminal ganglion; ov, otic vesicle; p, petrosal ganglion; pll, posterior lateral line ganglion.

Figure 3.3. Neural crest markers, *SoxE2*- and *TwistA* are expressed in cells surrounding cranial ganglia.

SoxE2 (A,B) and Twist (C,D) expression in control gRNA injected embryos surrounding mmV and the otic vesicle (arrows), (B,D) Horizontal sections through (A) and (C). Dashed lines outline the mmV and/or opV ganglia in (B) and (D). Arrows indicate *SoxE2* and *TwistA* expression surrounding the opV and/or mmV ganglia. (E, F, I, J) *SoxE2* expression surrounding mmV is lost in *FoxD-A* (E, F) and *SoxE1* (I, J) mutants at stage T25, while expression in the otic vesicle remains unaffected. *TwistA* expression is abolished from area surrounding the cranial ganglia in *ΔFoxD-A* (G, H) and *ΔSoxE1* (K, L) compared with controls (C, D). Asterisks indicate the loss of expression relative to controls. Dashed lines in B, D, F, H, J, L outline the mmV and/or opV ganglia. Abbreviations: mmV, maxillomandibular trigeminal ganglion; opV, ophthalmic trigeminal ganglion; OV, otic vesicle; p, petrosal ganglion.

Figure 3.4. Deletion of neural crest gene expression does not prevent early placode development in lamprey.

Expression of *Six1/2* at stage T25 (A, B) and *Pax3/7* at T24 (C) in control embryos. (D-F) *SoxE2* CRISPR mutants (*ΔSoxE2*); *Six1/2* expression (C) remains unaffected ventral to the otic cesicle (OV), and in the petrosal (p) and posterior lateral line ganglia(pll) at T25 while *Pax3/7* expression (D) is retained in the opV at T24. (E) Horizontal section through D with Six1/2 expression in OV and pll. Similarly, in *ΔFoxD*–A mutants (G-I) *Six1/2* (G, H) and *Pax3/7* (I) expression remain unchanged relative to control embryos (A-C). (J-L) Deletion of *DlxB* sequence (*ΔDlxB*) resulted in reduced expression of *Six1/2* in the otic, petrosal and posterior lateral line ganglia at T25 (J,K), and abolished expression of *Pax3/7* from the opV (asterisk in L). Abbreviations: opV, ophthalmic trigeminal ganglion; ov, otic vesicle; p, petrosal ganglion; pll, posterior lateral line ganglion.

Figure 3.5. HuC/D immunolabeling of control and mutant cranial sensory ganglia. (A-C) control T26 embryo. (A) lateral view of anterior region highlights morphology of cranial and epibranchial ganglia. (B) inset B' in A shows neurons present in the eye (e). ophthalmic (opV) and maxillomandibular (mmV) branches of the trigeminal, and fused geniculate (g) and anterior lateral line (all) ganglia. (C) inset C' in A highlights the petrosal (p), posterior lateral line (pll) and the anteriormost (n1) epibranchial ganglion. (D) Lateral view of T26 *ΔSoxE2* mutant embryo. Insets in D (E' and F') show a narrow elongate opV and mislocated g/all (E) compared to the control (B), while the petrosal (p) is located in a more ventral position relative to the pll (compare position of p in F and C). (G) Lateral view of T26 *ΔFoxD-A* mutant embryo. Insets in G (H' and I') show fused g/all in H is larger and elongate, relative to the control (B) while the petrosal (p) is

located in a more ventral position and the 1st and 2nd epibranchial ganglia appear to be fused into a single aggregate (n1+n2? In I). (J) Lateral view of T26 *ΔDlxB* mutant. Cranial ganglia are missing (asterisks in K) but a small number of individual HuC/D positive neurons are localized where epibranchial ganglia would be present (arrows in L). Abbreviations: all, anterior lateral line ganglion; g, geniculate ganglion; mmV, maxillomandibular trigeminal ganglion; opV, ophthalmic trigeminal ganglion; ov, otic vesicle; p, petrosal ganglion; pll, posterior lateral line ganglion; n1-n6, epibranchial ganglia 1-6. Scale bar: 50μm, same magnification in B,C,E,F,H,I,K and L.

Figure 3.6. Model for the evolution of cranial sensory ganglia.

