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EVOLUTIONARY AND DEVELOPMENTAL ORIGINS OF THE VERTEBRATE NEURAL CREST: INSIGHTS FROM LAMPREYS INTO 500 MILLION YEARS OF VERTEBRATE EVOLUTION

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EVOLUTIONARY AND DEVELOPMENTAL ORIGINS OF THE VERTEBRATE NEURAL CREST: INSIGHTS FROM LAMPREYS INTO 500 MILLION YEARS OF VERTEBRATE EVOLUTION

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ABSTRACT

Much of what distinguishes the vertebrates from their invertebrate relatives is a small group of embryonic cells known as the neural crest. In all vertebrate embryos the neural crest gives rise to a dazzling array of cell types and structures, including cartilage and bone of the head, face and neck, many of the sensory neurons throughout the head and trunk, striking patterns of pigmentation, the covering of teeth, major vessels that circulate blood from the heart, and much more. Despite their importance, the evolutionary origins of neural crest cells have remained enigmatic. My dissertation research is structured to address a set of fundamental questions concerning the evolutionary origins of this important cell type. To this end, I focused on deciphering the molecular, cellular and genetic features underpinning neural crest embryology in lampreys, a group of primitively jawless vertebrates that are derived from some of the earliest branching lineage of vertebrate animals. I then compared these mechanisms in lampreys with similar ones in jawed vertebrates. Because all vertebrates are either jawed or jawless, this comparative embryology method allows one to infer the nature of neural crest cells in the last common ancestor of these two vertebrate groups—in this case, the last common ancestor of all vertebrates. This method not only provides insights into early vertebrate history, but also tells us how changes in neural crest cell function over time could have driven major evolutionary transitions in the vertebrate lineage.

My dissertation is divided into six chapters (summarized below) that discuss and address fundamental, unresolved issues in the field of neural crest and vertebrate development and evolution. These include: the origin and evolution of vertebrates and vertebrate neural crest cells (chapter 1), mechanisms of neural crest cell migration (chapters 2, 3); evolution of developmental mechanisms that guide and organize neural crest cells into anatomical structures

and organs (chapter 4); evolution of neural crest gene function (chapter 5); and the origins of the neural crest cell population in early vertebrates (chapter 6). Taken together, my work provides important insights into the evolution of neural crest cells and the nature of our earliest vertebrate ancestors.

In chapter 1, I review ideas on the evolutionary origins of the vertebrates, vertebrate neural crest cells, and the neural crest gene regulatory network. I then discuss how comparative embryology between jawed and jawless vertebrate systems, coupled with modern molecular genetic tools such as CRISPR/Cas9, can be leveraged to address long-standing hypotheses related to early vertebrate evolution.

One of the most striking features of neural crest cells is their ability to embark on long-distance migration throughout vertebrate embryos. However, the evolutionary-genetic basis for this migratory feat is unknown. In chapter 2, I survey the neural crest literature and describe cellular and genetic features controlling migration that are common to neural crest cells in all vertebrates. I then describe similar features operating in a variety of migratory cells in invertebrates and propose that neural crest cells share a common molecular genetic "signature" with several other migratory cell types. This new synthesis predicts that neural crest cells evolved their impressive migratory capabilities by activating a core genetic toolkit for cell migration that originated in the last common ancestor of all animals.

In chapter 3, I explore the evolutionary origin of genetic mechanisms controlling neural crest migration in vertebrates. To this end, I analyze in lamprey the embryonic function of a gene called *Snail*, which initiates the earliest stages of neural crest migration in jawed vertebrates. I show that lampreys use a fundamentally different mechanism to initially detach neural crest cells from the neural tube before migrating, but that *Snail* gene activity is still required start the

physical process of cell migration. This work is important because it sheds light on the ancestral nature of neural crest migratory mechanisms in the first vertebrates.

Neural crest cells migrate extensively throughout vertebrate embryos, but how did this population of cells become organized into the structures and organ systems that have made the vertebrates an evolutionary success? In chapter 4, I show that the appearance of a new cellular communication system known as *Sema3F-Nrp* was a pivotal event in early vertebrate and neural crest evolution. This cellular system is active in lamprey embryos when neural crest cells are migrating and gradually sculpts specific groups of neural crest cells into key anatomical structures and organs in the lamprey head (e.g., head skeleton, sensory nerve cells). Based on similarities with other vertebrates, I propose that *Sema3F-Nrp* evolved in the first lineage of vertebrates. The origins of this cell-cell communication system allowed our early vertebrate ancestors to organize and pattern neural crest cells for the first time into entirely new structures and may have been an important mechanism for continually generating evolutionary change in the vertebrate body during the past 500 million years.

A major challenge in the field of neural crest biology is to identify how genes in invertebrates, which lack neural crest cells, evolved new roles for neural crest development in vertebrates. For example, the gene *Snail* is a key regulator of neural crest cells in jawed vertebrates and CNS neurons among invertebrates, but the fact that these two very different cell populations both use *Snail* genes is thought to be purely coincidental, rather than suggestive of a common evolutionary origin. In chapter 5, I show in lamprey—a jawless vertebrate spanning the invertebrate-jawed vertebrate divide—that the *Snail* gene regulates lamprey neural crest development as in other vertebrates, but also regulates CNS development similar to invertebrates. Thus, lampreys seem to bridge the evolutionary-genetic gap between invertebrates

and jawed vertebrates by using *Snail* for the simultaneous development of both of these cell populations. This study provides evidence that the genetic control of neural crest development by *Snail* genes in vertebrates likely evolved from an ancient function for CNS development among invertebrates.

In chapter 6, I address the evolutionary origins of the neural crest cell population and its migratory properties. The paradigm in the field of neural crest biology for the past 150 years has been that these cells form within and then migrate from the dorsal part of the embryonic neural tube. Because this process occurs the same way in all vertebrate embryos studied to date it is also thought to be an accurate reflection of what neural crest cells were like in our earliest vertebrate ancestors. In contrast to this paradigm, I demonstrate in lampreys that multiple transcriptional regulators of neural crest identity are expressed throughout much of the entire embryonic neural tube, not just the dorsal-most region. Using cell lineage tracing and live microscopic imaging, I show that neural crest cells expressing these genes can migrate from almost any position along the neural tube dorsal-ventral axis. In light of these findings, I propose a new evolutionary model in which the first vertebrate neural crest cells formed within and migrated from almost any position in the embryonic neural tube. This new model suggests that the neural crest that forms only in the dorsal neural tube of jawed vertebrates is not an ancestral vertebrate feature as has been thought, but should instead be viewed as a relatively recent evolutionary innovation.

PREFACE

The goal of this dissertation is to test a wide range of hypotheses related to the origin and evolution of vertebrates and vertebrate neural crest cells. Each research project (i.e., dissertation chapter) consisted of numerous molecular, cellular, genetic, and embryological techniques, including but not limited to: *in situ* hybridization, immunohistochemistry, cell lineage tracing, cell culture, and CRISPR/Cas9 genome editing. All of the projects contained in this dissertation were conceived by me under the mentorship of Dr. David McCauley in the Department of Biology at the University of Oklahoma.

Chapter one is an introduction to vertebrate evolution, neural crest development and the importance of lampreys in addressing evolutionary hypotheses for each of these topics. I wrote this chapter with editorial assistance from Dr. McCauley.

In chapter two, I conducted a literature review on neural crest migratory mechanisms and synthesized this literature to develop a new theoretical framework for understanding the evolution of neural crest migration. This review was written by me with editorial assistance from Dr. McCauley.

In Chapter three, I investigated the molecular and genetic mechanisms controlling neural crest migration in lamprey embryos. This project was conceived by me, under the mentorship of Dr. McCauley. I conducted all of the experiments myself with some assistance from Dr. Tian Yuan, who assisted with genotyping mutant embryos, and Kevin Zehnder, an undergraduate I trained in the lab who helped with gene expression analysis on wildtype lamprey embryos. This chapter was written by me, with editorial assistance from Dr. McCauley.

Chapter four is focused on understanding how neural crest cells in the developing lamprey head become patterned into specific morphological structures. I conceived this project and performed the majority of experiments, with help from Dr. Yuan and Dr. Lakiza in genotyping mutant embryos. This chapter was written by me and Dr. McCauley provided editorial critiques.

In chapter five, I investigated the molecular mechanisms that led to the integration of the gene *Snail* into the ancestral neural crest gene regulatory network. This project was conceived by me and I performed most of the experiments. Dr. Yuan and Dr. Lakiza assisted in genotyping mutant embryos and Kevin Zehnder helped with gene expression analysis of *Snail* on wildtype embryos. This chapter was written by me with editorial assistance provided by Dr. McCauley.

In chapter six, I investigated the nature of the neural crest cell population in early vertebrates. I conceived of the project and performed most of the experiments. Dr. Yuan contributed reagents (*in situ* riboprobes), Dr. Lakiza provided critique and advice on embryo slice culture experiments, and Kevin Zehnder helped with gene expression analysis on wildtype embryos. This chapter was written by me with editorial assistance by Dr. McCauley.

CHAPTER 1: THE VERTEBRATE NEURAL CREST: DEVELOPMENTAL AND EVOLUTIONARY PERSPECTIVES

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MAIN TEXT

The origin and early evolution of the vertebrates: a developmental perspective

For almost 200 years, the origin and early evolution of the vertebrates has remained a central issue in the fields of comparative biology and natural history (for recent reviews see Gee, 1996; Gee, 2018). Throughout much of the 19th and early 20th centuries, the field of comparative embryology strongly influenced studies on vertebrate evolutionary history. Early work by Haeckel, Müller, Kowalevsky, Balfour, Garstang, and others, represented some of the earliest attempts to resolve vertebrate origins within the framework of Darwinian evolutionary theory (Balfour, 1875; Balfour, 1880; Dohrn, 1875; Garstang, 1894; Garstang, 1896; Gegenbaur, 1878; Geoffroy Saint-Hilaire, 1830; Haeckel, 1860; His, 1868; Kovalevskij, 1866; Müller, 1869). However, with the rediscovery of Mendelian genetics in the early 20th century, and a shift toward an increasingly quantitative, gene-centric view of biology, evolutionary embryology soon gave way to population genetics and the "modern synthesis" as the new foundations for evolutionary biology (Bowler, 1989; Dobzhansky, 1937; Fisher, 1930; Huxley, 1943; Needham, 1959). Consequently, the aims of evolutionary embryologists, including the search for and reconstruction of putative vertebrate ancestors, as well as other types of macroevolutionary change, were viewed as anachronistic by a new generation of biologists working under the modern synthesis, compared to the more rigorous statistical approaches offered by evolutionary genetics (Amundson, 2005; Laubichler and Maienschein, 2008; Love and Raff, 2003; Pigliucci and Muller, 2010; Raff, 1996; Wilkins, 2002). Yet in the past 30 years the fusion of molecular biology, genetics, embryology and modern evolutionary theory—referred to as evolutionary developmental biology, or "evo-devo"—has brought embryology once again to the fore of

evolutionary studies (Carroll, 2000; Carroll et al., 2005; Gilbert, 2003a; Gilbert, 2003b; Gilbert et al., 1996; Hall, 2012; Love and Raff, 2003; Raff, 1996; Wallace, 2002). The evo-devo research program has offered a wealth of new and exciting findings showing how changes in developmental-genetic programs over time can drive the evolution of morphological, physiological and behavioral adaptations, as well as the origin and evolution of animal body plans—including that of the vertebrates.

The vertebrate body plan can be thought of as a developmental patchwork of phylogenetically integrated parts. Several of these parts can be traced back to the last common chordate or even deuterostome ancestor and have served as a scaffold upon which many new characteristic vertebrate features have evolved. Others appear abruptly in the vertebrate lineage with no obvious forerunners found among invertebrates, whether extinct or extant (Lowe et al., 2015; Satoh, 2016). The list of characters diagnostic of vertebrates is vast (for a recent inventory see Gee, 2018), but perhaps the most widely recognized of these are: a large tri-partite brain, including the vertebrate-specific telencephalon; a sophisticated peripheral nervous system with paired sensory organs; inner ear with vestibular apparatus and semicircular canals; muscular pharynx for pump-based respiration; head skeleton of cartilage and/or bone; epibranchial, hypobranchial and external eye muscles; a chambered, muscular heart; pharyngeal arteries supported by endothelium; and a segmented renal filtration system, among others (Gee, 1996; Gee, 2018; Janvier, 1996b; Janvier, 2003; Kardong, 2002).

One of the most fascinating features about the vertebrates is that many of their hallmark traits are derived largely from a single embryonic cell population known as the neural crest, discovered by Wilhelm His over 150 years ago (for comprehensive reviews refer to Hall, 2008;

Le Douarin and Kalcheim, 1999; Sauka-Spengler and Bronner-Fraser, 2008a; Sauka-Spengler et al., 2007; Trainor, 2013). The neural crest is a vertebrate-specific, migratory stem cell population that gives rise to many structures that define much of what it means to be a vertebrate, including most of the peripheral sensory nervous system, pigmentation, parts of the heart and teeth, as well as the vertebrate "new head"—the cartilage and bone that form the craniofacial skeleton (Fig. 1) (Gans and Northcutt, 1983; Green et al., 2015; Northcutt, 2005; Northcutt and Gans, 1983). In gnathostome (jawed) vertebrates the head skeleton has been substantially modified to give rise to articulated biting jaws that bear rows of sharp teeth (Brazeau and Friedman, 2015; Gans and Northcutt, 1983; Green et al., 2015; Kuratani, 2004; Miyashita, 2016; Northcutt, 2005). All of these features allowed early vertebrate fishes to colonize new ecological niches and acquire novel life history features, such as new and diverse modes of feeding, including active predation in some lineages (Denison, 1961; Gans and Northcutt, 1983; Janvier, 1996b; Mallatt, 1984a; Mallatt, 1984b; Mallatt, 1985; Purnell, 2002). This process led to vertebrates distinguishing themselves morphologically, physiologically and behaviorally from their closest relatives, the invertebrate chordates (Gans and Northcutt, 1983; Northcutt and Gans, 1983). Even now the neural crest is implicated in the continual morphological evolution among recent vertebrate groups (Fondon and Garner, 2004; Prescott et al., 2015; Sánchez-Villagra et al., 2016; Wilkins et al., 2014). What all of this reveals is that the neural crest is not only responsible for helping to shape much of the vertebrate body plan, but also continues to serve as a potent source for the developmental evolution of novel traits.

Neural crest developmental genetics

The neural crest forms in all vertebrate embryos in a highly stereotyped manner, at both molecular and cellular levels, in the form of a complex gene regulatory network (GRN, Fig. 2). The first genetic events that generate the neural crest occur as early as gastrulation and are concomitant with the establishment of the dorsal-ventral axis of the embryo. At this time, conserved intercellular signaling events involving antagonistic interactions between ventrally expressed bone morphogenetic proteins (BMPs) and dorsal mesoderm-derived BMP inhibitors (e.g., *Noggin*, *Chordin*, *Follistatin*) establish a gradient of BMP expression that is highest ventrally and laterally where the presumptive epidermis forms, and lowest at the dorsal midline (Betancur et al., 2010) where the presumptive central nervous system (i.e., neural plate) forms (Marchant et al., 1998). The neural crest is established in a region of intermediate BMP expression between these two domains, referred to as the neural plate border (Groves and LaBonne, 2014). It is within this border region that the combined signaling inputs and interaction between neural crest inducers such as *BMPs*, *Wnts*, *FGFs*, and *Delta-Notch* activate a core set of genes known as neural plate border specifiers (*Zic1*, *Dlx5*, *Msx1/2*, *Zic1*, *Pax3/7*, among others) which carve out a specific embryonic territory lateral to the neural plate that confers competence on cells within this region to respond to downstream signals that will specify their fate as bona fide neural crest (Betancur et al., 2010; Sauka-Spengler and Bronner-Fraser, 2008b). Such neural crest specifiers are activated by expression of neural plate border specifiers, combined with reiterated expression of the neural crest inducers. These specification factors are numerous and include members of the *SoxE* family, *Tfap2α*, *Id*, *Snail*/*Slug*, *nMyc*, *Twist*, *Ets*, *Myb* and a host of others that are directly responsible for establishing the hallmarks of neural crest cells (described

in detail below), including maintenance of "stem-ness", conversion from an epithelial to a mesenchymal cell type, and activation of genes that promote survival in the hostile embryonic environment (Baker, 2008; Meulemans and Bronner-Fraser, 2004; Sauka-Spengler and Bronner-Fraser, 2006; Sauka-Spengler and Bronner-Fraser, 2008b; Taylor and LaBonne, 2007).

After being specified in the dorsal neural tube, premigratory neural crest cells in gnathostomes undergo a dramatic change in cell structure and function, known as an epithelial-mesenchymal transition (EMT) (Chen et al., 2012; Kalluri and Weinberg, 2009; Morales et al., 2005; Nakaya and Sheng, 2013; Savagner, 2010). This process is responsible for transforming specified neural crest cells from a static, epithelial cell type to one that is individualized, mesenchymal, and capable of migrating. EMT involves both gene activation and repression programs. In most cases, repression precedes activation and is characterized by a subset of zinc finger and bHLH transcription factors, including *Snail*/*Slug* (Bolos et al., 2003; Manzanares et al., 2001; Nieto et al., 1994), *Twist* (Lander et al., 2011; Yang et al., 2004; Yang and Wu, 2008), *Sip1*/*Zeb2* (Comijn et al., 2001; Rogers, 2013; Vandewalle et al., 2005), *Zeb1* (Sánchez-Tilló et al., 2010; Vannier et al., 2013), *LMO4* (Ochoa and Labonne, 2011; Ochoa et al., 2012), *E12*/*E47*(Moreno-Bueno et al., 2006; Perez-Moreno et al., 2001; Zheng and Kang, 2014), among others. The primary function of these genes in the neural crest domain in gnathostomes is to directly bind and repress transcription of pro-epithelial gene batteries. As these repressive events are occurring there is concomitant upregulation of pro-mesenchymal genes, including Cad6/7/11(Coles et al., 2007; Pla et al., 2001), RhoB (Liu and Jessell, 1998; Perez-Alcala et al., 2004), *nMyc* (Wakamatsu et al., 1997; Zhang et al., 2016), FoxD3 (Simões-Costa et al., 2012; Stewart et al., 2006), Myb (Betancur et al., 2014; Betancur et al., 2009; Karafiat et al., 2005) and

others that are directly or indirectly responsible for activation of downstream effectors that mobilize and reorganize the actin cytoskeleton. In doing so, premigratory neural crest cells establish cell polarity and a leading edge, and, with the activation of contractile proteins, begin coordinated and directed migration out of the neural tube, often along chemotactic gradients (Shellard and Mayor, 2016).

Upon exiting the dorsal neural tube, neural crest cells in the head segregate into three distinct migratory populations, or "streams", along the anterior-posterior axis (mandibular, hyoid and branchial). In most cases the cells that exit from a discrete point in the neural tube migrate more or less along straight pathways ventrally to occupy structures in the pharyngeal arches, jaws or cranial ganglia (Birgbauer et al., 1995; Collazo et al., 1993; Epperlein et al., 2000; Kulesa and Gammill, 2010). Similar features can be found among migratory trunk neural crest, with a key difference being that the migratory trunk neural crest consists of small numbers of individual migratory cells rather than large streams. Throughout the head and trunk very little spatial deviation of the migratory pathways along the anteroposterior axis is observed. These clean and coordinated migratory movements and pathways are dictated by reciprocal intercellular communication between receptors located on the surface on neural crest cells (Eph, Neuropilin, CXCR4) and corresponding ligands (ephrins, semaphorins, CXCL12) secreted by other nearby cells into the extracellular matrix (Kulesa and Gammill, 2010). These interactions can be attractive, repulsive, or a combination of attraction-repulsion, thereby effectively biasing the migratory routes of neural crest cells into specific, narrow regions, such as the serially repeated pharyngeal arches. One example to demonstrate this point is the repulsive interaction between Neuropilin2 receptors and Semaphorin3 ligands (Kulesa et al., 2010; Kulesa and

Gammill, 2010). At the onset of migration, neural crest cells express the Neuropilin2 receptor on their surface and soon thereafter begin migrating (Osborne et al., 2005). Meanwhile, cells in the extracellular matrix actively secrete Sema3 ligand (Casazza et al., 2007). During the earliest phases of migration the neural crest cells will often deviate away from their initial path slightly but are soon set back into the proper migratory route by a repulsive interaction with Sema3 ligand on either side (anterior, posterior) of the migratory path, generating the stereotypical, cleanly separated streams of migrating cells throughout the pharynx (Kulesa et al., 2010; Kulesa and Gammill, 2010).