Representative chordate phylogeny; invertebrate chordate (left), jawless vertebrate lamprey (middle), jawed vertebrate (right). In invertebrate chordates, proto-placodal ectoderm (PPE)-derived sensory neurons are scattered in the head region. Lamprey, possesses paired cranial ganglia with clustered sensory neurons (red circles), surrounded by neural crest cells (green). In jawed vertebrates, placode cells contribute to sensory neurons in the lateral line, and the distal portion of the trigeminal (V), geniculate (VII), vestibuloacoustic (VIII) and epibranchial (IX) ganglia, while neural crest-derived sensory neurons are positioned in the proximal region of these ganglia.

Figure 3.7. Neighbor joining analysis of lamprey Six1/2.

Sea lamprey (*P. marinus*) PmSix1/2 (boxed) is clustered with but basal to other vertebrate Six1 and Six2 group. Amino acid sequences used in this analysis were obtained from the National Center for Biotechnology Information (NCBI) website

(accession numbers in parentheses) and included: *Branchiostoma floridae* Six (EEN45170), *Ciona intestinalis* Six12 (XP 018673379), *Ciona intestinalis* Six45 (NP 001072005), *Danio rerio* Six1a (NP 001009904), *Danio rerio* Six1 (AAO83592), *Danio rerio* Six2 (NP 571858), *Danio rerio* Six3 (BAA31752), *Danio rerio* Six4 (NP 571793), *Danio rerio* Six5 (NP 571795), *Danio rerio* Six6 (NP 957399), *Gallus gallus* Six1 (NP 001038150), *Gallus gallus* Six2 (NP 001038160), *Gallus gallus* Six3 (NP 989695), Gallus gallus Six4 (XP 003641490), *Gallus gallus* Six6 (NP 990325), *Homo sapiens* Six1 (CAA62974), *Homo sapiens* Six2 (AAK16582), *Homo sapiens* Six3 AAD15753, *Homo sapiens* Six4 (BAA84223), *Homo sapiens* Six5 (NP 787071), *Homo sapiens* Six6 (031400), *Mus musculus* Six1 (NP 033215), *Mus musculus* Six2 (BAA11825), *Mus musculus* Six3 (NP 035511), *Mus musculus* Six4 (NP 0355512), *Mus musculus* Six5 (NP 035513), *Mus musculus* Six6 (NP 035514), *Xenopus laevis* Six1 (NP 001082027), *Xenopus laevis* Six2 (XP 018120523), *Xenopus laevis* Six3 (AAF63242), *Xenopus laevis* Six4 (XP 017952201), *Xenopus laevis* Six6 (NP 001081933).

Figure 3.8. Genotyping sequences from five randomly pooled CRISPR injected embryos at stage T26.

Lamprey *DlxB* (A), *FoxD-A* (B), *SoxE1* (C) and *SoxE2* (D) wildtype sequences are listed at the top line of each panel, with the target sites in red and PAM sites in blue. Twenty (*DlxB*, *FoxD-A*) or forty (*SoxE1*, *SoxE2*) clones were sequenced from the total genomic DNA isolated from each pool of injected embryos. Numbers of nucleotide deletions, insertions or unchanged (Green et al.) are listed at 3’ end of each sequence. In

the sequences, deleted nucleotides are shown as "-", while insertion or substitutions are in green. Mutagenesis efficiencies of each CRISPR injection are 100%, 100%, 90% and 87.5% for *DlxB*, *FoxD-A*, *SoxE1* and *SoxE2* respectively.

FIGURES

Figure 3.1. Cell lineage tracing to determine neural crest and placodal contributions to cranial sensory ganglia.

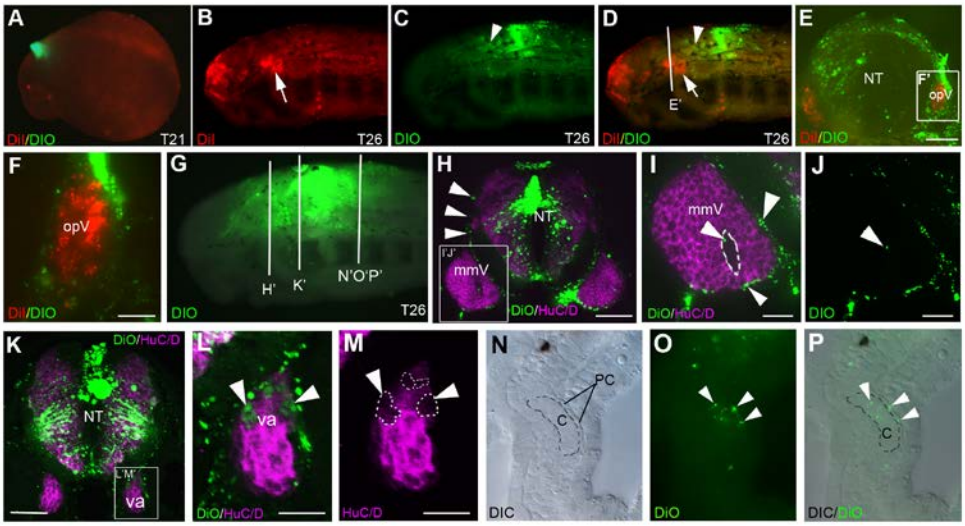


Figure 3.2. The transcription factors *SoxE2* and *TwistA*, are expressed in cells surrounding lamprey cranial ganglia.

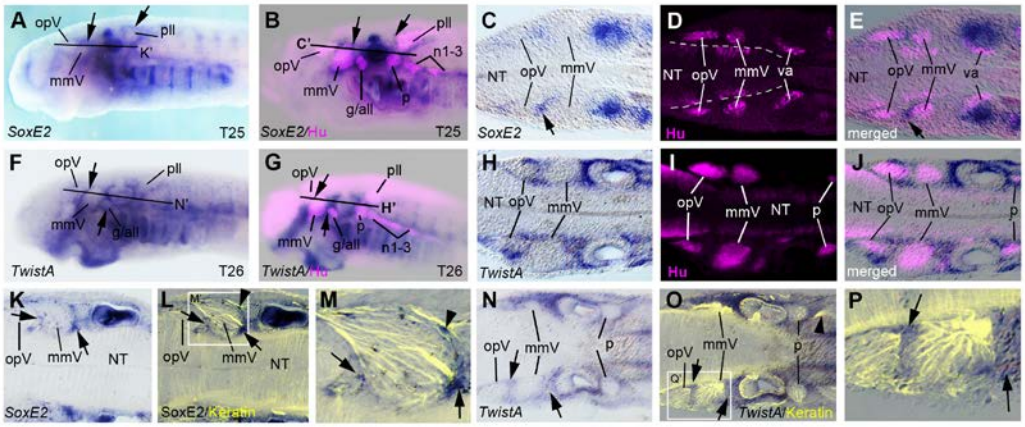


Figure 3.3. Neural crest markers, *SoxE2*- and *TwistA* are expressed in cells surrounding cranial ganglia.

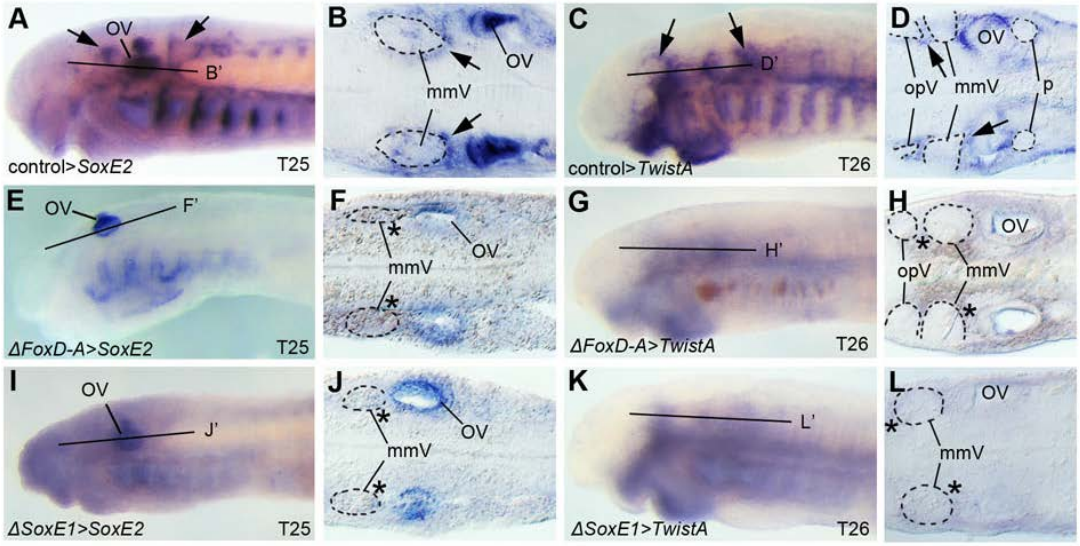


Figure 3.4. Deletion of neural crest gene expression does not prevent early placode development in lamprey