After becoming organized into a specific region of the embryo, migratory neural crest cells soon undergo a mesenchymal-epithelial-transition (MET), in which they lose their migratory phenotype and then differentiate into specific derivatives (e.g., cartilage, bone, neuron, glia, etc.). Recent work combining lineage tracing and single cell transcriptomics *in situ* reveals that populations of migratory crest undergo gradual commitment to particular cell types by "choices" made among a series of bi-potential fate options (e.g., ecto- versus non-ectomesenchyme, and neural versus melanocyte) (Soldatov et al., 2019). Once committed to a particular lineage, a cascade of transcription factors, signaling molecules and enzymes then drives differentiation into a wide range of cell types and structures that make up a substantial part of the vertebrate body plan (Figs. 1, 2).

Jawless vertebrates as "evo-devo" models for understanding neural crest evolution

The choice of traditional versus non-traditional model systems

How did many of the key features that characterize vertebrates, such as the neural crest and its underlying GRN, arise? And what are their molecular, cellular and genetic origins in the embryo? To begin to address these types of questions, it is important first to emphasize the significance of the model system that one chooses to work with. Much of our knowledge of vertebrate embryonic development comes from model systems (e.g., mouse, chick, zebrafish, frog; Gilbert, 2006) that enable fine-scale dissection of embryonic development. This is due in large part to the fact that these systems 1) are amenable to the establishment of genetic lines or are at least available throughout most of the year for experiments; 2) have high-throughput biochemical and molecular tools readily available; 3) have high-quality, fully annotated genomes, transcriptomes, etc.; 4) are capable of consistently yielding embryos for experimental work; 5) have well-described and vetted protocols for successful and efficient maintenance of adults and embryos.

Answering questions of evolutionary origin, however, often requires a completely different approach and set of criteria (Hall, 2012; Hall, 1999; Wallace, 2002). In the past several years the field of vertebrate evo-devo has witnessed an explosion of research into so-called "non-model" systems, including several chondrichthyans (sharks and other cartilaginous fishes), actinopterygians (ray-finned fishes), and agnathan (jawless) fishes—lampreys and hagfishes (Fig. 3) (Adachi et al., 2016; Braasch et al., 2015; Dahn et al., 2007; Gillis and Hall, 2016; Gillis and Tidswell, 2017; Green and Bronner, 2014; McCauley et al., 2015; Modrell et al., 2017a; Modrell et al., 2017b; Oisi et al., 2013a; Oisi et al., 2013b; Ota et al., 2007; Ota and Kuratani,

2007; Pasquier et al., 2017; Shapiro et al., 2004; Tarazona et al., 2016). Unfortunately, these animals often lack one or more of the features (described above) that make the mainstream developmental models appealing to most embryologists. Why focus on these systems then? Far from being ideal for insights into developmental mechanisms *per se*, their appeal is instead based largely on phylogenetic position. Each occupies an important node in vertebrate phylogeny and is therefore ideally suited for addressing specific evolutionary questions. For example, sharks are useful for understanding the evolution of dermal skeleton and paired fins; paddlefish and gar (basal actinopterygians) provide insight into the fin-to-limb transition and evolution of special sense organs (Adachi et al., 2016; Dahn et al., 2007; Gillis et al., 2017; Gillis et al., 2013; Gillis et al., 2012).

The cyclostomes: lampreys and hagfishes

For questions concerning the origin of the vertebrates and vertebrate-specific traits such as the neural crest, the ideal models are lampreys and hagfishes, which are the only surviving members of a lineage of primitively jawless fishes, the so-called "agnathans" (Fig. 3; Shimeld and Donoghue, 2012). Lampreys and hagfishes for the most part have a typical vertebrate body plan as well as neural crest cells in the head and trunk that contribute to a wide range of cell types, tissues and structures, including a cartilaginous head skeleton, pigment, and peripheral sensory neurons. Historically, the phylogenetic relationships among hagfishes, lampreys and gnathostomes have been controversial, with competing hypotheses placing lampreys as sister to gnathostomes with hagfishes as outgroup (agnathan paraphyly), or a grouping of hagfishes and lampreys together as sister to gnathostomes (cyclostome monophyly) (Hardisty, 1982; Heimberg

et al., 2010; Janvier, 1996a; Løvtrup, 1977; Miyashita et al., 2019a; Yalden, 1985). Resolution of this issue has important implications for vertebrate evolutionary biologists because each hypothesis has a very different take on the nature of early vertebrates and the assembly and modification of the vertebrate body plan. Current evidence from molecules and morphology now firmly places lampreys and hagfishes together as a monophyletic cyclostome group, originating from an ecologically diverse group of jawless fishes that were some of the first vertebrates to appear on the planet nearly half a billion years ago (Heimberg et al., 2010; Miyashita et al., 2019a; Miyashita et al., 2019b; Oisi et al., 2013b; Shimeld and Donoghue, 2012; Stock and Whitt, 1992). Unfortunately, lampreys and hagfishes are also the only extant representatives of this group. The rest of the jawless vertebrates and various stem lineages between the cyclostomes and crown group gnathostomes died out over 300 million years ago (Donoghue and Keating, 2014; Donoghue and Purnell, 2005). The importance of the cyclostomes like many other "non-model" systems lies almost entirely in their unique phylogenetic position (Green and Bronner, 2014; Kuratani et al., 2002; McCauley et al., 2015; Medeiros, 2013; Shimeld and Donoghue, 2012). Because they are sister to the jawed vertebrates (gnathostomes), comparison of embryonic development between these two groups, along with a suitable outgroup, allows evolutionary biologists to infer what features were present in their last common ancestor—the ancestor of all vertebrates (node "a", Fig. 4 and Fig. 5).

As mentioned above, both lampreys and hagfishes are cyclostomes and form a monophyletic sister group (node "b", Fig. 4) to jawed vertebrates (node "c", Fig. 4). Lampreys and hagfishes are then both, by definition, equally distant from all jawed vertebrates, so in principle each should be equally important for obtaining insights into origin and evolution of

neural crest cells. However, this fact ignores important practical concerns that must also be considered. Hagfish embryos have been challenging to obtain because the adults live and spawn in relatively deep sea waters that are difficult to access, their embryos take several months to develop, and they require very precise conditions to live and reproduce in the laboratory (Kuratani and Ota, 2008; Ota et al., 2007; Shimeld and Donoghue, 2012). To put these difficulties into context, a paper describing hagfish neural crest development, published in 2007, was the first such description of this animal's development in over 100 years (Dean, 1899; Kuratani et al., 2016; Kuratani and Ota, 2008; Ota et al., 2007; Ota and Kuratani, 2008; Shimeld and Donoghue, 2012).

Lampreys as tractable models for understanding vertebrate and neural crest evolution

Given the practical bottlenecks that restrict work with hagfishes, much of our knowledge of neural crest development in cyclostomes has instead come from lampreys. Compared to hagfishes, lampreys are much easier to work with. Adult lampreys migrate annually to spawn in shallow streams and rivers in and around North America, South America, Europe, Asia, and Australia and are fairly easy to capture (Docker, 2015; McCauley et al., 2015; Potter et al., 2015). Captured sea lamprey can be held in tanks of circulating water maintained at temperatures that have been shown to promote spawning (~18–20ºC) (reviewed in Moser et al., 2019). When the animals are mature, gametes can be stripped manually and mixed in small bowls of water, with the resulting embryos being reared successfully around 18ºC (Moser et al., 2019; Piavis, 1961; York et al., 2019).

Nonetheless, there are several features of lamprey biology and life history that have kept them from rising to the status of a traditional model system in developmental biology. One important limitation is that lampreys are seasonal animals and therefore only produce live embryos for experimental biology during a few months in the summer. This obviously limits the scope of investigation. In principle, one can plan to experiment using live animals for the few months they are available annually. In practice, however, variation in sea lamprey egg quality and adult survival often yields only a few short weeks of access to embryonic stages. From personal experience, we have found that this drawback can be ameliorated in part by keeping immature animals captured in late winter-early spring in chilled holding tanks and then gradually raising the temperature to create a series of maturing animals throughout summer and early fall (Moser et al., 2019; York et al., 2019). Other serious issues include the fact that because lampreys are semelparous, the adults cannot be kept for more than a single season for breeding and their larvae take several years to reach maturity (Dawson et al., 2015; Hardisty, 2013; Johnson et al., 2015), and the prolonged post-metamorphic phase of parasitic feeding in sea lamprey in particular exacerbates this problem (Potter et al., 2015). Thus, the unique life history of lampreys in general, and sea lamprey in particular, effectively prohibits the establishment of genetic lines, one of the key advantages of mainstream developmental models. Because of these difficulties, much of the early developmental work on lampreys was limited in scope. The earliest work on the embryology of lampreys was descriptive in nature, although this soon gave way to experimental techniques such as ablation and transplantation, and, with the advent of evo-devo, techniques such as gene expression analysis via *in situ* hybridization or immunohistochemistry, cell lineage tracing, and pharmacological application (Damas, 1943;

Damas, 1951; Gaskell, 1908; Horigome et al., 1999; Horstadius, 1950; Kuratani et al., 2004; Langille and Hall, 1988; McCauley and Bronner-Fraser, 2003; Murakami et al., 2001; Newth, 1950; Newth, 1951; Newth, 1956; Nyut, 1955; Tomsa and Langeland, 1999; Ueki et al., 1998). Recently, however, there has been a steady shift toward studying lamprey embryonic development by experimentally determining the function of individual genes or groups of genes (i.e., functional genetic analysis) during lamprey embryogenesis, a goal aided largely by sequencing and annotation of the sea lamprey somatic and germline genomes (Smith et al., 2013; Smith et al., 2018). The most recent technological advance in functional genomics is the **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats/Cas9 (CRISPR/Cas9) system, a revolutionary genome editing technology that has paved the way forward for significant advances in functional genomics in almost any developmental model, including non-traditional models such as lampreys. (Guo et al., 2014; Hwang et al., 2013; Irion et al., 2014; Jao et al., 2013; Qi et al., 2013; Square et al., 2015; Wang et al., 2016; Zu et al., 2016).

Descriptive embryological work in lampreys has provided a firm foundation for comparative studies. However, it is becoming increasingly important to shift the focus toward an evolutionary-developmental framework of functional genomics and genetics in order to test important macroevolutionary hypotheses regarding the origin of vertebrates and vertebrate neural crest cells. Adopting this strategy will help to identify key points of divergence, as well as conservation, of gene regulatory mechanisms in neural crest cells that illuminate our understanding of evolutionary changes to the vertebrate body plan and the evolution of novel cell types and gene regulatory networks. It is within this emerging functional genetic framework that I have designed and conducted my dissertation research. To this end, in the following chapters I

described my use of classical and experimental embryology, gene expression analysis, and functional genetics in the sea lamprey (*Petromyzon marinus*) to test key evolutionary hypotheses for multiple aspects of the embryonic development of neural crest cells reviewed above, including: the origins of neural crest migratory behavior, the evolution of regulatory mechanisms in the neural crest GRN, the construction of neural crest-derived structures, and the very origins of the neural crest cell population in the earliest vertebrates.

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Figure Legends

Fig. 1. Cartoon schematic of a neural crest cell which has stem cell properties (capable of self-renewal, circular arrow) and is multipotent by generating diverse cell types that make up numerous vertebrate structures and tissues.

Fig. 2. Network diagram from Simoes-Costa and Bronner (2015) of the neural crest GRN from studies on jawed vertebrate model systems. Solid lines indicate known direct interactions, whereas dashed lines indicate possible direct interactions.

Fig. 3. Jawless vertebrates. Lampreys (A, B, *Petromyzon marinus* pictured parasitizing a fish) and hagfish (C, *Eptatretus hexatrema*; D, *Eptatretus stoutii*) are the only surviving jawless vertebrates or "agnathans". Images used with permission from Wikipedia commons.

Fig. 4. Lampreys and hagfish occupy a key phylogenetic position for understanding vertebrate developmental evolution. Chordate lineages are diagramed with approximate divergence times (based on Donoghue and Purnell, 2005). While most early vertebrate lineages diverged from their common ancestor (node "a") around 400—450 million years ago, only a fraction are still with us today. In particular, because the stem lineages between cyclostomes (node "b") and crown group gnathostomes (node "c") died out over 300 million years ago, the jawless vertebrates—lampreys and hagfish—are the only extant systems we have to study early vertebrate history. Images were obtained from PhyloPic (phylopic.org).

Fig. 5. The comparative embryological method applied to extant models. Despite the fact that most lineages between the cyclostomes and crown group jawed vertebrates are extinct (see Fig. 4), comparisons of embryonic development among extant jawed vertebrates (e.g., zebrafish, top), jawless vertebrates (lamprey, middle), and an outgroup (invertebrate chordates represented by amphioxus, bottom) allow for inferences concerning the evolution of developmental mechanisms in early vertebrates.

Figure 1

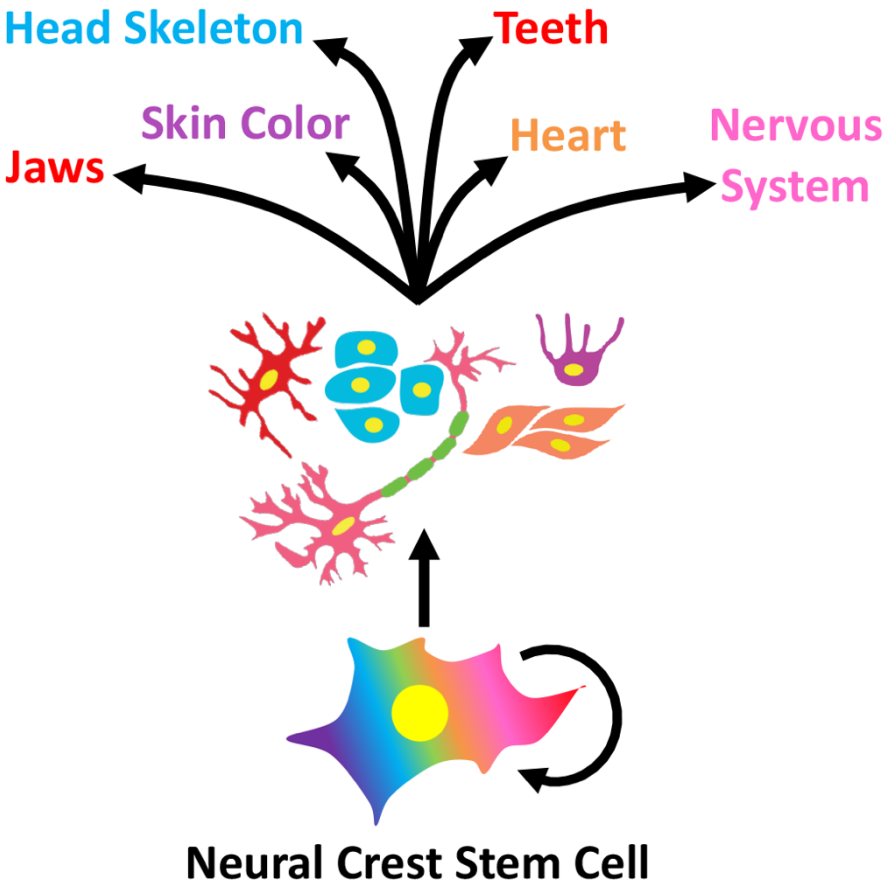

Head Skeleton
Teeth
Skin Color
Heart
Nervous System
Jaws
Neural Crest Stem Cell

Figure 2

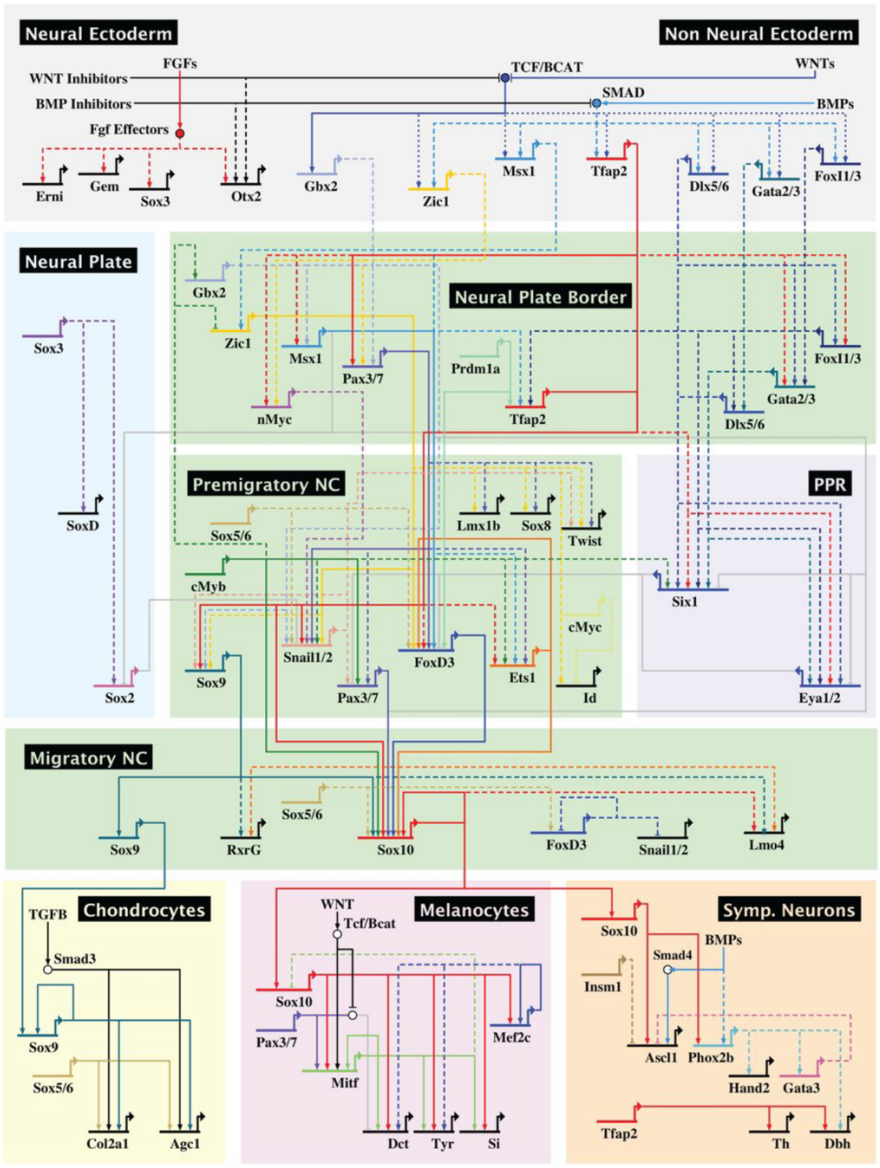

Neural Ectoderm
Non Neural Ectoderm
FGFs
WNT Inhibitors
BMP Inhibitors
Fgf Effectors
TCF/BCAT
WNTs
SMAD
BMPs
Erni
Gem
Sox3
Otx2
Gbx2
Zic1
Msx1
Tfap2
Dlx5/6
Gata2/3
FoxI1/3
Neural Plate
Neural Plate Border
Sox3
Gbx2
Zic1
Msx1
Pax3/7
nMyc
Prdm1a
Tfap2
FoxI1/3
Gata2/3
Dlx5/6
SoxD
Premigratory NC
PPR
Sox5/6
Lmx1b
Sox8
Twist
cMyb
Six1
cMyc
Snail1/2
FoxD3
Ets1
Sox9
Pax3/7
Id
Sox2
Eya1/2
Migratory NC
Sox9
RxrG
Sox5/6
Sox10
FoxD3
Snail1/2
Lmo4
TGFB
Chondrocytes
Smad3
Sox9
Sox5/6
Col2a1
Agc1
WNT
Tcf/Bcat
Melanocytes
Sox10
Pax3/7
Mef2c
Mitf
Dct
Tyr
Si
Symp. Neurons
Sox10
BMPs
Smad4
Insm1
Ascl1
Phox2b
Hand2
Gata3
Tfap2
Th
Dbh