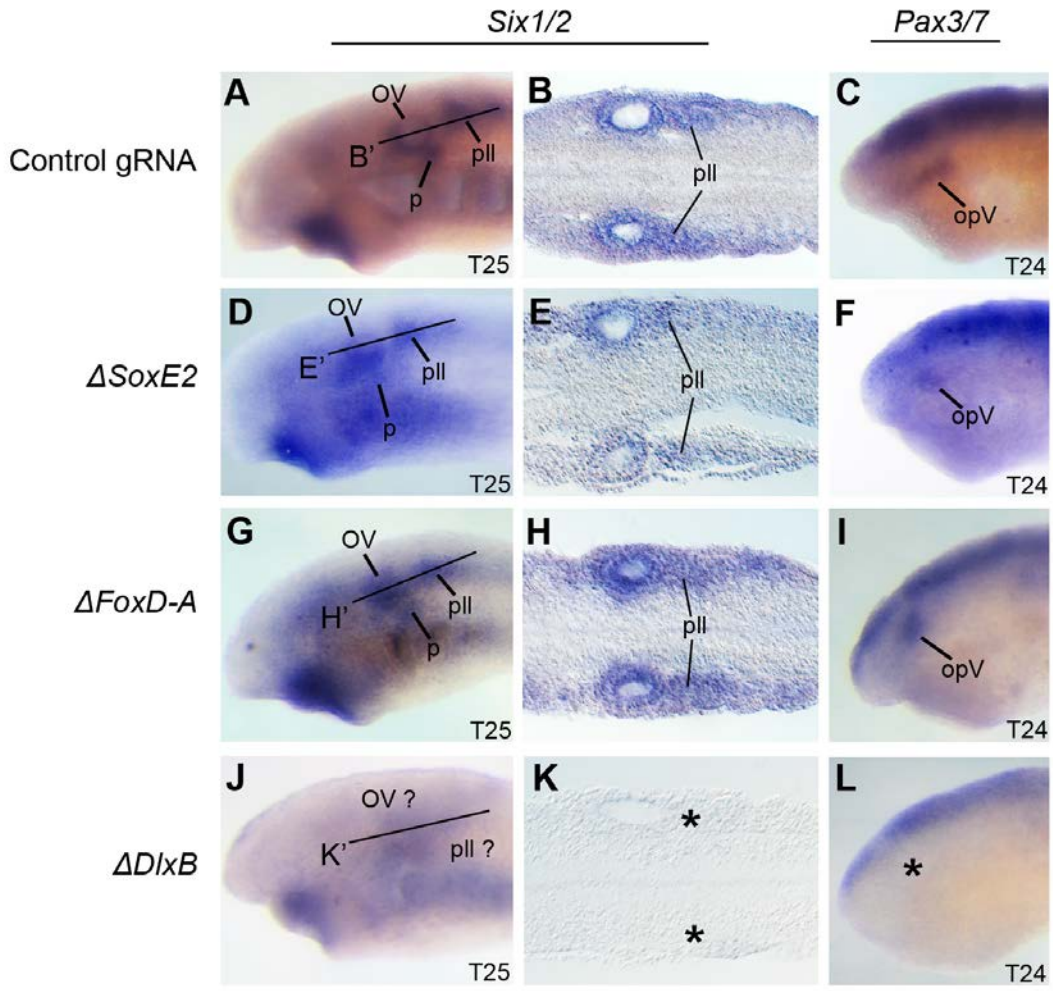


Figure 3.5. HuC/D immunolabeling of control and mutant cranial sensory ganglia.

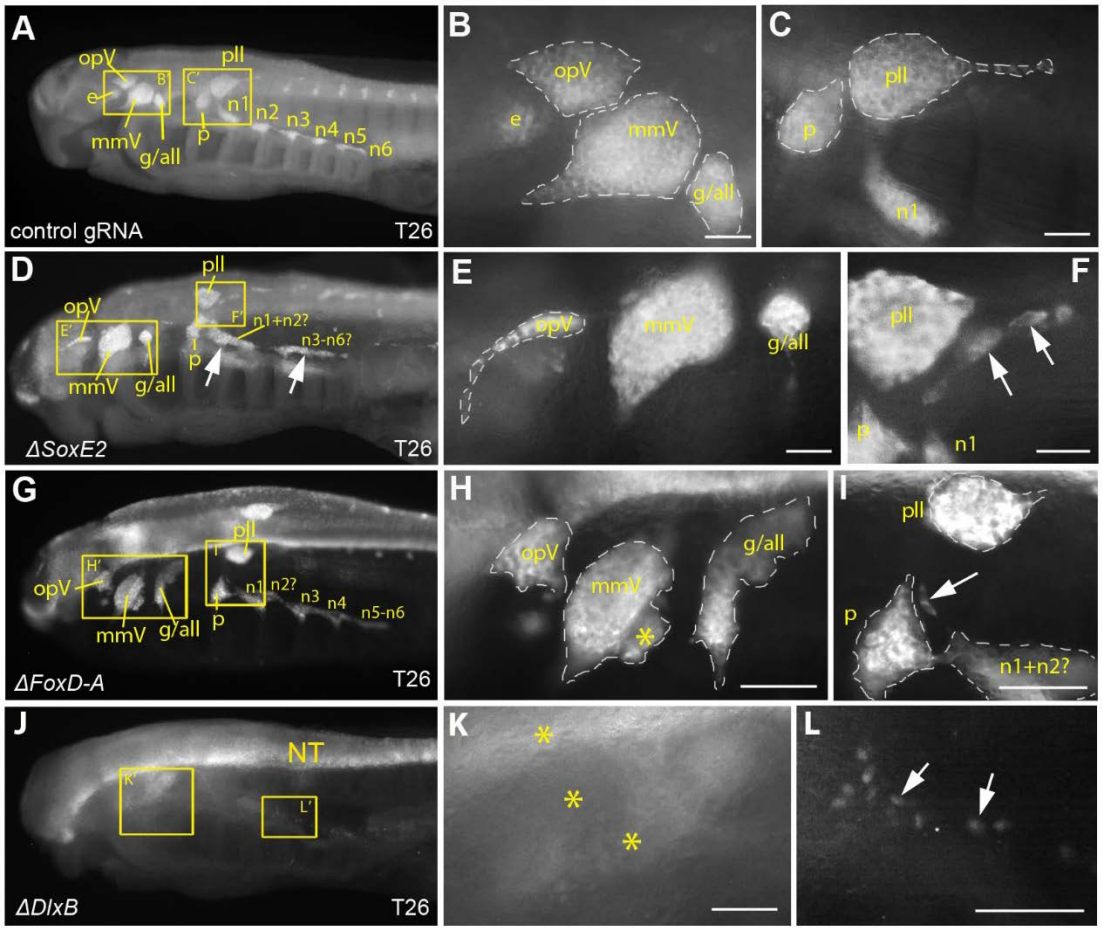
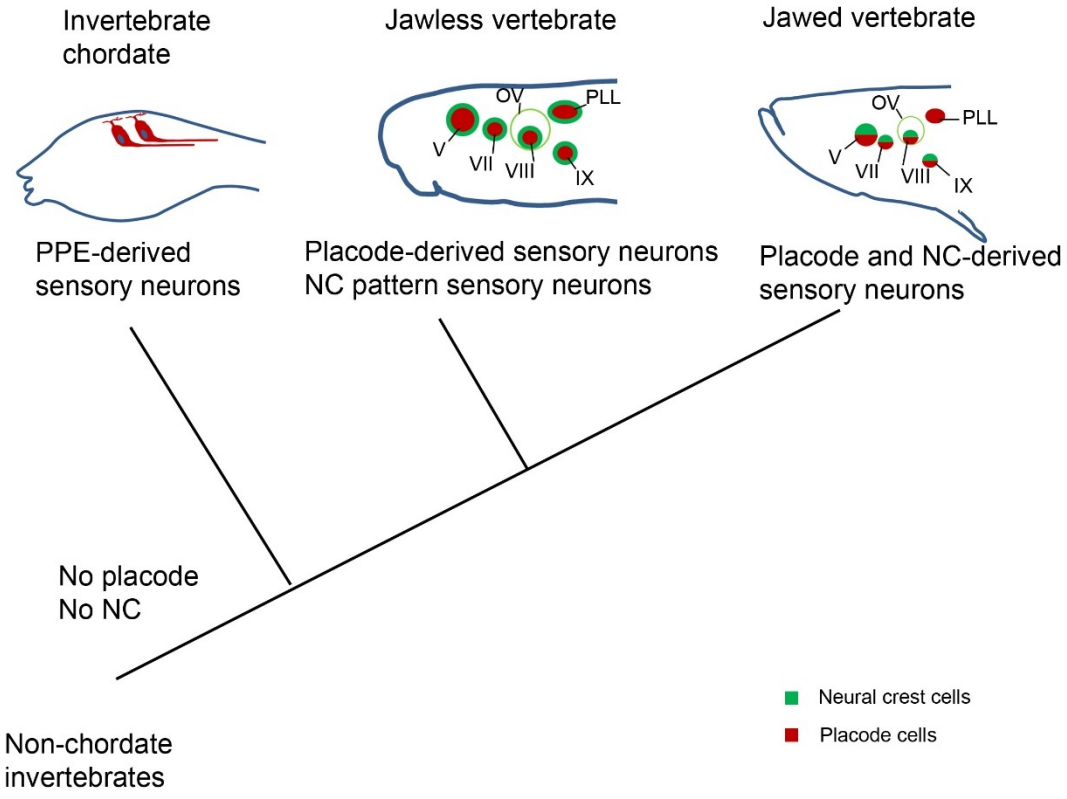