Figure 3

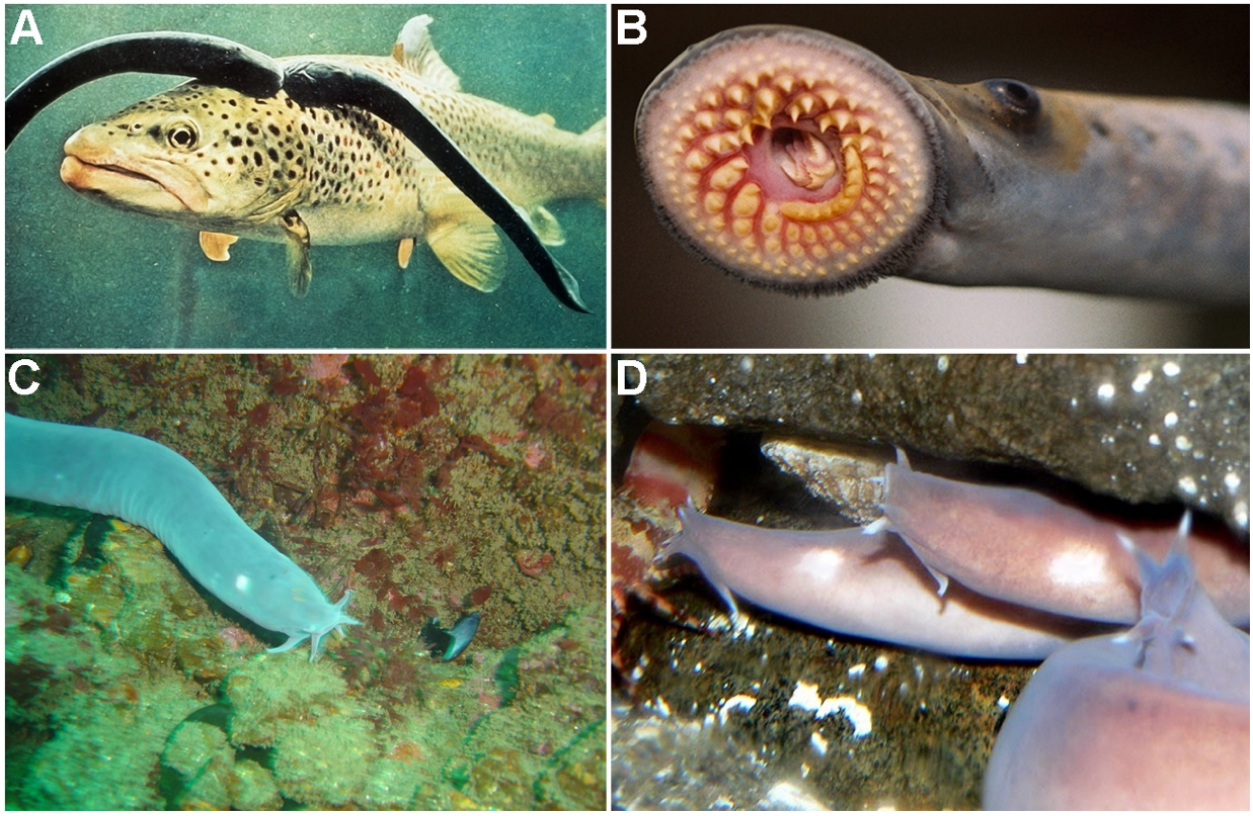


Figure 4

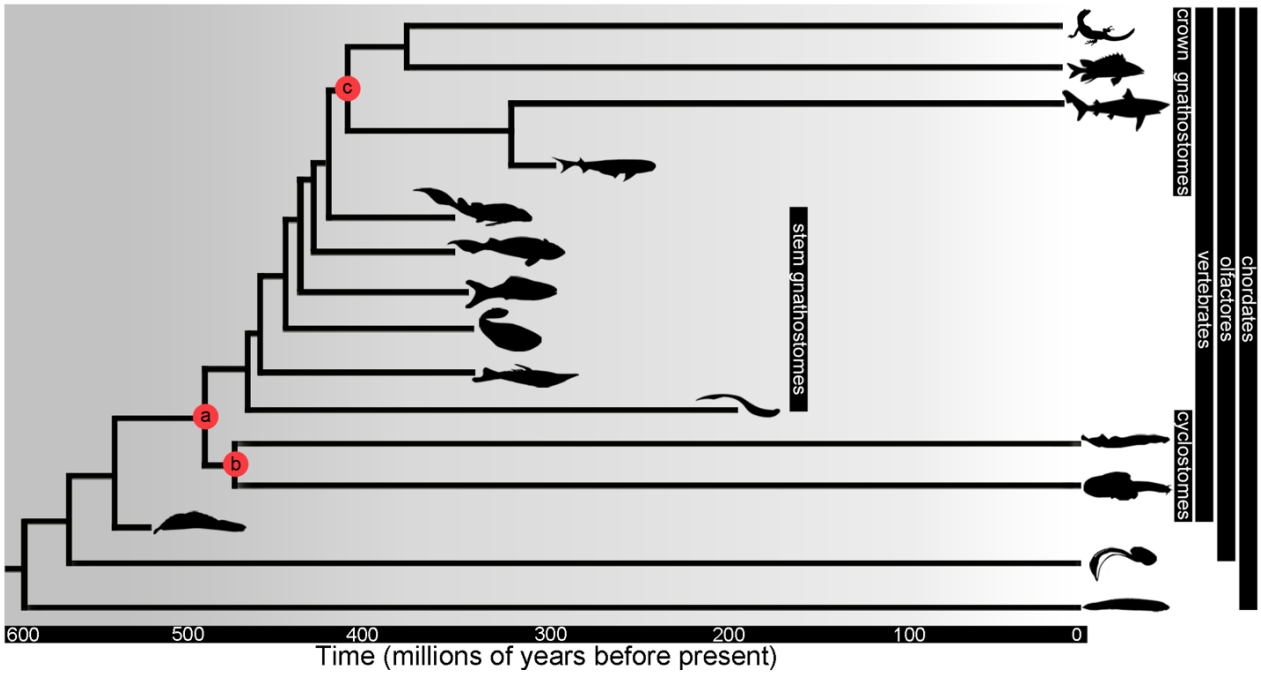
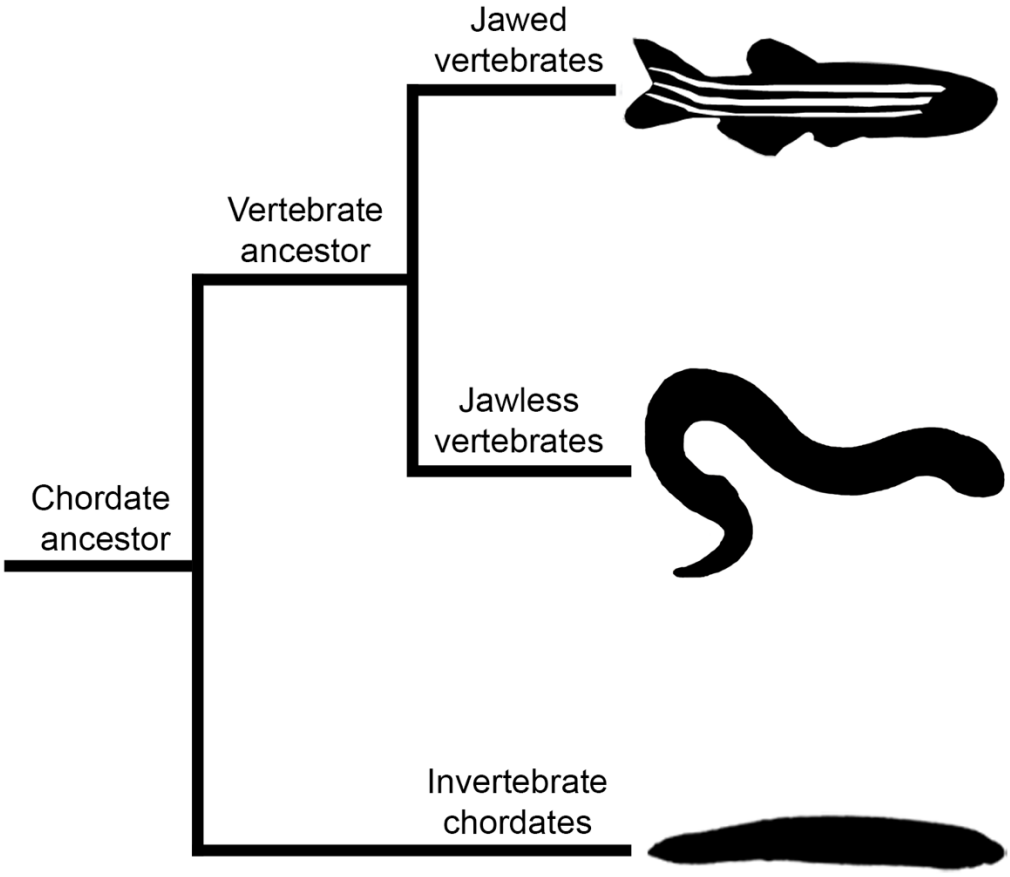

c
a
b
stem gnathostomes
crown gnathostomes
vertebrates
olfactores
chordates
cyclostomes
600
500
400
300
200
100
0
Time (millions of years before present)

Figure 5


Jawed
vertebrates
Vertebrate
ancestor
Jawless
vertebrates
Chordate
ancestor
Invertebrate
chordates

CHAPTER 2: THE EVOLUTION OF NEURAL CREST CELLULAR EMT, DELAMINATION AND MIGRATION

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I. Introduction

During early vertebrate evolution, the acquisition of a novel cell type—the neural crest—was seminal in establishing much of the vertebrate body plan and provided the substrate for diversification of vertebrate morphology (Gans and Northcutt 1983). Neural crest cells form in the dorsal-most part of the embryonic neural tube, from which they detach and then migrate throughout much of the embryo where they give rise to many tissues and organ systems (Le Douarin 1999) (Figures 1, 2 show a generic model for neural crest migration in vertebrate embryos). For example, they comprise the core of the vertebrate head, including much of the cartilage, bone and muscle of the skull and jaws, as well as contribute to paired sensory organ systems (Trainor 2013). Importantly, many of these structures fundamentally distinguish the body plan of vertebrates from that of their closest relatives, the invertebrate protochordates (Gans and Northcutt 1983, Northcutt and Gans 1983). Thus, neural crest cells are a prime example of how a key developmental and evolutionary innovation can drive the origin and diversification of a major animal clade.

The neural crest forms in all vertebrate embryos in a highly stereotyped manner at both molecular and cellular levels. The neural crest is established in a region of intermediate BMP expression between the medial neural plate and lateral epidermal ectoderm, referred to as the neural plate border (Milet and Monsoro-Burq 2012, Groves and LaBonne 2014). Within this border region, intercellular inductive signaling by BMP, WNT, FGF, and Delta-Notch activates a core set of genes known as neural plate border specifiers (e.g., Zic1, Dlx5, Msx1/2, Pax3/7). These, in turn, carve out a specific embryonic territory that confers competence on cells within the neural plate border to respond to downstream signals that will eventually specify their fate as

bona fide neural crest (Monsoro-Burq, Wang, and Harland 2005, Betancur, Bronner-Fraser, and Sauka-Spengler 2010). Such neural crest specifiers are activated by expression of neural plate border specifiers, combined with reiterated expression of neural crest inducers. These specification factors include members of the SoxE family, Tfap2α, Id, Snail1/Snail2, Myc, Twist, Ets, and a host of others that are directly responsible for establishing neural crest identity. One of the hallmarks of neural crest cells is their ability to undergo a dramatic change in cell shape and molecular architecture, resulting in what is known as an epithelial-to-mesenchymal transition (EMT), a feature that confers on neural crest cells an ability to embark on long-distance migrations to specific locations throughout the vertebrate embryo (Savagner 2010, Bronner 2012, Cheung et al. 2005a, Duband et al. 1995, Kerosuo and Bronner-Fraser 2012, Morales, Barbas, and Nieto 2005, Cordero et al. 2011, Kulesa et al. 2010) (Figure 3 shows a simple model for molecular and cellular features of neural crest EMT). Although all metazoans have cells that undergo EMT and migrate at some point during development (Kee et al. 2007, Hay 1995, 2005, Fritzenwanker, Saina, and Technau 2004b), neural crest cells in vertebrates are unique in several respects. For example, the neural crest possesses a unique "molecular anatomy" that is defined by combinatorial expression of a unique suite of transcription factors and signaling molecules (Green, Simoes-Costa, and Bronner 2015, Sauka-Spengler and Bronner-Fraser 2008a, b). In addition, the extent to which neural crest cells migrate as a proliferative, stem cell-like population throughout the embryo, while managing to avoid succumbing to cell death and finding their often distant target regions in the developing vertebrate embryo, has no parallel among other metazoan cell types (Kulesa, Ellies, and Trainor 2004, Cordero et al. 2011, Trainor 2013).

Given the central role of EMT and migration during neural crest development, an outstanding question in vertebrate evolutionary-developmental biology is how these cells acquired this ability, and what the patterns and mechanics were like when they appeared in the first vertebrates over 500 million years ago (Muñoz and Trainor 2015). Currently, we understand very little about the evolutionary history of the core features of the EMT and migration module of the neural crest GRN and therefore have no context for how it was assembled and/or co-opted from molecular circuitry that most likely originated from simpler precursors among invertebrates. Addressing these issues is critical both to understand neural crest evolution specifically and vertebrate evolution more broadly, because it would identify the basal cellular and molecular control mechanisms that allowed the spread of multipotent progenitor cells throughout the vertebrate embryo and contributed to the developmental origin of novel morphological features.

In this Chapter, we review the molecular, cellular and genetic mechanisms controlling EMT and migration of neural crest cells across vertebrates, in an attempt to highlight key features that can be generalized for a range of vertebrate clades. We also compare and contrast similar mechanisms mediating EMT and cell migration in diverse metazoans in order to better understand the phylogenetic context in which neural crest cells acquired the ability to break free from the neural tube and migrate. Finally, using a comparative approach we lay out a scenario for the origin of EMT and migratory properties in vertebrate neural crest cells, and speculate on the assemblage and topology of the EMT-migration module of the neural crest GRN, and how this module has been re-wired across almost 500 million years of vertebrate evolution.

II. Neural Crest EMT and Migration in Vertebrates

There is a large volume of literature describing the molecular, cellular and genetic underpinnings of neural crest EMT and migration in vertebrates. Many of these studies have been conducted in established model systems (e.g., mouse, zebrafish, chick, *Xenopus*), especially those that have genetic lines available and/or are relatively easy to obtain and manipulate in laboratory settings (Barriga et al. 2013, Linker, Bronner-Fraser, and Mayor 2000, Vallin et al. 1998, Carl et al. 1999, Tucker 2004, Morales, Barbas, and Nieto 2005, Coles, Taneyhill, and Bronner-Fraser 2007, Willems et al. 2015, Vannier et al. 2013, Scarpa et al. 2015, Kubota and Ito 2000). However, although this provides an extensive catalogue of the transcription factors, signaling cascades and downstream effectors involved in neural crest EMT and migration, there is very little context for how these studies can shed light on the evolutionary origin of neural crest EMT, how these factors were integrated into the neural crest EMT-migration module of the ancestral GRN, and how elaboration and modification of this module helped shape the vertebrate body plan. Below, we first describe the developmental genetics of EMT, delamination and migration across jawed (gnathostome) vertebrates, with an emphasis on features that are shared across multiple gnathostome taxa, and hence are likely to be evolutionarily conserved. We then compare and contrast these mechanisms operating in gnathostomes with what we currently know from basal jawless (agnathan) vertebrates to gain insight into what may be ancient features of vertebrate neural crest EMT versus those that are elaborations on the ancestral EMT program in more derived clades.

A. Gnathostome Neural Crest EMT and Migration

1. *Intercellular Signaling Pathways in Neural Crest EMT*

Similar to induction of the neural crest at the neural plate border, the initiation of neural crest EMT occurs by receptor-ligand interactions and the subsequent activation of intracellular signaling pathways in the dorsal neural tube. These pathways, which include TGFβ/BMP (Sela-Donenfeld and Kalcheim 1999), FGF, PDGF and WNT signaling (De Calisto et al. 2005, Burstyn-Cohen et al. 2004) are highly conserved across metazoans and have been "plugged in" to diverse GRNs (Davidson and Erwin 2006). During induction in the dorsal neural tube, presumptive neural crest cells often express the receptors for these signaling molecules (e.g., FGFR2/3, PDGFR-α) on their cell surface and activate intracellular signaling events once bound to their corresponding ligands, which emanate from surrounding tissues (Huang and Saint-Jeannet 2004, Sauka-Spengler and Bronner-Fraser 2008b, Knecht and Bonner-Fraser 2002). The result is signal transduction to the nucleus (e.g., by β-catenin and Smads) and subsequent regulation of gene expression, often by activating expression of key transcription factors that will repress epithelial fate and promote the conversion of premigratory neural crest to mesenchyme (Sauka-Spengler and Bronner-Fraser 2008b) (described below). For example, canonical WNT signaling, together with the neural plate border specifiers Pax3 and Zic1 coordinately activate expression of Snail1 (Sato, Sasai, and Sasai 2005), a key transcriptional regulator of neural crest EMT. Similarly, BMP4 signaling stimulates transcriptional activity that leads to an increased number of premigratory neural crest cells undergoing EMT (Shoval, Ludwig, and Kalcheim 2007).

2. *Transcriptional Control of Neural Crest EMT*

As described above, early intercellular inductive signaling converges on the regulation of expression of several transcription factors that are directly responsible for initiating neural crest EMT, delamination and early migration (Sauka-Spengler and Bronner-Fraser 2008b, Thiery and Sleeman 2006). Many of these transcription factors also control specification of bona fide neural crest, including SoxE (McKeown et al. 2005, Cheung et al. 2005a), FoxD (Fairchild et al. 2014) (Stewart et al. 2006), Snail1/Snail2 (Nieto et al. 1994, Manzanares, Locascio, and Nieto 2001, Bolos et al. 2003), Twist (Yang et al. 2004, Yang and Wu 2008, Lander, Nordin, and LaBonne 2011), Sip1 (Vandewalle et al. 2005, Comijn et al. 2001), Zeb1(Sánchez-Tilló et al. 2010, Vannier et al. 2013), LMO4 (Ochoa and Labonne 2011, Ochoa, Salvador, and Labonne 2012), E12/E47 (Perez-Moreno et al. 2001, Moreno-Bueno et al. 2006, Zheng and Kang 2014), among many others. The transcriptional control of neural crest EMT involves coordination of both gene activation and repression programs, which collectively inhibit expression of pro-epithelial gene batteries and upregulate those promoting mesenchymal state (Coles, Taneyhill, and Bronner-Fraser 2007, Pla et al. 2001, Perez-Alcala, Nieto, and Barbas 2004, Liu and Jessell 1998).