Figure 3.6. Model for the evolution of cranial sensory ganglia.



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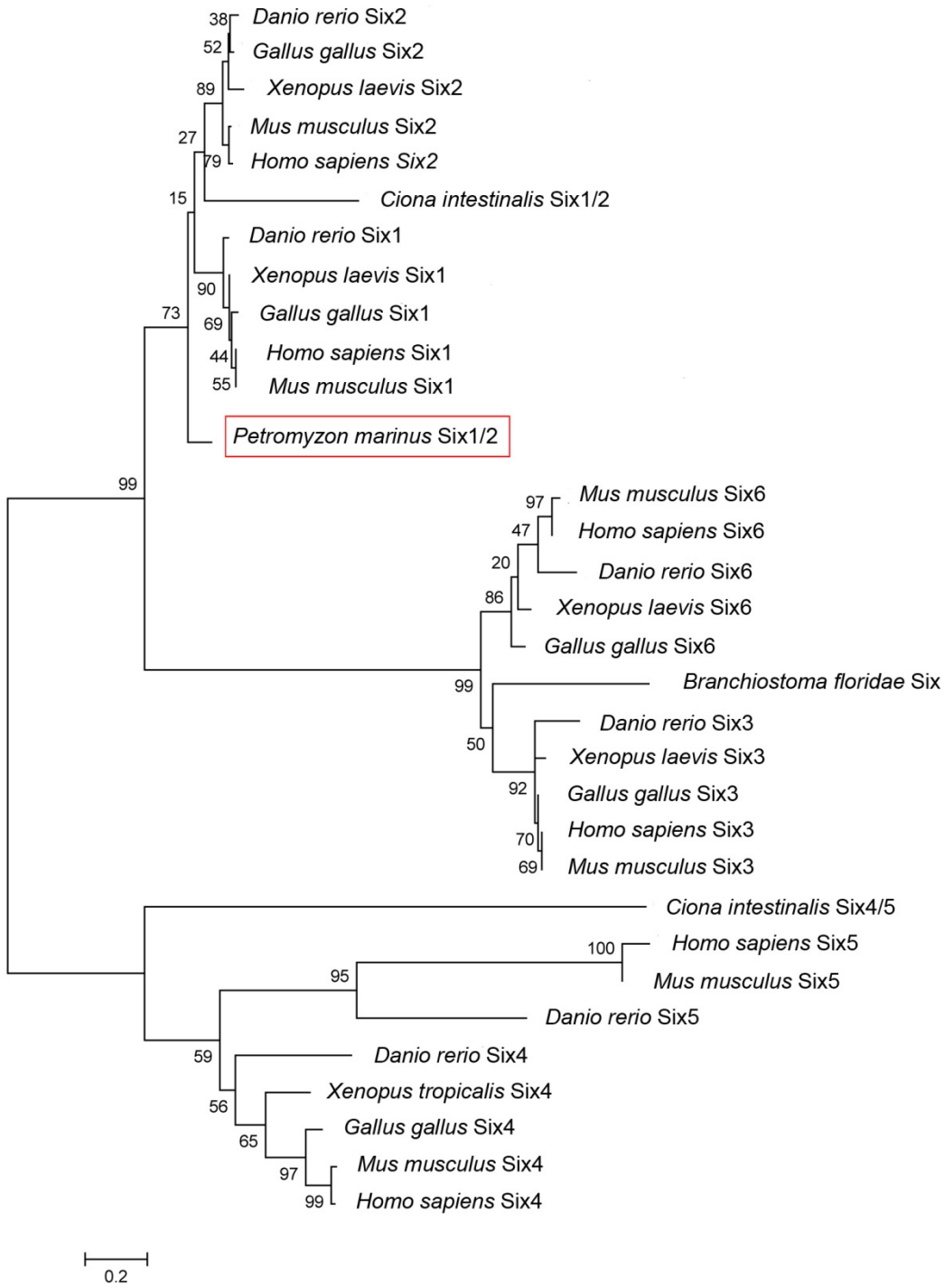
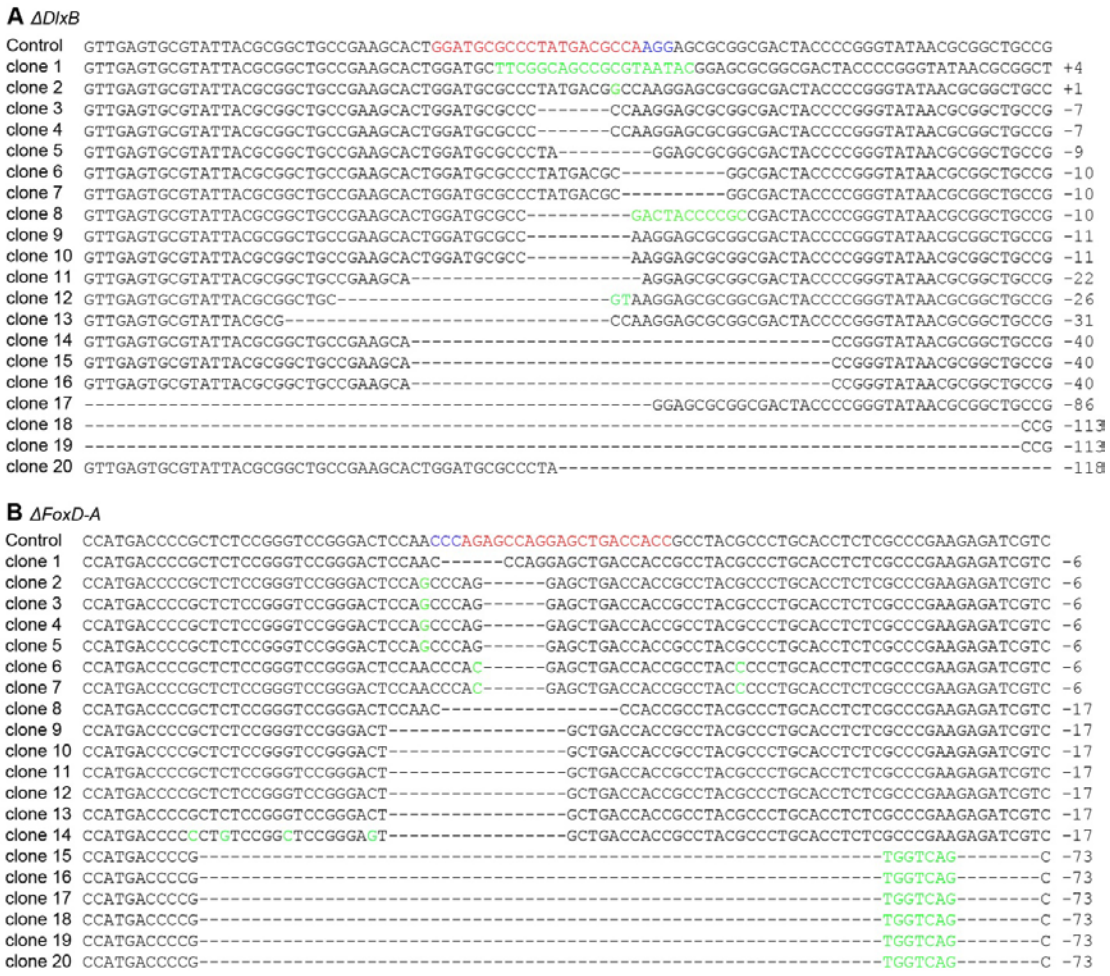