In gnathostomes, the HMG box transcription factor sub-family, SoxE, consists of three paralogues, Sox8, Sox9, and Sox10 (Kim et al. 2003, Lee et al. 2016, Cheung and Briscoe 2003, Heeg-truesdell and Labonne 2004). Each of these genes is involved in some capacity early in neural crest specification, although their exact functions may vary and be redundant among taxa (Heeg-truesdell and Labonne 2004). However, after specification SoxE factors are also important transcriptional regulators of neural crest EMT and migration. In the trunk neural crest of chick embryos, for example, Sox9 together with activity of Snail1 and/or Snail2 (formerly identified as

Snail and Slug respectively) proteins is sufficient to induce EMT and delamination, and there is evidence that Sox9 promotes Snail1/Snail2 expression for neural crest EMT in a BMP-dependent manner (Sakai et al. 2006). Similarly, Sox10 is expressed in delaminating neural crest and its upregulation is concomitant with decreased expression of pro-epithelial genes (Cheung et al. 2005b). In addition, forced expression of Sox10 alone is sufficient not only to specify more neural crest, but also to promote ectopic migration of cells from the entire dorsal-ventral axis of the neural tube (McKeown et al. 2005).

The forkhead box transcription factor FoxD3 is another critical upstream specifier of neural crest identity that is also required later for trunk neural crest EMT and migration in both fish and birds. For example, FoxD3 appears to function primarily in controlling differential expression of intercellular adhesion proteins, a prerequisite for neural crest EMT in gnathostomes (Dottori et al. 2001, Cheung et al. 2005b) (described below). Current evidence suggests that FoxD3 represses expression of certain epithelial cadherins and other epithelial gene batteries, whereas it may promote expression of intercellular adhesion proteins that facilitate neural crest delamination and migration (Fairchild and Gammill 2013, Fairchild et al. 2014). The zinc finger transcription factors Snail1 and Snail2 occupy key nodes in the vertebrate neural crest GRN, and play important roles early in neural crest specification (Nieto 2002, Hemavathy, Ashraf, and Ip 2000). Both Snail1 and Snail2 control the onset of neural crest EMT, as functional perturbation of either gene may inhibit delamination of premigratory crest from the neural tube, whereas overexpression promotes ectopic migration (del Barrio and Nieto 2002). There is also strong evidence from both embryos and cell culture that Snail1/2 factors are direct transcriptional repressors of epithelial genes, including type I and type II cadherins (Bolos et al. 2003,

Taneyhill, Coles, and Bronner-Fraser 2007, Guaita et al. 2002). The mechanism by which this occurs—binding of Snail proteins to consensus E-box (CANNTG) elements in the target gene promoter—is thought to down-regulate transcription (Nieto 2002). Although transcriptional repression may be directly related to Snail promoter occupancy, there is evidence that Snail1/2 recruit other proteins that inhibit transcription, such as histone deacetylases (Peinado et al. 2004) and/or other pro-EMT transcription factors such as LMO4 (Ferronha et al. 2013), Sox9 (Cheung and Briscoe 2003, Liu et al. 2013) and LIM homeodomain proteins (Langer et al. 2008). Interestingly, despite widespread evolutionary conservation of Snail1/2 in neural crest development, there have been changes in which specific Snail gene is recruited for neural crest EMT across gnathostome lineages (Locascio et al. 2002). For example, in anamniotes Snail1—rather than Snail2—is the primary regulator of neural crest EMT, and this is likely to be the ancestral condition in vertebrates. Near the origin of amniotes, however, Snail1 activity in the neural crest was swapped for Snail2, and there was yet again an apparent secondary reversion back to Snail1 activity in the neural crest with the evolution of mammals. These data have provided evidence that significant shuffling of Snail1/Snail2 activity has occurred during the evolution of the neural crest EMT module, although the significance of this remains unknown (Locascio et al. 2002).

In addition to the SoxE, FoxD, and Snail families, the transcription factors Twist, Sip and Zeb have emerged as key players in neural crest EMT in gnathostomes (Lander, Nordin, and LaBonne 2011, Vandewalle et al. 2005, Hopwood, Pluck, and Gurdon 1989). Similar to Snail1/2, the mechanism of action by these proteins is thought to be direct transcriptional repression of genes that promote an epithelial state, often achieved by coordinated activity with co-repressors

acting on the promoter of the target gene (Lehmann et al. 2016, Connerney et al. 2006). On the other hand, there may be context-dependent roles for transcriptional activation, as nuclear localization of Twist results in upregulation of N-cadherin and migration in cell culture (Alexander et al. 2006). In chick embryos, Sip1 is thought to promote neural crest delamination since loss of Sip1 function impairs delamination and EMT in premigratory crest (Rogers, Saxena, and Bronner 2013). Finally, although less well-studied, there is growing evidence for the importance of other transcription factors in neural crest EMT, including Ets-1 in cranial neural crest migration in chick (Théveneau, Duband, and Altabef 2007, Tahtakran and Selleck 2003), LMO4 (Ochoa, Salvador, and Labonne 2012), HIF1α (Barriga et al. 2013) in neural crest EMT in *Xenopus*, and E12/E47 during EMT in cultured cells (Perez-Moreno et al. 2001).

3. *Intercellular Adhesion Proteins*

Mesenchymal cells such as migratory neural crest are distinguished from epithelia by their behavior, overall morphology and gene expression profiles (Hay 2005, Thiery 2003, Savagner 2001, Hay 1995, Duband et al. 1995). Mesenchymal cells have unique markers that characterize their affinities for other cells and provide a reliable means of distinguishing them from epithelia (Mani et al. 2008, Fendrich et al. 2009, Kalluri and Weinberg 2009). The classical cadherins have long been recognized as diagnostic of epithelia versus mesenchyme and are divided into type I (E-cadherin, N-cadherin, R-cadherin, P-cadherin) and type II (Cadherin-6–15) (Tepass et al. 2000, Peinado, Portillo, and Cano 2004, Kemler 1992) subgroups. Although other intercellular adhesion proteins have been implicated in neural crest EMT (e.g., occludins, claudins, connexins), we focus here on the role of classical cadherins. For detailed discussions of

other intercellular adhesion proteins involved in neural crest EMT please refer to Trainor (Trainor 2013).

Cadherins are transmembrane proteins that promote the ability of cells to adhere together (Tepass et al. 2000). In the classic model of EMT, premigratory neural crest cells undergo cadherin "switching", a process by which they downregulate type I cadherins (e.g., E- or N-cadherin) and upregulate type II cadherins (Cadherin 6b, Cadherin 7, Cadherin 11) (Wheelock et al. 2008, Nakagawa and Takeichi 1998, Pla et al. 2001). This outcome is achieved through direct repression of type I cadherin genes (and other pro-epithelial gene batteries) by transcription factors such as Snail1/2, Twist, Sip1, Zeb1 and E12/E47, to indirectly stimulate activation of type II cadherins. It was thought that cadherin switching was required for neural crest EMT and migration because the binding affinity of type I cadherins is much greater than that of type II cadherins, and consequently type I cadherins restrict cell movement (Katsamba et al. 2009). However, it is now thought that the concept of a singular type I-type II cadherin switch oversimplifies the complex process of neural crest EMT. In both frog and chick embryos, for example, the type I "pro-epithelial" cadherins such as N-Cadherin and E-Cadherin are expressed in and may even be required for cranial neural crest migration, whereas type II "pro-migration" cadherins, such as Cadherin 6b in the chick midbrain, are repressed in order to allow neural crest cells to migrate (Taneyhill, Coles, and Bronner-Fraser 2007, Huang et al. 2016, Rogers, Saxena, and Bronner 2013). What these findings suggest is that regulation of type I/ type II cadherins in the neural crest is likely to involve subtle yet complex shifts in gene expression—relative to persistent type I cadherin expression in the rest of the neural tube proper—that promote the individuation of the neural crest from the rest of the embryonic neural tube.

4. *Reorganization of the Cytoskeleton*

The marked shift in intercellular adhesion proteins during neural crest EMT is accompanied by an equally important series of changes to the cellular cytoskeleton (Hill et al. 2008, Duband et al. 1995). Although there are numerous ways in which cytoskeletal changes promote EMT, the most thoroughly characterized of these involve a fundamental reorganization of the structural properties of actin filaments to establish cell polarity with a leading edge that allows directed migration (Savagner 2001). Cytoskeletal reorganization is regulated in large part by the Rho family of small GTPases, a subfamily of the Ras superfamily of small G-protein signaling molecules (Clay and Halloran 2011, Sadok and Marshall 2014). The Rho subfamily includes RhoA/B/C, and each Rho protein has distinct developmental functions in cell polarity and migration. Among the Rho A/B/C group, RhoB has figured prominently in studies of neural crest EMT and migration (Liu and Jessell 1998). Through interactions with the Rho associated kinase (ROCK), Rho GTPases are responsible for regulating the spatial-temporal assembly of actin microfilaments, as well as their contractility (Lai et al. 2005). These functions are most obvious in Rho-mediated organization of stress fibers and actin filaments in filapodia and lamellipodia of migratory cells (Nobes and Hall 1995). At the transcriptional level, the HMG box transcription factor SoxD is known to regulate expression of RhoB; loss of SoxD-mediated RhoB expression prevents premigratory neural crest from exiting the neural tube (Perez-Alcala, Nieto, and Barbas 2004). BMP and WNT signaling also helps reorganize the cytoskeleton to enhance neural crest migration by promoting RhoB expression in premigratory and migratory crest, and almost certainly does so by regulating expression of transcription factors that directly promote neural crest EMT and migration (Burstyn-Cohen et al. 2004, Taneyhill and Bronner-Fraser 2005, Sela-

Donenfeld and Kalcheim 1999). Taken together, these findings demonstrate that cytoskeletal mobilization and reorganization—mediated in large part by Rho GTPases—is instrumental to neural crest EMT and migration in gnathostomes.

5. *Breakdown of the Basal Lamina and Early Migration*

After neurulation, the basal lamina—a specialized extracellular matrix (ECM) of fibrous protein—forms on the basal surface of the neural tube. The basal lamina has many different functions, including inhibition of EMT and cell migration to maintain structural integrity of the neuroepithelium (Tyler 2003b). Neural crest cells, in order to emigrate from the neural tube, must overcome the barriers imposed by the neural tube basal lamina (Erickson 1987). In some cases, the default state is that the dorsal neural tube delays production of a basal lamina until all neural crest cells have migrated, rendering this problem obsolete (Martins-Green and Erickson 1987). In other cases, however, premigratory neural crest cells undergoing EMT must actively break down and degrade the basal lamina to fully emigrate from the neural tube (Kerosuo and Bronner-Fraser 2012).

Neural crest cells express several different proteases that help to break down the basal lamina and alter the structural properties of the surrounding ECM. Together, these processes create a favorable environment for migration and are necessary for a proper EMT. The two most widely studied of these proteins are ADAMs (A Disintegrin And Metalloprotease) and MMPs (Matrix MetalloProteases) (Neuner et al. 2009) (Cai et al. 2000) (Alfandari et al. 2001). Both proteases cleave cell surface proteins and signaling molecules, with the resulting fragments serving to drive the EMT program by transcriptional regulation. *Xenopus* ADAM13 is required

for migration of cranial neural crest (Alfandari et al. 2001, McCusker et al. 2009), and ADAM19 is expressed prominently in the neural crest (Neuner et al. 2009). MMP2 is expressed in chick migratory neural crest (Cai et al. 2000, Anderson 2003, Duong and Erickson 2004). These proteases also degrade and remodel ECM proteins surrounding neural crest cells such as fibronectin, in order to create an ECM pathway favorable to neural crest migration (Thiery and Sleeman 2006, Trainor 2013). In chick, for example, transcriptional regulation of ADAMs and MMPs is mediated by Ets1, leading to breakdown of the basal lamina and early neural crest migration (Théveneau, Duband, and Altabef 2007).

B. Cyclostome Neural Crest EMT and Migration

The cyclostomes (lampreys and hagfish) are the only surviving relicts of an ancient and ecologically dominant group of jawless fish (agnathans) from the Paleozoic. Because they occupy the basal-most phylogenetic position among extant vertebrates, and are the sister group to gnathostomes (Heimberg et al. 2010), comparative studies of cyclostome biology have strong potential to offer insights into the genetic and morphological innovations likely present in the vertebrate ancestor. Given the relative ease of obtaining, culturing, and manipulating embryos (McCauley et al. 2015), as well as the availability of an annotated genome (Smith et al. 2013) and modern molecular-genetic tools (York et al. 2017, Zu et al. 2016, Square et al. 2015, Parker et al. 2014), lampreys have emerged as the leading cyclostome model system for studying the evolutionary-developmental biology of basal vertebrate traits (McCauley et al. 2015). Similar to gnathostomes, lampreys have neural crest cells that contribute to the head skeleton (Cattell et al. 2011, Jandzik et al. 2015, McCauley and Bronner-Fraser 2006, Lakiza et al. 2011), pigment

(McCauley and Bronner-Fraser 2003, Lakiza et al. 2011), and cranial sensory and enteric neurons and glia (Green, Uy, and Bronner 2017, Modrell et al. 2014). Early cell labeling and gene expression studies showed that many defining properties of neural crest cells are conserved in lamprey (McCauley and Bronner-Fraser 2003). For example, lamprey neural crest cells form in the dorsal neural tube, delaminate, and then migrate in three streams in the head, similar to that in gnathostomes (McCauley and Bronner-Fraser 2003). Moreover, gene expression and functional analysis has shown that much of the neural crest GRN in agnathans is very similar overall to that of gnathostomes, suggesting that the molecular features of vertebrate neural crest cells may be conserved to the base of vertebrates (Sauka-Spengler et al. 2007). Notably, however, very little work has been done on specific modules within the broader neural crest GRN, including the regulatory circuit that controls defining features of the neural crest—EMT, delamination and cell migration.

1. *Intercellular Signaling and Transcriptional Control*

In gnathostomes, BMP2 and BMP4 are expressed in the dorsal neural tube and are crucial for activating early transcriptional regulators of neural crest EMT, such as Snail1/2 (Sauka-Spengler and Bronner-Fraser 2008b, Raible and Ragland 2005). By contrast, despite having three BMP paralogues (BMP2/4a, BMP2/4b, BMP2/4c), lamprey never expresses any of these genes during the onset of neural crest EMT and migration, localizing instead to the neural plate, neural plate border, endoderm and post-migratory crest (Sauka-Spengler et al. 2007, McCauley and Bronner-Fraser 2004). Expression of the intercellular inducer WNT8 occurs in both the neural plate border and dorsal neural tube, suggesting a possible WNT-mediated signaling role during

lamprey neural crest development that is conserved with gnathostomes (Sauka-Spengler and Bronner-Fraser 2008c). However, functional analysis is needed to tease apart inductive signaling of the neural crest in general from a possible direct role for WNTs and other intercellular signaling molecules during lamprey neural crest EMT.

The transcriptional control of lamprey neural crest EMT and migration shows both evolutionary conservation, as well as important differences, compared to gnathostomes. Lamprey has three SoxE group transcription factors (SoxE1, SoxE2, SoxE3), and phylogenetic analysis shows that SoxE2 and SoxE3 are likely to be homologues of gnathostome Sox10 and Sox9, respectively (Lee et al. 2016). Both SoxE1 and SoxE2 are expressed in neural crest cells undergoing EMT and migration from the neural tube (Lakiza et al. 2011). However, functional knockdown of either gene results in nearly complete loss of premigratory neural crest, rather than an arrest of EMT and migration, making it unclear whether or not SoxE factors actually regulate EMT independent of an earlier role in neural crest specification (McCauley and Bronner-Fraser 2006, Lakiza et al. 2011). Although lamprey does not have a strict paralogue to gnathostome FoxD3, a homologue of this gene, FoxD-A, is expressed in lamprey premigratory and migratory neural crest, and functional perturbation of FoxD-A results in loss of migratory neural crest and neural crest derivatives (Sauka-Spengler et al. 2007). In contrast to SoxE and FoxD-group genes, the transcription factors Twist (Betancur, Bronner-Fraser, and Sauka-Spengler 2010, Hopwood, Pluck, and Gurdon 1989) and Ets (Betancur, Sauka-Spengler, and Bronner-Fraser 2009), which repress epithelial cell fate and promote cranial neural crest delamination in gnathostomes, respectively, have no apparent role in neural crest EMT and

migration in lamprey, but are both expressed in post-migratory cranial neural crest-derived cartilage and embryonic vasculature, respectively (Sauka-Spengler et al. 2007).

Preliminary studies on lamprey Snail, a "master" regulator of neural crest EMT and migration in gnathostomes, suggested that this factor was not expressed at all in lamprey premigratory or migratory neural crest cells (Rahimi et al. 2009). However, it has recently been shown that Snail is indeed critical for early neural crest development in lamprey, as Snail mutants show defects in neural crest migration, fail to express a type II cadherin (CadIIA) in premigratory and migratory neural crest, and lose neural crest derivatives such as cartilage and cranial sensory neurons (York et al. 2017). It was also shown that lamprey has homologues of both *Sip1* and *Zeb1* that are expressed in domains overlapping with *Snail*, *CadIIA*, and *Pax3/7* in the dorsal neural tube during the onset of neural crest migration, raising the possibility that Sip and Zeb transcription factors are evolutionarily conserved regulators of neural crest EMT across vertebrates (York et al. 2017).

2. *Intercellular Adhesion Proteins*

In contrast to gnathostomes, lampreys have a relatively simple genomic complement of classical cadherin adhesion proteins, with only a single representative of the type I (*CadIA*) and type II (*CadIIA*) cadherins, similar to the condition in invertebrate chordates (York et al. 2017, Hulpiau and Van Roy 2009, Gallin 1998). Interestingly, *CadIA* is never expressed anywhere in the neural tube during the early stages of neural crest EMT/delamination, whereas the pro-mesenchymal *CadIIA* localizes to early premigratory and migratory cranial neural crest (York et al. 2017). This stands in stark contrast to the situation in gnathostomes in which type I cadherins are first

expressed in the dorsal neuroepithelium and then are gradually replaced by pro-mesenchymal type II cadherins to facilitate migration ("cadherin switching", described above). This suggests that neural crest cells in early vertebrates may not have required modulation of cadherin intercellular adhesion proteins to facilitate neural crest migration, and that cadherin-switching-mediated neural crest EMT and migration may be a gnathostome novelty (York et al. 2017).

3. *Cytoskeletal reorganization and breakdown of basal lamina*

Compared to detailed analysis both *in vivo* and *in vitro* of the downstream mechanics of cellular EMT and migration in gnathostomes (cytoskeletal changes, ECM remodeling), almost nothing is known regarding the operation of these processes in agnathans. For example, it is unclear what homologues of ECM remodeling proteins (e.g., MMPs, ADAMs) or cytoskeletal regulators (Rho GTPases) are present in agnathan genomes, much less where these genes may be expressed and what their functional relationships are to neural crest EMT, delamination and migration.

C. Summary

Taken together, studies of neural crest development in lamprey suggest that the topology of the EMT/migration-specific module in agnathans shows evolutionarily conserved features, but is also quite different from that of gnathostomes. These differences raise the possibility that the molecular-genetic and cellular mechanisms of neural crest EMT in gnathostomes may have diverged from the ancestral condition in early vertebrates. For example, a lack of expression of key transcriptional regulators of neural crest EMT, as well as no apparent switching between intercellular adhesion proteins such as classical cadherins in agnathans, may indicate that the

EMT of ancestral vertebrate neural crest cells may have involved alternative means to initiate migration from the neural tube. It is clear that a more detailed analysis of the functional roles of the neural crest EMT module during agnathan development is needed, in order to clarify what the ancestral state of the vertebrate EMT module may have been and how this module has been altered over the course of vertebrate evolution.

III. Ancient Origin of Cellular EMT and an EMT Gene Regulatory Network

Vertebrate neural crest cells are defined in part by their ability to undergo a coordinated EMT and initiate migration. However, this feature alone does not distinguish neural crest cells from other cell types. Indeed, cells undergoing EMT have been described for almost all Metazoan clades, and an ability to undergo EMT may in fact be a defining feature of Metazoans (Figure 4 summarizes diverse cellular EMTs in a wide range of Metazoan groups). The origin of EMT and mesenchyme, in conjunction with a complex ECM (Ereskovsky, Renard, and Borchiellini 2013, Hynes 2012), most likely facilitated the integration and communication of large groups of cells, thereby allowing the establishment of discrete structures, and eventually, organs and organ systems. The interface of epithelium and ECM facilitated the ability of individual cells or groups of cells to undergo morphogenetic movements and establish basic structural properties within animal embryos. However, it was the ability of a subset of cells to undergo EMT during Metazoan embryonic development that was largely responsible for generating the varied and complex developmental morphologies across groups as diverse as sponges and humans. By deploying EMTs and migrating into new regions of the embryo, these cell types could encounter new cellular environments and intercellular signaling cues, thereby potentiating the evolution of

novel cell types and embryonic structures. Below, we review several examples of EMTs across invertebrate Metazoans, with a focus on the molecular, cellular and genetic similarities to vertebrate neural crest cells in order to provide context for a program for cellular EMT that could have been incorporated into the ancestral neural crest GRN.

A. Basal Metazoans

1. *Sponges*

Sponges are almost entirely mesenchymal animals, being made up of a variety of loosely arranged cell types that appear to lack belt-like adhesion proteins and a basal lamina (Boute et al. 1996, Tyler 2003a). Nonetheless, sponge embryos perform gastrulation-like movements and there are several points during sponge development in which individual cells or groups of cells undergo EMT-based movements involving ingression, delamination and invagination (Nakanishi, Sogabe, and Degnan 2014, Ereskovsky 2010, Ereskovsky, Konyukov, and Tokina 2010). Interestingly, there are no classical cadherin homologues in sponge genomes, making it unclear if the EMT mechanisms operating during sponge development are like those operating in other animals. This is accentuated by the fact that genomic analysis of the *A. queenslandica* genome failed to uncover many key transcriptional regulators of EMT and migration, including Twist, Snail and Forkhead (Nakanishi, Sogabe, and Degnan 2014). Nonetheless, the downstream mechanics of cell polarity and migration (Rho, Rac, CDC42) are likely conserved, as these features appear to predate metazoans (Boureux et al. 2007).

2. *Cnidarians*

The origin of Cnidarians was a major milestone in animal evolution because they are the first grade of animals that have true epithelial cells (ectoderm, gastrodermis) and an ECM. The evolution of an interface between epithelia and ECM was important because it facilitated the detachment and migration into the ECM of individual cells and even whole groups of cells. Overall, cnidarian development is similar to that of bilaterians and primarily involves epithelial morphogenesis (Magie, Daly, and Martindale 2007, Magie and Martindale 2008). However, changes in cellular morphology reminiscent of EMT are observed during gastrulation, where invaginating cells of the gastrodermis express *Forkhead* and *Snail* (Fritzenwanker, Saina, and Technau 2004a). Presumably, these transcription factors function to apically constrict invaginating cells, but there is also evidence that individual cells can ingress and migrate shortly after invagination (Magie and Martindale 2008, Kraus and Technau 2006, Byrum 2001). Cnidarians therefore provide the first direct evidence of a simple EMT GRN involving Snail and Forkhead (Fox) transcription factors to control morphogenesis and cell ingression among basal metazoans.

B. Bilaterians

Bilaterians—which are divided into the ecdysozoans, lophotrochozoans, and deuterostomes—include all other metazoans above sponge and cnidarian-grade organisms (Aguinaldo et al. 1997, Halanych et al. 1995). With the advent of bilaterians, we see a dramatic increase in body plan complexity. This is commensurate with an increase in developmental complexity that was driven in large part by the evolution of novel cellular interactions. These interactions were in turn likely

facilitated by diverse cell types that could take advantage of an EMT program promoting cell migration.

1. *Ecdysozoans*

a. ***Insects***

In the fruit fly *Drosophila*, cadherin switching from *D*E-Cadherin to *D*N-Cadherin is observed during gastrulation, as the mesoderm invaginates and is eventually internalized (Oda, Tsukita, and Takeichi 1998, Hemavathy, Meng, and Ip 1997). This process involves deployment of both Twist and Snail protein activity to directly repress transcription of epithelial genes, thereby facilitating apical constriction and invagination. The internalized mesodermal cells eventually migrate throughout the embryo to generate body wall musculature (Wheelock et al. 2008). Migration of mesoderm requires Rho GTPase activity, as loss of the Rho guanine nucleotide exchange factor, *pebbles*, results in maintenance of epithelial traits and failure of mesodermal cells to create protrusions (Smallhorn, Murray, and Saint 2004). The exact *cis*-regulatory relationships remain elusive, but it seems that both Twist and Snail intersect with the gene regulatory program that activates Rho expression in delaminating mesoderm (Leptin 1999), providing a striking example of how EMTs in invertebrates deploy almost identical basic gene regulatory programs to initiate EMT and cell migration.

b. ***Nematodes***

In the nematode, *C. elegans*, many of the cellular adhesion proteins that mediate neural crest EMT and migration in vertebrates, such as cadherins (Hill et al. 2001), similarly regulate tissue

morphogenesis (Costa et al. 1998) and cell migration (Montell 1999). There is also evidence that cells of endodermal fate ingress individually (Leptin 2005). Yet, it is unknown if EMT-mediated cell migration during ingression involves the same regulatory control as that which occurs in other ecdysozoans. For example, although nematodes contain a Snail homologue (CES-1), this protein functions primarily to repress pro-apoptotic genes during cell fate determination (Metzstein and Horvitz 1999, Reece-Hoyes et al. 2009, Thellmann, Hatzold, and Conradt 2003). Snail family members across bilaterians are known to inhibit apoptosis during cell migration in numerous contexts (Barrallo-Gimeno and Nieto 2005, Vega et al. 2004), but it is not clear if maintenance of cell survival is the sole function of CES-1, or if it is also critical for the initiation of EMT and migration in ingressing endoderm. Zag-1, a zinc finger homeodomain transcription factor, is homologous to vertebrate Zeb1 and is expressed in and required for proper neural development in *C. elegans* (Clark and Chiu 2003). Loss of Zag-1 results in failure of neural progenitors to migrate and leads to loss of neural differentiation (Clark and Chiu 2003), but similar to CES-1, it is not clear if the lack of neural migration results from loss of regulatory mechanisms similar to those in the vertebrate neural crest.

2. Lophotrochozoans

a. ***Annelids and Molluscs***

Relatively little is known about the operation of EMT processes in lophotrochozoans apart from gene expression analysis. In polychaete annelids, one of two *Snail* paralogues is expressed in neuroectodermal derivatives, including migrating neuroblasts and maturing neurons in the central nervous system (Dill, Thamm, and Seaver 2007). In contrast to *Drosophila*, *Capitella sp*.

Twist is not expressed in migrating cells during gastrulation, but this may be due to functional compensation by *Snail* (Dill, Thamm, and Seaver 2007). Contrary to most other invertebrates, Snail genes in the gastropod mollusk, *Patella vulgata*, are never expressed in involuting mesoderm, and instead are expressed mostly in sensory neurons and in several parts of the early larva in which ectodermal clefts or folds are forming (Lespinet et al. 2002). This raises the possibility that Snail may drive apical constriction and EMT-like movements similar to that of ventral furrow formation in *Drosophila*.

b. ***Acoels***

The acoelomates (also known as the acoels or acoelomorpha), are worm-like creatures that have become increasingly important for understanding the origin of bilaterian traits (Philippe et al. 2011). Planarians in particular are an excellent model for studying cell migration and EMT as they are capable of regenerating many of their organs, which inevitably involves production of mesenchyme. Nevertheless, we currently lack insight into the molecular-genetic control of how these processes operate. Descriptions of embryogenesis in one planarian, *S. polychroa*, suggest that early developmental events involving EMT (e.g., gastrulation), coincide with expression of key EMT regulatory factors such as *Twist* and *Snail* (Martín-Durán, Amaya, and Romero 2010). More extensive gene expression and functional analyses are required to form a comparative framework for studying the evolution of EMT mechanisms in these organisms.

3. Deuterostomes

Although less diverse than their lophotrochozoan and ecdysozoan relatives, the deuterostomes—the third main division of bilaterians—include the vertebrates and their closest extant relatives: the echinoderms, hemichordates, and invertebrate chordates. It is within the deuterostomes that we see the appearance of cells within the central nervous system that begin to take on cellular and molecular features of EMT that are strikingly similar to that of vertebrate neural crest.

a. ***Echinoderms***

During gastrulation in sea urchins, a group of cells known as the primary mesenchyme undergoes EMT and detaches from epithelial cells of the vegetal plate (Shook and Keller 2003, Saunders and McClay 2014). As individual cells of the primary mesenchyme begin to ingress, they endocytose a pro-epithelial cadherin (Cad-1) (Miller and McClay 1997, Wu, Ferkowicz, and McClay 2007). This is mediated by Twist and Snail activity, as functional perturbation of Snail and Twist results in failure of primary mesenchyme ingression, and loss of mesenchyme-derived skeleton (Wu and McClay 2007, Wu, Yang, and McClay 2008).

b. ***Hemichordates***

Hemichordates have become increasingly important models for studying the evolutionary-developmental biology of deuterostome features such as the organization of the central nervous system, pharynx, and mesoderm (Green et al. 2013a, Gillis, Fritzenwanker, and Lowe 2012, Lowe et al. 2003). However, there is a paucity of detailed studies on hemichordate EMT or cell migration. Gene expression analyses show that key EMT regulators such as Snail and Forkhead

are expressed in early mesoderm (Green et al. 2013b), but the functions, if any, of these genes within the context of EMT/cell migration are unknown. Similarly, although the hemichordate *P. flava* has a type I (*Pf*Cad1) and type II (*Pf*Cad2) classical cadherin (Oda and Takeichi 2011), it is unknown how they influence EMT and cell migration.

c. ***Invertebrate Chordates—Amphioxus and Tunicates***

The embryonic development of the invertebrate chordates—amphioxus and tunicates—has occupied a central place in the study of vertebrate origins for nearly 150 years (Dohrn 1875, Gee 1996, Wada 2001, Lacalli 2010, Laubichler and Maienschein 2007). These animals are the closest extant relatives of vertebrates and share many embryological and genomic features with vertebrates (Putnam et al. 2008). Despite these similarities, however, it had been assumed throughout much of the history of neural crest research that invertebrate chordates do not have migratory neural crest cells, or even likely homologues of the vertebrate neural crest (Gans and Northcutt 1983). However, over the past two decades, analysis of gene expression and function, as well as cell lineage tracing has questioned this thinking, and in doing so provided new and exciting insights into the evolutionary origin of the migratory properties of the neural crest.

The cephalochordates, represented by amphioxus, are the basal-most invertebrate chordates and have retained many of the ancestral morphological and genomic traits of the last common chordate ancestor (Holland, Laudet, and Schubert 2004, Putnam et al. 2008). Although amphioxus development relies primarily upon morphogenetic, sheet-like cellular movements rather than individual or collective cell migration, there are a few known cases of migratory cells that appear to undergo EMT, such as sensory neurons that derive from the ventral epidermal

ectoderm and migrate a short distance before re-inserting into the epidermis (Kaltenbach, Yu Jr, and Holland 2009). Similarly, gene expression analysis revealed that *Distal-less* transcripts (homologous with vertebrate *Dlx*) localize in dorsal epidermal cells that move as a sheet toward the dorsal midline during neurulation (Holland et al. 1996). These cells have lamellipodial extensions and appear to detach from the underlying neuroepithelilum, leading the authors to speculate that these dorsal epidermal cells may be homologous with vertebrate neural crest. Yet, unlike the neural crest, these cells never completely delaminate, nor do they migrate away from their site of origin, and they never form any cell type other than epidermis. Thus, although gene expression patterns have been informative for outlining the ancestral framework for the neural crest GRN, there have been no key cell types identified in cephalochordates having the cellular and molecular properties that offer a compelling link to migratory neural crest.

It has been suggested that the lack of migratory neural crest in amphioxus is a consequence of their lacking much of the neural crest specifier and EMT program in the dorsal neural tube, lending support to the notion that these parts of the neural crest GRN were co-opted from other cell types (e.g., mesoderm, endoderm) to the neural tube early in the origin of the vertebrates (Hall 2008, Meulemans and Bronner-Fraser 2005). Although the notion of gene co-option has figured prominently in studies of neural crest evolution, there is one interesting example that runs counter to this trend. The transcription factor Snail—a key regulator of neural crest EMT and migration in gnathostome vertebrates—is expressed in the amphioxus neural tube (Langeland et al. 1998). In gnathostomes, forced expression of *Snail1 or Snail2* results in ectopic neural crest EMT and migration, suggesting that these factors are sufficient to induce cells to emigrate from the neural tube (Hemavathy, Ashraf, and Ip 2000) (del Barrio and Nieto 2002,

Guaita et al. 2002). Yet, amphioxus lacks migratory neural crest, raising questions as to why *Snail*-positive cells in the neural tube are unable to migrate. Although much of the neural crest regulatory apparatus controlling induction, neural border specification and cell differentiation are conserved among invertebrate chordates, amphioxus lacks expression of many of the downstream effectors that are largely responsible for downregulating epithelial state and promoting mesenchymal state in the neural tube (Yu et al. 2008). For example, only a single RhoA/B/C gene is present in the amphioxus genome in contrast to individual RhoA, RhoB, and RhoC genes in vertebrate genomes (Boureux et al. 2007), which implies that gene duplication may have been an important step in promoting the migration of "proto-neural crest cells" from the neural tube. In addition, the downstream effectors of migration, such as the classical cadherins, may be highly derived and not function in the same context as vertebrate classical cadherins that promote neural crest migration. For example, the classical cadherins in *B. belcheri* (Bb1C and Bb2C) are structurally similar to vertebrate E- and N-cadherins, respectively, but they have swapped functions because Bb1C is expressed in the neural tube and somites, whereas Bb2C is expressed in the epidermis—expression patterns exactly opposite to that of *E-Cadherin* and *N-Cadherin* in vertebrates (Oda et al. 2002, Oda, Akiyama-Oda, and Zhang 2004). Amphioxus cadherins also lack extracellular domains, yet are capable of holding cells together, suggesting that the fundamental mechanisms of cadherin-mediated adhesion in the amphioxus neural tube are substantively different from that in any other chordate (Oda, Akiyama-Oda, and Zhang 2004).

Molecular phylogenetic analysis places the tunicates as the sister group to vertebrates, and therefore makes them the best model system for studying the evolution of migratory neural

crest that appeared after the divergence of vertebrates from invertebrate chordates (Delsuc et al. 2006). As described above, it had often been assumed that migratory neural crest cells first appeared in early vertebrates, with little or no vestiges of a rudimentary neural crest among invertebrate chordates (Gans and Northcutt 1983). However, vital dye (DiI) labeling experiments in the mangrove tunicate (*Ecteinascidia turbinata*) were the first to reveal that cells originating near the dorsal neural tube of the tadpole larva were not only capable of migrating, but could also differentiate into pigment cells, a cellular derivative of vertebrate neural crest (Jeffery, Strickler, and Yamamoto 2004). Moreover, these cells expressed HNK-1 protein and *ZicA* mRNA, similar to that of migratory neural crest in vertebrates (Jeffery, Strickler, and Yamamoto 2004). These results were later confirmed in other ascidian species, suggesting that tunicates possessed so-called migratory "neural crest-like cells" (NCLCs) (Jeffery, Strickler, and Yamamoto 2004) (Jeffery 2006). Subsequent studies revealed that NCLCs originate near the neural plate border and express several markers of neural crest cells, including homologues of *Twist*, *Myc*, *FoxD*, type II *Cadherin* and *Rho A/B/C* GTPases(Jeffery et al. 2008). Although these cells are similar to bona fide neural crest, there are some important differences. For example, NCLCs do not exit immediately from the neural folds, but rather remain stationary for a prolonged period to proliferate prior to delamination. This is unlike the case in many vertebrates in which neural crest cells either migrate before or immediately after neural tube closure (Jeffery et al. 2008, Jeffery 2006). Also, the NCLCs of ascidians originate from the *a*7.6 trunk lateral cell lineage, which is not within the neural plate border and is also distinct from other types of NCLCs reported in different tunicate species (Jeffery et al. 2008) (described below).

There have been recent reports of other types of NCLCs in tunicates that are completely different from those described in the *a*7.6 cell lineage. In *Ciona intestinalis*, pigment cell precursors from the *a*9.49 lineage in the head contribute to cranial sensory structures such as the otolith and ocellus and are regulated by a conserved *Wnt7-FoxD* axis that operates in neural crest-derived melanocytes in vertebrates (Abitua et al. 2012). Although they normally delaminate and migrate only a short distance within the neural tube, cells from the *a*9.49 lineage can be induced to migrate out of the neural tube as mesenchymal cells upon forced expression of *Twist* (Abitua et al. 2012). This suggests that NCLCs with minimal migratory potential could have been directed to undergo a full EMT and migrate extensively throughout a chordate embryo simply by co-option of a single pro-migration transcriptional regulator such as Twist.

Yet another distinct NCLC has been recently identified in *Ciona*, a cell lineage that contributes to the bipolar tail neuron (BTN) (Stolfi et al. 2015). BTN precursors form within a *Snail-Pax3/7-Msx*-positive neural plate border region, delaminate and migrate (Stolfi et al. 2015). Eventually, these cells differentiate into neurons that express *Neurogenin*, which the authors argue is similar to development of neural crest-derived dorsal root ganglia in vertebrates. Interestingly, these cells also appear to downregulate Cadherin.b, a classical cadherin homologue found in epithelial cells of the neural tube, and forced expression of a proepithelial protocadherin in the neural tube prevented BTN precursor migration. Taken together, these results provide the first evidence that migratory NCLCs in invertebrate chordates control differential expression of intercellular adhesion molecules to facilitate migration, as occurs during EMT in gnathostome vertebrate neural crest cells (Stolfi et al. 2015).

IV. Evolutionary Emergence of Neural Crest EMT and Migration

Taking into account the use of a wide array of transcription factors and signaling molecules in both vertebrate neural crest cells and similar migratory cell types among invertebrates, it becomes clear that there is more than one way to execute cellular EMT during embryonic development. From our comparative analysis across diverse metazoans, we identify a highly conserved core gene regulatory network that is expressed in and is responsible for governing EMT and migration in several different developmental contexts and cell types (Figure 5 summarizes the most conserved features of cellular EMT across Metazoan clades). These can range from ingression of neuroblasts and primary mesenchyme in insects and echinoderms, to invagination and delamination of sensory cells and mesenchyme in cnidarians, nematodes and molluscs. We propose that this conserved network sets into motion a molecular chain of events that regulates diverse types of cellular EMT and has underlain the repeated evolution of EMTs in different metazoan cell types.

An important theme in evolutionary developmental biology is deep homology—the concept that similar developmental processes and embryonic structures may arise independently in different lineages by using common genes or even entire regulatory networks (Shubin, Tabin, and Carroll 2009). Classic examples of deep homology include the shared molecular circuitry controlling development of non-homologous limbs and eyes across bilaterians (Shubin, Tabin, and Carroll 2009). Thus, although these structures, or the cell types that comprise them, are not homologous *sensu stricto*, the regulatory interactions of the genes that control their development are, and have been deployed over and over again to accomplish the same developmental goal. Likewise, we propose that there is a deeply homologous metazoan EMT regulatory network for

cell delamination and migration (Figure 5). Although there are almost certainly clade-specific features that have been lost from or superimposed onto this core network, we suggest that this highly conserved EMT program is a recurring motif in EMTs that have evolved throughout metazoan evolution and formed the basis for the evolution of a neural crest EMT module in NCLCs in invertebrate chordates and in bona fide neural crest in vertebrates.