Figure 3.8. Genotyping sequences from five randomly pooled CRISPR injected embryos at stage T26.



C *ΔSoxE1*

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Control  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAGCCCCTGCGGGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG
clone 1  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAGCCCCTGCGGGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG WT
clone 2  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAGCCCCTGCGGGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG WT
clone 3  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAGCCCCTGCGGGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG WT
clone 4  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAGCCCCTGCGGGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG WT
clone 5  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCG----------GGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -10
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clone 7  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCG----------GGGCGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -10
clone 8  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAG-----------CGGACCCCCACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -11
clone 9  GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAG-----------CGGACCCC-ACGGAGGGATCTTCAAGGGGGTCCATGGCGAG -12
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clone 38 GGAAATCGGTCAAGGG-------------------------------------------------------------GGTCCATGGCGAG -63
clone 39 GGAAATCGGTCAAGGGCTCCGGCGACGGGGACGCGGCGAG------------------------------------------------ -96
clone 40 ------------------------------------------------------------------------GGGGGTCCATGGCGAG -124
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D *ΔSoxE2*

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Control  GCGCGGACGACCCCTTCTCGGAGAGCATCCAGGCGGCCGTGAGTCAAGTGCTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC
clone 1  CGGAGAGCATCCAGGCGGCCGTGAGTCAAGAGCATCCAGGCGGCCGTGAGTCAGTCGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC +18
clone 2  CGGAGAGCATCCAGGCGGCCGTGAGTCAAGAGCATCCAGGCGGCCGTGAGTCAGTCGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC +18
clone 3  CCTTCTCGGAGAGCATCCAGGCGGCCGTGAGTCAAGGTCGTCCGCGCCTACTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC +12
clone 4  GCGCGGACGACCCCTTCTCGGAGAGCATCCAGGCGGCCGTGAGTCAAGTGCTGGACGGCTACGACTGGTCGCTGTTGCCCGTGCCCGTGCGC WT
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APPENDIX

LIST OF ABBREVIATIONS

all	Anterior lateral line
AP	Anterior-posterior
bHLH	Basic helix-loop-helix
BLBP	brain lipid-binding protein
BMP	Bone morphogenetic protein
CC	Central canal
CNS	Central nervous system
dp(1-6)	Dorsal progenitor domains (1-6)
DRG	Dorsal root ganglion
DV	Dorso-ventral
EDNRB	Endothelin receptor-B
EMT	Epithelial-mesenchymal transition
ENS	Enteric nervous system
FABP7	Fatty Acid Binding Protein 7
FGFR3	fibroblast growth factor receptor 3
FP	Floor plate
g	Geniculate trigeminal ganglion
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GLAST	Astrocytic glutamate transporter
GP	Glia progenitor

GRN	Gene regulatory network
MBP	Myelin basic protein
M_D	Motor root dorsal branch
M_V	Motor root ventral branch
MEP	Motor exit point
ML	Mantle layer
mmV	Maxillomandibular trigeminal ganglion
MN	Motor neuron
MYA	Million years ago
MZ	Marginal zone
N	Notochord
NC	Neural crest
NP	Neuron progenitor
NT	Neural tube
NVZ	Neuoepithelial ventricular zone
OPC	Oligodendrocyte precursor
opV	Ophthalmic trigeminal ganglion
OV	Otic vesicle
p	Petrosal ganglion
p(0-3)	Ventral progenitor domains (0-3)
PDGFRα	platelet-derived growth factor receptor alpha
pll	Posterior lateral line

PLP	proteolipid protein
pMN	Motor neuron progenitor domain
PNS	Peripheral nervous system
RP	Roof plate
shh	Sonic Hedgehog