Some of the most ancient components of this EMT network include intercellular signaling pathways, such as those from TGFβ (e.g., BMPs) and canonical Wingless (WNT) signaling pathways (Figure 5). These signaling systems show little variation across metazoans and rely on conserved intracellular effectors to control expression of target genes (Davidson and Erwin 2006). During neural crest EMT, these signaling pathways converge on the activation of one or more transcription factors, including members of the Snail, Twist and Forkhead families, which are also activated for EMTs in diverse metazoan groups. Once expressed, these transcriptional regulators directly bind and repress gene batteries responsible for maintaining epithelial fate, such as certain cadherin intercellular adhesion proteins (Figure 5). These same factors then either directly or indirectly turn on cytoskeletal regulators that reorganize the cytoskeleton and prepare cells to detach and migrate.

Observations that migratory cells of various developmental and phylogenetic origins all activate this core EMT-migration program provide compelling evidence that it has been inserted *in toto* into diverse gene regulatory programs that define many cell types across metazoans, including NCLCs and vertebrate neural crest. During early chordate evolution, our comparisons suggest that there were perhaps a few cell types capable of undergoing EMT and migrating. This is approximated by the condition in amphioxus, in which some sensory neurons and a few other

cell types move a short distance away from their site of origin and then become epithelial (Lu, Luo, and Yu 2012). Notably, however, these cells never originate from the neural plate border, suggesting no affinity to NCLCs or neural crest.

With the appearance of Olfactoreans (Tunicates+Vertebrates) (Delsuc et al. 2006), we see the appearance of NCLCs in the head and trunk of chordate embryos (Abitua et al. 2012, Jeffery et al. 2008, Stolfi et al. 2015). Some of these cells appear within or nearby the neural plate border, undergo EMT, delaminate, and then migrate to form derivatives such as pigment and sensory neurons. Thus, NCLCs are strikingly similar to vertebrate neural crest and likely used the same or similar molecular mechanisms to accomplish EMT (Abitua et al. 2012, Stolfi et al. 2015). For example, it is likely that NCLCs in ascidians undergo EMT and migrate by modulating intercellular adhesion proteins such as cadherins (Stolfi et al. 2015), and these cells originate nearby or within the neural border and express transcription factors that promote EMT, including *Snail* and *Pax3/7* (Stolfi et al. 2015).

Although it remains unclear whether NCLCs are homologous to bona fide neural crest, these cells do not form major structures or organ systems as occurs in vertebrates, but rather form isolated cells or small cell populations such as sensory neurons or other sensory cell types (Stolfi et al. 2015, Abitua et al. 2012). It seems likely then that the EMT module in NCLCs was either co-opted multiple times by individual cells in the neural plate border, or more likely, by one or a few progenitor cell types that could divide and spread throughout the head and trunk, generating large groups of migratory cells reminiscent of migratory neural crest streams in vertebrates. Another important difference between NCLCs and vertebrate neural crest is that NCLCs are not multipotent, forming only single cell types (Stolfi et al. 2015). Thus, although

invertebrate chordate NCLCs would have established much of the core neural crest-GRN that operates in vertebrate neural crest, including the EMT/migration module, this program was not linked to multipotency. The acquisition of a multipotency program would have likely endowed migratory NCLCs with the ability to generate the diverse set of cellular derivatives that distinguish bona fide vertebrate neural crest (York et al. 2017).

During the origin of early vertebrates, the EMT module likely consisted of a very simple network, similar to that operating in invertebrate NCLCs and nonchordate deuterostomes. This is bolstered by analysis of the transcription factor repertoire of neural crest EMT/migration in agnathans, which suggests that many "key" signal transduction pathways and transcriptional regulators of neural crest EMT (e.g., Twist, Ets, BMPs), as well as cellular mechanisms (e.g., cadherin switching) are dispensable for neural crest EMT in basal vertebrates (York et al. 2017, Sauka-Spengler et al. 2007). Thus, early vertebrates—represented by extant agnathans—may offer key insights into the stepwise assembly of the EMT module of the neural crest GRN operating in higher (gnathostome) vertebrates.

Based on the apparent differences in their EMT modules, there was likely a large scale "re-wiring" of the EMT module after the divergence of agnathans and gnathostomes. Presumably, this would have occurred by *cis*-regulatory evolution that brought novel transcriptional regulators and intercellular signaling pathways such as Twist, Ets, BMP2/4, and others into the dorsal neural tube, which would have been facilitated by and integrated with additional lineage-specific gene duplications (Ohno 1970, Wada and Makabe 2006, Donoghue and Purnell 2005). Although the significance of such changes to the EMT module is not clear, one possibility is that these additional genes would have endowed migratory neural crest cells

with functional redundancy to ensure a properly timed and coordinated EMT. This can be seen in extant gnathostomes in which one of the seminal events of neural crest EMT—transcriptional repression of epithelial gene batteries—often involves coordinate repression by numerous proteins, including Snail, Twist, SoxE, Sip1, Zeb1, and many others (Thiery and Sleeman 2006).

V. Conclusions

Of the many molecular and cellular features that define vertebrate neural crest cells, an ability to undergo EMT, delaminate and migrate from the embryonic neural tube is one of their hallmark traits, yet the evolutionary origin of the neural crest EMT program has remained obscure. By comparing the molecular, genetic and cellular features of EMT in vertebrate neural crest cells with similar mechanisms in diverse invertebrate cell types, we identify a core conserved set of genes and cellular mechanisms that may constitute an ancient regulatory program that served as the basis for the independent origin of cellular EMTs during animal evolution. This network was likely deployed during the evolution of neural crest cells in early vertebrates, and has been elaborated upon significantly with the divergence of agnathans and gnathostomes.

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FIGURE LEGENDS

Figure 1. Neural crest migration in a generalized vertebrate embryo. A lateral view shows migratory neural crest streams that originate in the midbrain and hindbrain of the central nervous system (purple) descending ventrally into the head anteriorly and pharyngeal arches more posteriorly (arrows). Large and small brown ovals indicate eye and otic vesicle, respectively. Somites are indicated by pink rectangles. Anterior is left, dorsal is up.

Figure 2. EMT, delamination and early migration of neural crest from the dorsal neural tube in a generalized vertebrate embryo. Premigratory neural crest cells (blue shading) are specified in the dorsal-most aspect of the neural tube. Soon after specification, these cells undergo EMT, delaminate from the underlying neural epithelium and then begin to migrate laterally and ventrally, and in doing so invade surrounding tissues.

Figure 3. Model for canonical cellular EMT and delamination program to initiate neural crest migration. (a) Shortly after being specified in the dorsal neural tube, premigratory neural crest cells (red) activate a genetic program that directs changes at the cell surface of intercellular adhesion and junction proteins which allows neural crest cells to break free from neighboring neuroepithelial cells (purple cells) and the underlying basement membrane (b). Concomitant with changes in cell surface proteins is breakdown and reorganization of the actin cytoskeleton to establish a leading edge (c) and begin directed migration (d).

Figure 4. Examples of cellular EMTs in diverse metazoan embryos including: cranial neural crest in amphibians (a, *Xenopus*) and agnathans (b, lamprey); neural crest-like cells in tunicates (c, cranial melanocytes left, bipolar tail neurons, right); epidermal sensory neurons in amphioxus (d); primary mesenchyme in sea urchins (e); visceral mesoderm in *Drosophila* (f); neuroblasts in trochophore larvae of annelids (g); interstitial mesenchyme in planula larvae of Cnidarians (h); and migratory mesenchyme in sponges (i). Solid red shading indicates cells undergoing EMT in each embryo. Panels a-d, g show whole mount cartoons of the embryo, whereas e, f, h, i represent cross-sections showing the interior of the embryo.

Figure 5. Hypothetical gene regulatory network (GRN) showing conserved elements governing EMT during embryonic development across metazoans.

Figure 1

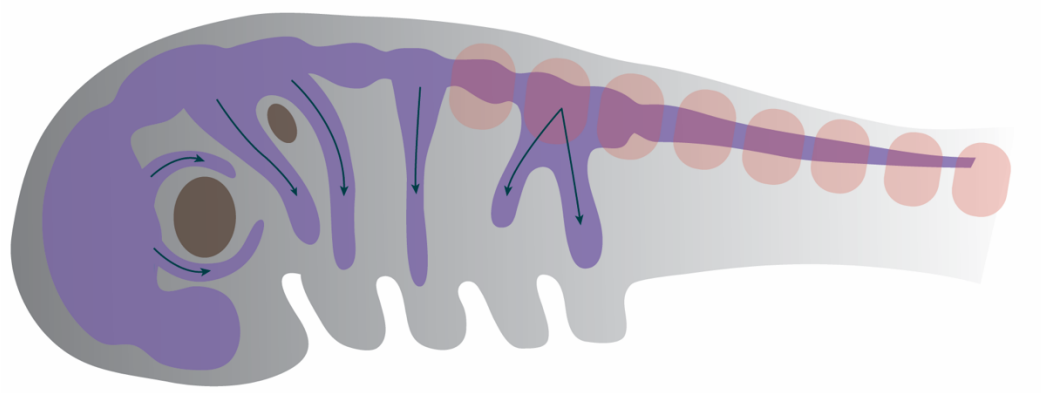


Figure 2

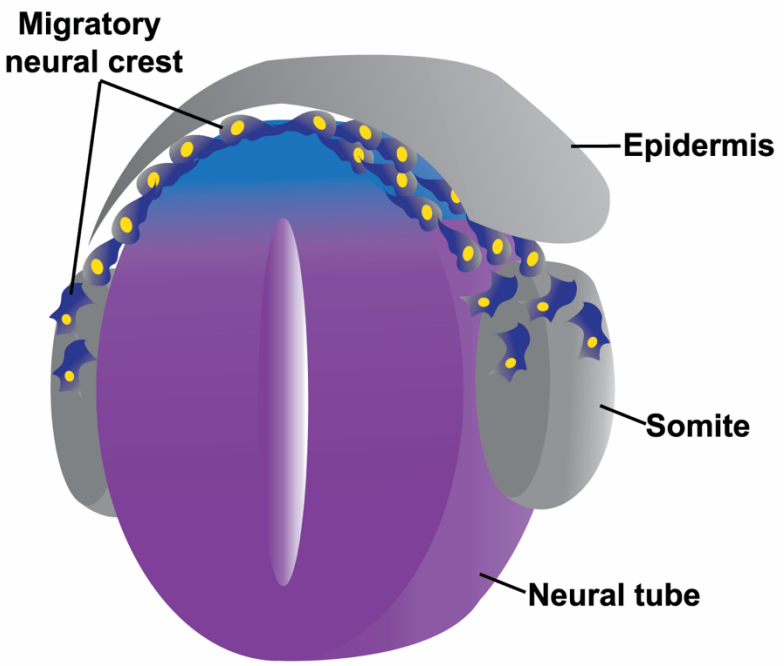


Figure 3

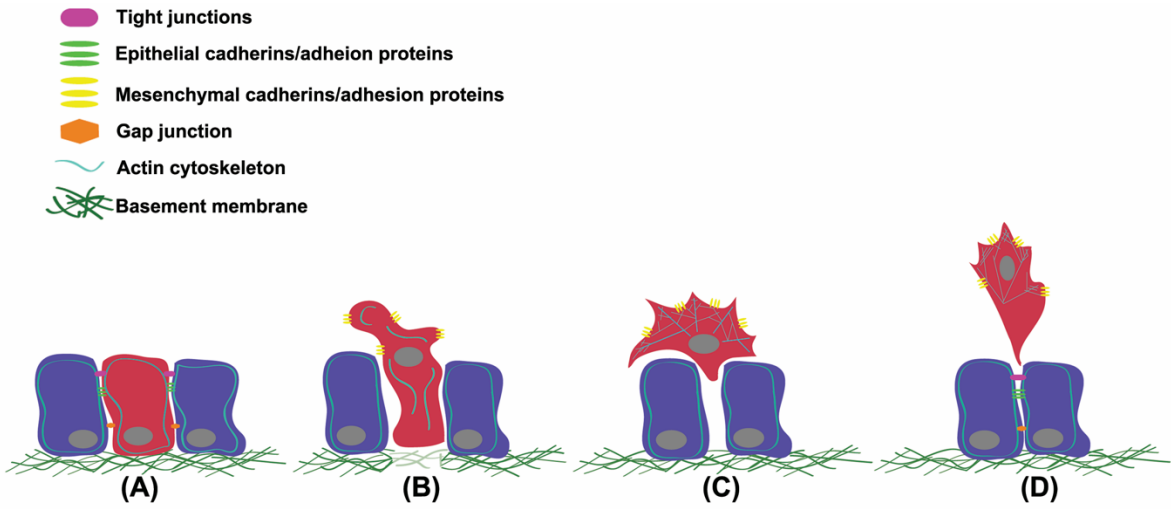

Tight junctions
Epithelial cadherins/adheion proteins
Mesenchymal cadherins/adhesion proteins
Gap junction
Actin cytoskeleton
Basement membrane
(A)
(B)
(C)
(D)

Figure 4

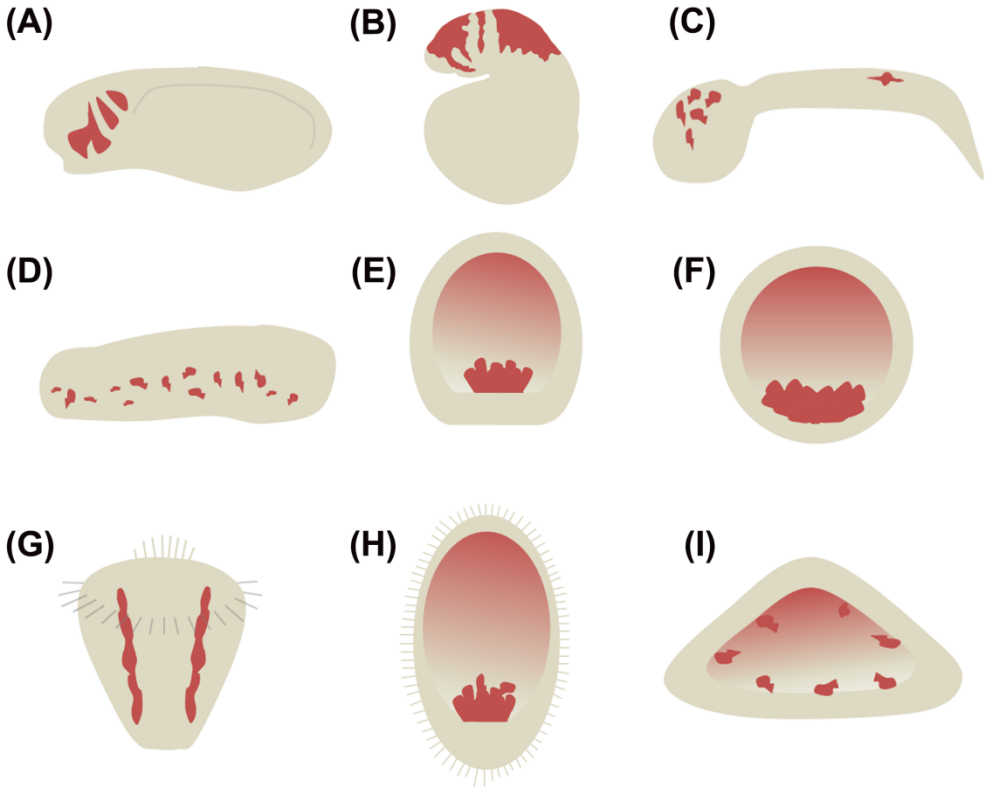
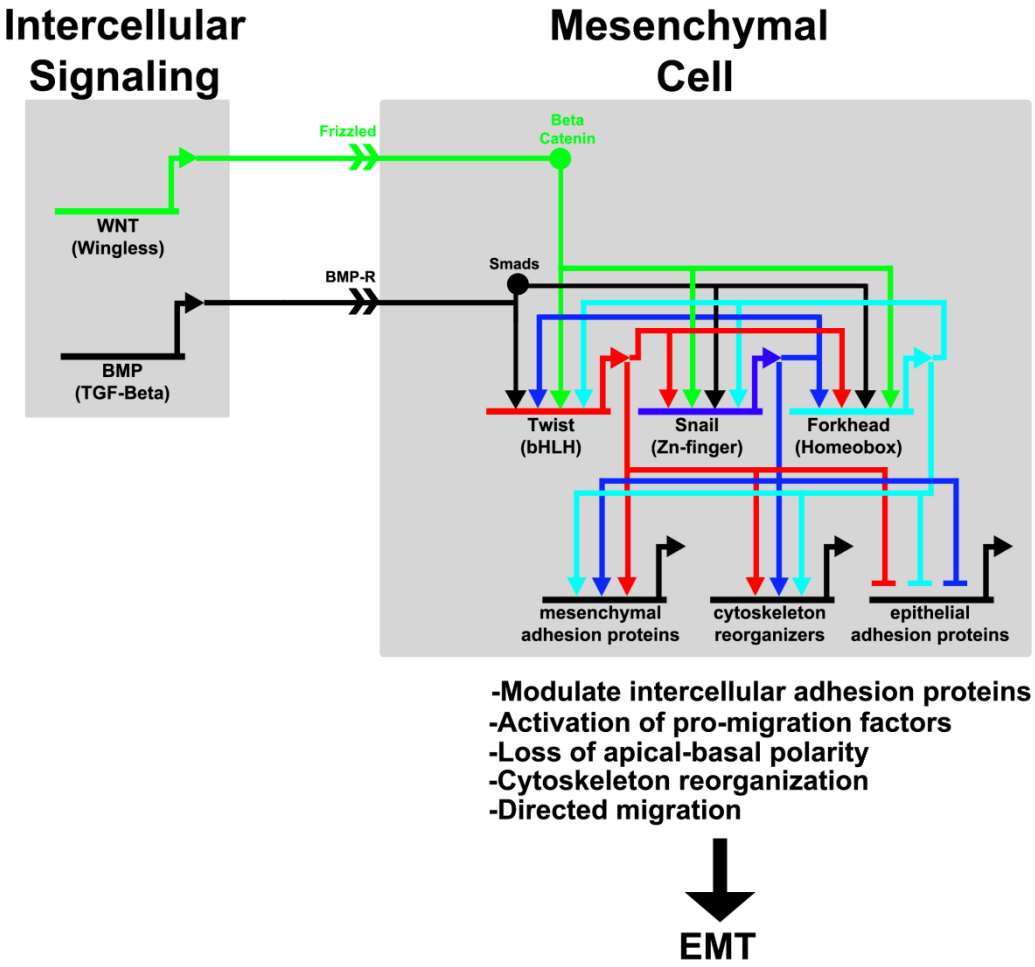


Figure 5


Intercellular Signaling
Mesenchymal Cell
Frizzled
Beta Catenin
WNT (Wingless)
BMP-R
Smads
BMP (TGF-Beta)
Twist (bHLH)
Snail (Zn-finger)
Forkhead (Homeobox)
mesenchymal adhesion proteins
cytoskeleton reorganizers
epithelial adhesion proteins
-Modulate intercellular adhesion proteins
-Activation of pro-migration factors
-Loss of apical-basal polarity
-Cytoskeleton reorganization
-Directed migration
EMT

CHAPTER 3: LAMPREY NEURAL CREST MIGRATION IS SNAIL-DEPENDENT AND OCCURS WITHOUT A DIFFERENTIAL SHIFT IN CADHERIN EXPRESSION

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ABSTRACT

The acquisition of neural crest cells was a key step in the origin of the vertebrate body plan. An outstanding question is how neural crest cells acquired their ability to undergo an epithelial-mesenchymal transition (EMT) and migrate extensively throughout the vertebrate embryo. We tested if differential regulation of classical cadherins—a highly conserved feature of neural crest EMT and migration in jawed vertebrates—mediates these cellular behaviors in lamprey, a basal jawless vertebrate. Lamprey has single copies of the type I and type II classical cadherins (*CadIA* and *CadIIA*). *CadIIA* is expressed in premigratory neural crest, and requires the transcription factor Snail for proper expression, yet *CadIA* is never expressed in the neural tube during neural crest development, suggesting that differential regulation of classical cadherin expression is not required to initiate neural crest migration in basal vertebrates. We hypothesize that neural crest cells evolved by retention of regulatory programs linking distinct mesenchymal and multipotency properties, and emigrated from the neural tube without differentially regulating type I/type II cadherins. Our results point to the coupling of mesenchymal state and multipotency as a key event facilitating the origin of migratory neural crest cells.

INTRODUCTION

The evolutionary origin of the vertebrates is linked to their acquisition of the neural crest, a multipotent, migratory embryonic cell population that contributes to the development of many vertebrate traits, including the peripheral nervous system, pigment cells, and components of the endocrine system (Donoghue et al., 2008; Green et al., 2015; Square et al., 2016; Trainor, 2013). The neural crest is also responsible for generating the core of the vertebrate "new head"—the cartilage, bone and muscle that forms the pronounced cranium and jaws, features that house the primary sense organs and are hypothesized to have facilitated the invasion of new ecological niches, and distinguish vertebrates morphologically and behaviorally from their closest relatives, the invertebrate chordates (Cattell et al., 2011; Gans and Northcutt, 1983; McCauley and Bronner-Fraser, 2006). The neural crest is therefore exemplary of a developmental and evolutionary innovation that correlates with the adaptive radiation of a major animal clade.

The neural crest forms in vertebrate embryos in a highly stereotyped manner. They become established in the neural plate border between the medial neural plate and lateral epidermal ectoderm (Figure 1A). A highly conserved gene regulatory network (GRN) of transcription factors and signaling molecules orchestrates the progressive specification of these cells to become bona fide neural crest (Betancur et al., 2010; Meulemans and Bronner-Fraser, 2004; Sauka-Spengler and Bronner-Fraser, 2008a). These specification factors include members of the SoxE family, Tfap2α, Id, Snail/Slug, Myc, Twist, Ets, Myb and several others that are directly responsible for establishing the hallmarks of neural crest cells (Sauka-Spengler and Bronner-Fraser, 2006; Simoes-Costa and Bronner, 2015; Simões-Costa and Bronner, 2013). One key feature of neural crest development is a dramatic change in cell shape and molecular

architecture that results in an epithelial-to-mesenchymal transition (EMT), a feature that enables these cells to migrate to specific locations throughout the vertebrate embryo (Bronner, 2012; Duband et al., 1995; Kerosuo and Bronner-Fraser, 2012) (Figure 1B, C). Although EMT is not specific to vertebrates (Kee et al., 2007) or neural crest *per se* (Nakaya and Sheng, 2013; Savagner, 2010), the extent to which neural crest cells migrate as a multipotent and proliferative cell population has no parallel in any other animal embryo. Neural crest EMT has been described at length in embryos of numerous jawed (gnathostome) vertebrates (Ahlstrom and Erickson, 2009; Barriga et al., 2013; Duband et al., 1995; Strobl-Mazzulla and Bronner, 2012), and is therefore thought to be an evolutionarily conserved process. The initiation of neural crest EMT requires fine-tuned control of the spatial and temporal expression of numerous genes (Savagner, 2001; Thiery and Sleeman, 2006), many of which also play important roles earlier in neural crest development (e.g., *Twist, Snail*). The proteins encoded by these genes mediate detachment of the neural crest from the underlying neural epithelium and initiate migration, primarily by promoting reorganization of the cytoskeleton (Clay and Halloran, 2011; Clay and Halloran, 2010). After migration, neural crest cells lose their mesenchymal morphology, and undergo terminal differentiation (Betancur et al., 2010; Hall, 2008) into many of the cell types that define vertebrates (Figure 1D).

Key to the process of neural crest EMT in jawed vertebrates is the concerted activity of a suite of signaling molecules and transcription factors to repress activity of genes that promote an epithelial phenotype and activate genes that promote migration (Savagner, 2001, 2010). At the molecular level, the onset of EMT is characterized by modulation at the cell surface of cadherin intercellular adhesion proteins that may correlate with epithelial versus mesenchymal states

(Dady et al., 2012; Taneyhill, 2008) (Figure 1A–C). The differential expression of type I and type II cadherins in the neural crest domain is thought to be one of the principal mechanisms that controls the onset of neural crest migration in gnathostomes. This can occur by direct transcriptional repression of certain cadherins in the dorsal neural tube, whereas the remaining non-migratory neuroepithelium retains uniform levels of type I cadherin (usually N-Cadherin) expression (Gheldof and Berx, 2013; Rogers et al., 2013; Scarpa et al., 2015; Taneyhill and Schiffmacher, 2013; Wheelock et al., 2008) (Figure 1A–C). At the transcriptional level, key factors that mediate these changes in cadherin expression include members of the Twist, Sip, Zeb, and Snail families, all of which play evolutionarily conserved roles in EMT among metazoans (Betancur et al., 2009; Fairchild et al., 2014; Lander et al., 2011; Linker et al., 2000; Theveneau et al., 2007). Classical models of neural crest EMT have often described switches from type I "epithelial" cadherins (*E-Cadherin*, *N-Cadherin*) to type II "mesenchymal" cadherins (*Cadherin-6*, *Cadherin-7*, and *Cadherin-11*) (Clay and Halloran, 2014; Coles et al., 2007; Nakagawa and Takeichi, 1998b; Vallin et al., 1998). However, there is now evidence that expression of both type I and type II cadherins occurs in both premigratory and migratory neural crest, and that certain cadherins may not always strictly correlate with epithelial or mesenchymal fates (Abbruzzese et al., 2016; Campbell and Casanova, 2015). For example, type I cadherins, such as E-Cadherin (Huang et al., 2016b), persist in and may even be required for early migration of cranial neural crest in *Xenopus*, whereas expression of a type II cadherin, Cadherin-6b, is repressed to facilitate neural crest emigration in the chick midbrain (Coles et al., 2007). By contrast, Cadherin-6 (Clay and Halloran, 2014) and Cadherin-6b (Park and Gumbiner, 2010b) are expressed in premigratory and early migratory neural crest in the fish hindbrain and chick

trunk, respectively. Taken together, these findings indicate that modulation of cadherin expression relative to the rest of the neural tube, rather than a singular cadherin "switch" *per se*, may be an important step in driving neural crest EMT and migration. Thus, regardless of clade-specific variation in exact mechanisms and expression patterns, the regulation of cadherin expression in the dorsal neural tube allows neural crest cells to detach from the neighboring neuroepithelium, mobilize their cytoskeleton, and begin collective migration (Kuriyama and Mayor, 2008; Liu and Jessell, 1998a; Liu and Jessell, 1998b; Perez-Alcala et al., 2004).

The regulation of EMT by differential cadherin expression is a highly conserved feature of neural crest development. However, it is not clear when this key regulatory step evolved. Evolutionary-developmental studies in a basal jawless (agnathan) vertebrate, the sea lamprey, *Petromyzon marinus*, suggest that some key steps in the control of neural crest development might not be conserved across vertebrates. In particular, there are marked differences in the neural crest GRN of lamprey and gnathostomes which suggest that the ancestral state of the EMT/migratory network module in the first vertebrates might have been very different from that described in gnathostomes. In lamprey, *Twist* and *Ets* are not expressed in premigratory or migratory neural crest (Sauka-Spengler et al., 2007), whereas they are critical for EMT and neural crest migration in gnathostomes (Hopwood et al., 1989; Linker et al., 2000; Theveneau et al., 2007). Similarly, an earlier expression analysis of lamprey Snail, a key transcriptional regulator of neural crest EMT in gnathostomes, suggested that this factor may not function in lamprey premigratory or migratory neural crest (Rahimi et al., 2009). These studies highlight important regulatory differences within the migration-specific module of the lamprey neural crest GRN that are distinct from those of gnathostome vertebrates and raise the possibility that

the molecular-genetic and cellular mechanisms of neural crest EMT and migration in gnathostomes may have diverged substantially compared to ancestral conditions operating in the first vertebrates.

In an attempt to better understand the evolutionary origin and diversification of the EMT and migration module of the neural crest GRN, we studied these processes during embryonic development of lampreys. Because lampreys occupy the most basal phylogenetic position among extant vertebrates, they have strong potential to offer crucial insights into the genetic and morphological innovations likely present in the vertebrate ancestor (Green and Bronner, 2014; McCauley et al., 2015). As such, lampreys have emerged as important evolutionary-developmental models for studying the origin of vertebrate traits, including neural crest cells and their migratory properties (McCauley and Bronner-Fraser, 2003; McCauley et al., 2015; Medeiros, 2013). Given the central role of differential cadherin expression in the EMT of gnathostome neural crest cells, we examined whether similar mechanisms are conserved to the base of vertebrates or constitute an evolutionary novelty of gnathostomes by examining expression of classical cadherin orthologues and transcriptional regulation of cadherin expression in lamprey embryos during key stages of neural crest migration. We confirm that lamprey has single orthologues of both type I (*CadIA*) and type II (*CadIIA*) cadherins that are basal to their gnathostome cognates (Sauka-Spengler et al., 2007). Whereas we find that *CadIIA* is expressed early in premigratory and migratory neural crest, we never observe expression of *CadIA* in the neural tube throughout neural crest development. This suggests that, unlike in gnathostomes, lamprey neural crest cells never undergo a substantial shift in type I-type II cadherin expression relative to the rest of the neural tube to initiate migration. Despite these early differences,

expression patterns of the two cadherins are largely conserved later in development (Hatta et al., 1987; Quinlan et al., 2004). Using CRISPR-Cas9-mediated deletions of lamprey Snail DNA sequences, we also show that functional perturbation of this key regulator of neural crest EMT and migration, results in downregulation of expression of key genes involved in neural crest migration and loss of multiple neural crest derivatives. Taken together, our results suggest that differential regulation of cadherin expression during neural crest development is an innovation of gnathostome vertebrates, and support the notion that neural crest cells evolved by a heterochronic shift of multipotent-mesenchymal gene regulatory programs acting in early stages of embryonic development (Buitrago-Delgado et al., 2015).

MATERIALS AND METHODS

Embryo collection and gene cloning

To collect embryos, gravid adult sea lampreys were obtained from the Hammond Bay Biological Station, Millersburg, MI, and shipped to the University of Oklahoma. Adults were housed at 14°C in a recirculating water system. Sperm and eggs were stripped manually from gravid males and females into *ca*. 200 ml of water (Nikitina et al., 2009) and eggs were observed for the presence of a fertilization membrane. Excess sperm was washed from fertilized zygotes which were held undisturbed through gastrulation (2 days post fertilization) in 0.05X Marc's Modified Ringers solution (MMR) chilled to 18 °C. Embryos were then transferred to modified Pyrex dishes (236 ml, or 472 ml) to be reared in turbulence under constant flow, which enabled high-density rearing of embryos in a confined volume (*ca.* ~4000 embryos/236 ml dish) with high survival to hatching (~90%). All procedures using adult lampreys were conducted with approval

from the University of Oklahoma Institutional Animal Care and Use Committee (IACUC, R15-027).

Previous analysis (Sauka-Spengler et al., 2007) revealed that lamprey has orthologues of a type I cadherin (*CadIA*) with sequence homology to gnathostome *N-cadherin*, *E-cadherin* and *R-cadherin*, and a type II cadherin (*CadIIA*) that is similar in sequence to *Cadherin6*, *Cadherin7*, and *Cadherin11*. *CadIA*, *CadIIA, and Neural Cell Adhesion Molecule* (*NCAM*) gene fragments were uncovered from the 2010 version of the *Petromyzon marinus* genome assembly (Smith et al., 2013), amplified from a sea lamprey cDNA library (kindly provided by Dr. James Langeland), ligated into pGEM-T easy vector, and sequenced.

Phylogenetic Analysis

Multiple sequence alignment of 34 chordate classical cadherin amino acid sequences was performed using ClustalW. A JTT+G model of protein evolution was used for neighbor joining analysis, with robustness estimated after 1000 bootstrap replicates. Maximum likelihood analysis did not change the relatedness of either CadIA or CadIIA to their respective clades. Sequences analyzed, and corresponding accession numbers (in parentheses) included: Bb, *Branchiostoma belcheri* (CadII: BAD12592.1, CadI: BAC06835); Ci, *Ciona intestinalis* (BAA12592); Cs, *Ciona savignyi* (CadII: BAB68345); Dr, *Danio rerio* (Cad10: ABC12758, Cad6: BAD66654, Cad7: NP001070916, Cad11: NP571289, ECad: AAK52054, NCad: AAI33732, RCad: AAY41878); Gg, *Gallus gallus* (Cad6: NP001001758, Cad10: NP99938, Cad7: NP989518, Cad11: AAC33675, ECad: NP001034347, NCad: NP001001615, RCad: NP001004391); Mm, *Mus musculus* (Cad6: EDL03251.1, Cad10: AAH62962.1, Cad7: AAD01278.1, EDL11194.1, ECad:

AAH98501.1, NCad: AAH22107.1, RCad: EDL07297.1); Pm, *Petromyzon marinus*; Xl, *Xenopus laevis* (Cad6: AAG30809, Cad11: NP001165705, ECad: 001165703); Xt, *Xenopus tropicalis* (Cad10: XP002935630, Cad7: XP017950519, NCad: AAI66196, RCad: AAI61139).

In situ **hybridization, immunohistochemistry and imaging**

To determine developmental expression patterns, anti-sense RNA probes to detect *CadIA*, *CadIIA*, *NCAM*, *SoxE1* and *SoxE2* gene sequences were synthesized (sequences for *CadIA* and *CadIIA* and *NCAM* are provided in Supplementary Fig. S1; *SoxE1* and *SoxE2* from McCauley and Bronner-Fraser, 2006), and immunohistochemistry was used to detect Hu C/D (Invitrogen) and cleaved Caspase3 (Promega) protein, according to previously described protocols (Nikitina et al., 2009; Sugahara et al., 2015). For double chromogenic *in situ* hybridization experiments, digoxigenin and fluorescein-labeled riboprobes were hybridized in embryos and detected using anti-digoxigenin and anti-fluorescein alkaline phosphatase (AP)-conjugated antibodies, respectively. The first AP-conjugated probe was detected using BCIP staining, and then inactivated by incubation for 45 min in 0.1M Glycine-HCl (pH 2.2) and washed several times in MABT. Detection of the anti-fluorescein AP-conjugated antibody was by NBT/BCIP staining according to standard methods. Whole mount embryos were photographed on a Zeiss V8 stereomicroscope. Selected embryos were embedded in 5% agarose and Vibratome sectioned (20 μm). Sections were mounted in 75% glycerol on a coverslipped glass slide and photographed on a Zeiss Axioimager Z1 using Zeiss Axiovision software (v4.7). Figures were assembled using Adobe Photoshop CS5.5.

CRISPR-Cas9 injections, and genotyping

For CRISPR-Cas9 perturbations, lamprey zygotes were microinjected (~5 nl) with 1 ng ul^{-1} Cas9 protein (PNA Bio), 500 pg guideRNA (gRNA) matching the targeted genomic locus, and 10% fluorescein dextran tracer, in nuclease-free water (Doudna and Charpentier, 2014; Hwang et al., 2013; Square et al., 2015; Zu et al., 2016). Two different gRNAs were tested separately and in combination to target the first exon of the *Snail* coding sequence (gRNA1: 5′ TCCAGTGCACAAGGTGCGGGG 3′; gRNA2: 5′ GCACGTGCGTACCCACACTGG 3′; Protospacer adjacent motif (PAM) sequence is underlined). Analysis of negative controls was performed to show specificity of Snail mutant phenotypes by using a single gRNA (5′CTCGACGGGAATCTTAGGAGG 3′) to target the homeobox transcription factor NKX2.2, which is expressed in the lamprey neural tube, but does not regulate early development of the neural crest (Sugahara et al., 2011). All gRNA constructs were carefully selected to recognize only a single region of the targeted gene and to avoid off-target cleavage effects based on the following stringency criteria described previously for lamprey (Square et al., 2015): 50–80% GC content; targeted regions as close as possible to the presumptive start codon (or 5′ end of available genomic sequence); no potential non-specific/off-target hits to the known *P. marinus* genome that had greater than 80% similarity by BLAST analysis, or with fewer than three mismatches in the ten base pairs closest to the PAM sequence. Injected embryos lacking fluorescence after 4 days post fertilization were discarded. Embryos were reared to desired stages, fixed in 4% MEMFA, dehydrated, and stored at -20ºC in 100% methanol.

The genotypes of putatively mutant embryos were validated in order to link genotypes to phenotypes in specific putatively mutant embryos. Genomic DNA was isolated, amplified and

sequenced from individual putative CRISPR mutants after phenotypic analysis by *in situ* hybridization or immunostaining. Briefly, following gene expression analyses and imaging, embryos were incubated 24–48h with 0.1 mg ml^{-1} proteinase K prior to extraction of genomic DNA (Sive et al., 2000). Oligonucleotides (Sigma) flanking the *Snail* (forward: 5′ GACGGAGCAGCAGAACGATGGT 3′; reverse: 5′ ACCGCTCCCCATAAAACACGC 3′) and *NKX2.2* (forward: 5′ CGCAGACGTTCGAGCTGGAG 3′, reverse: 5' GCGCACGTGTTCACTTCATC 3') genomic CRISPR target sites were used to PCR amplify and sequence 673 bp and 696 bp, respectively, of each genomic locus to establish a direct relationship between a specific deletion genotype within an individual embryo and observed gene expression and morphological phenotypes in the same animal. Our criteria for gRNA selection (Square et al., 2015), direct validation of mutant genotype-phenotype relationships within individual embryos, consistent phenotypes whether using single or multiple gRNAs, and confirmed specificity of Snail and control mutant phenotypes (see "Results"), make it unlikely that observed mutant phenotypes are attributable to off-target effects.

To estimate the efficiency of CRISPR-Cas9-mediated mutagenesis at the Snail locus, we sequenced six or seven putatively mutant embryos after analysis by *in situ* hybridization or immunohistochemistry, randomly selected from embryos that showed complete or near-complete loss of expression of *CadIIA* expression at T22, *SoxE2* expression at T22, *CadIIA* expression at T26, Hu expression at T26, *SoxE1* expression at T26, and Caspase3 expression at T22 (Table 1; Figs. 5, S2). Thus, a total of 37 individual embryos were sequenced to detect CRISPR-mediated DNA deletions out of 103 putatively mutant embryos analyzed for gene expression phenotypes by *in situ* hybridization or immunohistochemistry (Table 1). The number of embryos with a Snail

mutant genotype was determined from the total number of mutants observed for each gene-expression phenotype examined, in order to calculate the efficiency of CRISPR/Cas-mediated deletions targeting the Snail locus. (Fig. 5BB, Table 1).

RESULTS

A previous study showed that lamprey appears to have only a single gene copy of each of the type I and type II classical cadherins, referred to as *CadIA* and *CadIIA*, respectively (Sauka-Spengler et al., 2007). We conducted additional BLAST searches against the 2007 and 2010 sea lamprey genome assemblies (https://genome.ucsc.edu/cgi-bin/hgGateway) (Smith et al., 2013) using lamprey *CadIA* and *CadIIA* sequences, and type I and type II cadherin sequences from several gnathostomes, but failed to identify any additional classical cadherin orthologues. Although we cannot exclude the possibility that additional classical cadherins exist in the lamprey genome, our results, coupled with previous analyses (Sauka-Spengler et al., 2007) strongly suggest that *CadIA* and *CadIIA* are likely to be the only representatives of the classical cadherins. Neighbor-joining and maximum likelihood analysis of lamprey *CadIA* and *CadIIA* amino acid sequences confirmed their orthology with type I and type II classical cadherins, respectively in gnathostomes, (Fig. 1E), with lamprey *CadIIA* positioned basal to gnathostome Type II cadherins, and lamprey *CadIA* equidistant from gnathostome Type I E-Cad and N-Cad/R-Cad genes.

To better understand the evolutionary origin of mechanisms that regulate EMT in vertebrate neural crest cells, we examined the expression patterns of lamprey *CadIA* and *CadIIA* during key stages of neural crest development as these cells prepare to migrate. We first

examined cadherin expression during the earliest stages of neural crest development (Tahara stage (=T) 17) (Tahara, 1988), as the neural crest domain is established in the neural plate border region (see Figure 1A). Neither *CadIA* nor *CadIIA* are highly expressed in the neural plate or neural plate border (Fig. 2A, E), although *CadIIA*-positive cells appear to involute into the blastopore near the end of gastrulation (arrowhead in Fig. 2E). After the establishment of the neural crest domain in the neural plate border region, neurulation elevates the border regions and brings them together as neural folds that fuse in the dorsal midline to form the neural tube (Figure 1B, C). At this stage in gnathostomes the ventral neuroepithelium retains strong, uniform expression of type I cadherins, whereas neural crest cells in the dorsal neural tube differentially regulate expression of both type I and type II cadherins (Figure 1B, C). This shift in expression of cadherins in the dorsal neural tube allows the pool of premigratory and migratory neural crest to become isolated and phenotypically and genetically distinct from the rest of the neural tube. In contrast to gnathostomes, lamprey never expresses *CadIA* anywhere in the neural tube (Fig. 2B, C), but does express *CadIIA* in much of the neural tube by stage T20, including the dorsal-most region where premigratory neural crest forms (Fig. 2F, inset in 2F). Slightly later (T21), neural crest cells expressing *CadIIA* exit from the neural tube (Fig. 2G, inset in 2G) and by T22 migratory neural crest cells are visible as three streams descending toward the pharynx (Fig. 2H). Double *in situ* hybridization indicates that *CadIIA* expression in the dorsal neural tube overlaps with *Pax3/7* (Fig. 2I–L), a known marker of premigratory neural crest in lamprey and jawed vertebrates (Nelms and Labosky, 2010; Sauka-Spengler and Bronner-Fraser, 2008a; Sauka-Spengler et al., 2007). Notably, throughout these key stages of neural crest development, we

never observed *CadIA*-positive cells anywhere in the embryonic neural tube (Fig. 2B, C), despite continuous expression of *CadIIA* in premigratory and migratory neural crest (Fig. 2F–L).

Previous work (Sauka-Spengler et al., 2007) together with our phylogenetic analysis (Fig. 1E) and exhaustive search of the lamprey genome, diminish the possibility that a different classical cadherin regulates the premigratory state of lamprey neural crest. However, one possible explanation for the apparent lack of differential cadherin expression during neural crest development is that lamprey instead downregulates an alternative intercellular adhesion protein other than *CadIA*, which leads to neural crest migration. NCAM is a common intercellular adhesion protein that maintains epithelial integrity in the neural tube of gnathostomes (Weledji and Assob, 2014). We identified a lamprey orthologue of *NCAM*, and examined its expression during neural crest migratory stages. Similar to *CadIA*, *NCAM* is also not expressed in the neural tube during neural crest migration (Fig. 2D). Together, these findings show that lamprey does not downregulate expression of *CadIA* or *NCAM*—two intercellular adhesion proteins—before the onset of neural crest migration. In addition, *CadIIA* is already expressed in premigratory neural crest in lamprey and its expression persists throughout migration. These results suggest that, unlike gnathostomes, lamprey neural crest cells may not undergo a canonical change in intercellular adhesion molecules to facilitate neural crest migration.

Since we observed no expression of *CadIA* or *NCAM* during early development of premigratory and migratory neural crest in lamprey (Fig. 2), we examined their expression at later developmental stages in order to validate probe sensitivities and determine late stage neural crest expression domains. We observed conserved expression of these genes between gnathostomes and lamprey at later embryonic stages. For example, well after neural crest

migration has occurred (E10–E15), the same riboprobes revealed *CadIA* transcripts enriched in the otic vesicle, oral mesenchyme, pharyngeal arches and heart, all of which are domains of expression conserved with gnathostome type I cadherins (Fig. 3A, B) (Levi et al., 1991; Novince et al., 2003; Quinlan et al., 2004). *CadIIA* shows conserved expression domains in neural crest-derived cranial ganglia (Fig. 3C, D) (Borchers et al., 2001; Liu et al., 2006; Nakagawa and Takeichi, 1998b). Despite the lack of expression during neural crest EMT and migration stages, *NCAM* is expressed throughout the central nervous system and in condensing cranial ganglia (Fig. 3E, F) (Bally-Cuif et al., 1993; Cremer et al., 1997). Thus, despite the differences observed in the expression of *CadIA*, *CadIIA*, and *NCAM* during early neural crest specification in lamprey, these genes show conserved patterns of expression with gnathostomes at later developmental stages. This suggests that early observed expression differences we observed may reflect differences in the regulation of cell migration.

Some of the key transcriptional regulators of EMT and neural crest migration in gnathostomes include members of the Sip, Zeb and Snail/Slug family of zinc finger transcription factors (Peinado et al., 2007). Functional perturbation of Snail or Slug in gnathostomes correlates with loss of neural crest precursors and/or an inability of neural crest cells to detach and migrate from the neural tube (Barrallo-Gimeno and Nieto, 2009; Nieto, 2002; Powell et al., 2013; Rogers et al., 2013), and loss of Sip/Zeb protein function correlates with an inability of neural crest cells to undergo a complete EMT. We cloned and analyzed the expression of lamprey cognates of *Sip1* (Fig. 4B, H, N) and *Zeb1* (Fig. 4C, I, O) and found that they, too, are expressed in *CadIIA*-like domains (Fig. 4A, G, M) in the neural tube. Although it was previously interpreted that *Snail* is not expressed in early neural crest development in lamprey (Rahimi et al., 2009), we find that

Snail transcripts do indeed localize early to the dorsal neural tube and are also expressed in prominent bilateral stripes throughout the neural dorsal-ventral axis (Fig. 4D, J, P). Furthermore, double *in situ* hybridization confirmed that *Snail* expression overlaps in the dorsal neural tube with that of *Pax3/7* (Fig. 4E, K, Q), a pan-vertebrate marker of early neural crest cells (Nelms and Labosky, 2010; Sauka-Spengler et al., 2007). Moreover, we found that *Snail* and *CadIIA* are also expressed in overlapping patterns in the dorsal neural tube, and appeared to maintain similar expression domains throughout much of the neural dorsal-ventral axis (Fig. 4F, L, R). Taken together, these results strongly support a revised view that Snail is expressed in early developing neural crest cells in lamprey.

Our results showing overlapping expression of *Snail* and *CadIIA* in the lamprey neural tube during stages of neural crest migration raise the possibility that Snail-mediated control of neural crest EMT and migration may be an ancient feature of vertebrate neural crest cells. To test this notion, we studied the functional relationship between Snail protein activity and development of lamprey neural crest cells using the CRISPR-Cas9 system (Doudna and Charpentier, 2014; Square et al., 2015; Zu et al., 2016). We found that CRISPR-mediated deletion of lamprey *Snail* sequences caused severe defects in neural crest development. Analysis of *CadIIA* expression at T22 in one set of *Snail*$^{-}$ CRISPR mutants revealed complete loss of both premigratory and migratory neural crest populations (Fig. 5C n=16/31 embryos; Table 1) compared to wildtype (Fig. 5A, G) and negative controls (Fig. 5E, K), suggesting regulatory linkage between *Snail* and *CadIIA*. We also observed hypomorphic *Snail*$^{-}$ phenotypes, with weakened expression of *CadIIA* in the premigratory crest cells in the neural tube, but no *CadIIA*-positive migratory neural crest (Fig. 5I and inset panel, n=14/31 embryos; Table 1). This

suggests that perturbation of *Snail* function prevents *CadIIA*-expressing cells from exiting the neural tube in these embryos. Similarly, we noted a reduction in *SoxE2*-positive migratory neural crest in Snail mutants (compare Fig. 5B, F, H, L with D, J; *n*=11/11 embryos, Table 1). The reduction of both pre-migratory and migratory neural crest in *Snail* mutant embryos resulted in loss of neural crest-derived structures later at T26, including *CadIIA* (*n*=14/17) and Hu (*n*=15/17) positive cranial and dorsal root ganglia (Fig. 5M-R), as well as *SoxE1*-positive prechondrocytes in the pharyngeal arches (Fig. 5S-U, *n*=23/24) (Table 1). Because *Snail* is known to inhibit apoptosis in migratory neural crest (Vega et al., 2004), we reasoned that loss of neural crest in Snail mutants may be attributed to increased cell death. Caspase3 immunostaining revealed increased cell death, localized primarily to the dorsal-most region of the neural tube where *SoxE2*- and *CadIIA*-positive premigratory neural crest cells were found (Fig. 5V-AA, compare with Fig. 5G,H; *n*=10/13; Table 1). To link Snail deletion phenotypes to specific genotypes within individual embryos, genomic DNA was extracted from individual embryos after analysis by *in situ* hybridization or immunohistochemistry. The *Snail* locus encompassing the deletion site was then PCR-amplified, sequenced, and compared against the lamprey Snail gene sequence to confirm mutagenesis for representative mutant embryos (Fig. 5BB, see also Supplementary Information Figure S2). The efficiency of CRISPR-mediated Snail mutagenesis was further confirmed by sequencing the Snail locus from 37 randomly sampled individual putative mutant embryos after phenotypic analysis by *in situ* hybridization or immunostaining (see "Materials and Methods"). We found that 100% (37/37) of selected putative mutant embryos had a corresponding genomic lesion (Table 1) and 78% (29/37) of the genotypes were unique (see Supplementary Fig. S2 for full list of individual embryo sequences). Of the 37

mutant genotypes, 23 and 14 embryos had in-frame and out-of-frame mutations, respectively, suggesting that both types of mutations are effective in generating strong phenotypes.

To control for non-specific effects of CRISPR-mediated deletions, we targeted the homeobox gene *NKX2.2*, which is expressed in the lamprey neural tube, but does not regulate development of the neural crest (Sugahara et al., 2011). Negative control deletions of *NKX2.2* showed no appreciable changes in gene expression of *CadIIA*, T22, *n*=10; *SoxE2*, T22, *n*=7; *CadIIA*, T26, *n*=5; *SoxE1*, T26, *n*=5; Hu, T26, *n*=5; Caspase3, T22, *n*=8) compared to wildtype embryos (Fig. 5). Putative NKX2.2 control mutants were also confirmed by sequencing (Fig. 5BB). Finally, to determine if the loss of Snail activity in CRISPR mutants is related to transcriptional or translational perturbation, we performed *in situ* hybridization for *Snail* mRNA in putative mutants. *Snail* transcription was largely unaffected (Supplementary Fig. S3), suggesting that phenotypes observed in Snail mutants are likely attributable to loss of functional Snail protein, rather than inhibition of transcription at the Snail locus.

DISCUSSION

The molecular and cellular control of EMT and migration was a key step in the origin of the vertebrate neural crest, yet how the gene regulatory network controlling this process was assembled and what the ancestral state of these control mechanisms looked like remains a major unresolved issue in evolutionary developmental biology. Our results suggest that differential expression of classical cadherins, an important and highly conserved event during neural crest EMT and migration in gnathostome vertebrates, is not required to initiate neural crest migration in lamprey. This provides evidence that the key cellular and molecular events governing core

features of neural crest cells in the first vertebrates may have been very different from that described in gnathostomes, and suggests that the canonical model of EMT and cadherin regulation during neural crest migration may be a derived feature of gnathostome neural crest biology.

Comparative analysis of classical cadherins across chordates reveals conservation and divergence of both structure and function (Gallin, 1998; Hulpiau and Van Roy, 2009, 2011). The closest extant relatives of the vertebrates, the tunicates (Delsuc et al., 2006), appear to have only two classical cadherins, similar to the condition in lamprey. These cadherins are orthologous to type I and type II cadherins and are expressed in epithelial tissues, including the central nervous system, endoderm, notochord and sensory structures (Noda and Satoh, 2008). Amphioxus has two described cadherins as well, but their structural properties are unique and these genes appear to have resulted from an independent tandem duplication event (Oda et al., 2002). Moreover, both cadherins in amphioxus lack the calcium-binding extracellular repeat domains that mediate homo- and heterophilic interactions of cadherins between cells in other animals, yet in amphioxus, cells expressing these cadherins still adhere together (Oda and Takeichi, 2011). This suggests that either some as-yet undiscovered cadherins mediate these interactions, or there is an unknown mechanism whereby amphioxus cadherins can mediate intercellular adhesion without a conserved extracellular domain. Based on the cadherin repertoire present in both invertebrate chordates and vertebrates, the most parsimonious explanation for this pattern is that the last common chordate ancestor likely possessed a single type I and a single type II classical cadherin gene (Hulpiau and van Roy, 2011). However, given the peculiar structural and functional properties of amphioxus cadherins, and the likelihood that these resulted from an independent

gene duplication event, these cadherins are likely to be unique to cephalochordates. Thus, tunicates may be a better model for studying the evolution of cadherin function in chordates.

Based on the genomic and functional data of classical cadherins across chordates, we also propose that the last common chordate ancestor possessed a single type I and a single type II cadherin, each of which likely was expressed in discrete epithelial tissues (Fig. 6, node A). Notably, tunicates express a type II cadherin orthologue (Noda and Satoh, 2008) in the central nervous system during early tadpole stages similar to lamprey, which suggests that proper regulatory control of type II cadherin expression in the neural tube was already established at the base of vertebrates. These observations in invertebrate chordates, taken together with our data in lamprey, suggest that the first vertebrates that evolved neural crest cells did not initially deploy differential regulation of classical cadherin expression during neural crest cell migration, as occurs in gnathostomes (Fig. 6, node B). During the earliest stages of lamprey neural crest development, a sharp upregulation of the type II cadherin, *CadIIA*, occurs in the putative neural crest domain and in much of the neural tube. This expression pattern in the dorsal neural tube is largely similar to that of duplicated type II cadherin paralogues in gnathostomes (e.g., Cadherin-6, Cadherin-7, Cadherin-11), suggesting an ancient evolutionary origin of type II cadherin expression in premigratory and migratory vertebrate neural crest. On the other hand, expression of *CadIIA* in the rest of the neural tube may indicate a simultaneous but distinct function from promoting neural crest migration in the dorsal neural tube. For example, a pan-neural *CadIIA* expression domain may instead promote epithelial integrity of the neural tube mid-ventrally, while facilitating neural crest migration dorsally. A potential pro-epithelial function of *CadIIA* may explain the lack of, and compensate for, *CadIA* expression in the rest of the neural tube.

Based on the already-present domain of *CadIIA*-positive cells in the neural tube of lamprey, we suggest that the migratory neural crest cells in the dorsal neural tube of ancestral vertebrates did not require modulation of type I-type II cadherins, but rather were maintained ontogenetically as a population "ready-to-migrate" multipotent cells (Fig. 6, node B). Although there is evidence that modulation of type I and type II cadherins is a key step in controlling EMT in various Metazoan cell types, our findings indicate that neural crest cells in early vertebrates may not have required such a mechanism. Integration of pro-migration transcriptional and cytoskeletal regulators (e.g., *Snail*, *Sip1*, *Zeb1*) into the neural crest domain (Jandzik et al., 2015; Meulemans and Bronner-Fraser, 2005) with type II cadherins would have coordinately facilitated the migration of these cells from the neural tube, as indicated by similar expression of these transcriptional regulators in the lamprey neural tube (Fig. 4). Interestingly, each of the transcriptional regulators we identified (Sip1, Zeb1, Snail) is not restricted to the dorsal neural tube, as would be expected for genes restricted to premigratory neural crest. Instead, they are all expressed throughout the dorsal-ventral axis of the neural tube (Fig. 4), but the significance of this observation for neural crest migration and/or neurogenesis in lampreys has not been established.

Recent work has suggested that the neural crest represents a form of "cellular neoteny", in which a multipotent gene regulatory program was maintained by a heterochronic shift into the proto-neural crest population (Buitrago-Delgado et al., 2015) (Fig. 6, node B). Our findings in lamprey support this idea, and suggest that this heterochronic shift involved maintenance of a gene regulatory program promoting mesenchymal state that was coupled to multipotency (Mani et al., 2008; Schmidt et al., 2015), two of the hallmark traits of neural crest cells. Next, after

diverging from agnathans, we propose that in gnathostomes type I cadherins may have been co-opted or their expression domains may have expanded into the neural tube during early embryonic development, thereby excluding and replacing early type II cadherin expression to create an epithelial cellular domain. This earlier expression of pro-epithelial cadherins would then require that neural crest cells deploy differential expression of cadherins to mediate EMT in the dorsal neural tube to endow this cell population with migratory properties (Fig. 6, node C).

Following the evolution of differential cadherin expression for EMT in gnathostome neural crest cells, there was an expansion of cadherin paralogues, as indicated by numerous type I and type II cadherin genes present in gnathostome genomes (Gallin, 1998; Hulpiau and van Roy, 2011) (Fig. 6, node C). There is evidence that these additional cadherin genes have acquired specialized functions by subfunctionalization and neofunctionalization. For example, in chicken embryos, Cadherin 6b functions early in neural crest development by segregating the neural crest population from the underlying neuroepithelium (Nakagawa and Takeichi, 1998a; Van Roy, 2013). In the chick midbrain repression of Cadherin 6b by *Snail2* appears to be required for neural crest cells to exit the neural tube (Taneyhill et al., 2007), whereas Cadherin 7 is expressed in migratory neural crest (Van Roy, 2013). On the other hand, Cadherin 6b expression is expressed in neural crest cells undergoing EMT in the chick trunk (Park and Gumbiner, 2010a) and fish hindbrain (Clay and Halloran, 2014). These findings suggest that in gnathostomes, Cadherin 6b and Cadherin 7 have acquired distinct, but complementary, functions to orchestrate neural crest segregation and migration. Diversification of type I cadherins has similarly resulted in new functions during gnathostome development. Retinal cadherin (R-cad) has acquired tissue-specific roles in inner ear and lens development (Novince et al., 2003),

whereas E-cadherin, which usually operates as a pro-epithelial type I cadherin, is sometimes expressed in mesenchymal tissues, including early migrating neural crest in chick and frog (Dady et al., 2012; Huang et al., 2016a). There is also evidence for the evolution of new signaling roles during neural crest EMT for type I and type II cadherins in gnathostomes. For example, proteolytic cleavage of classical cadherins by ADAM and MMP proteases results in small intracellular fragments that regulate neural crest EMT and migration. Some fragments may be extracellular (Abbruzzese et al., 2016), whereas intracellular fragments can localize to the nucleus and exert fine-tuned control over gene expression patterns requisite for neural crest EMT (Abbruzzese et al., 2016; McCusker and Alfandari, 2009; Schiffmacher et al., 2014; Schiffmacher et al., 2016). These and other types of regulatory linkages show how the evolution of complex inter- and intracellular control mechanisms was likely an important driver for the establishment of novel linkages in the migration module of the neural crest GRN in gnathostomes.

A comprehensive analysis of neural crest regulation in lamprey established that many of the transcription factors and signaling molecules controlling neural crest development in gnathostomes were also conserved in lamprey (Sauka-Spengler et al., 2007). This suggested that the core of the neural crest GRN was fixed in ancestral vertebrates over 500 million years ago and has undergone relatively little change since that point. However, it is difficult to reconcile this model of widespread conservation with the incredibly diverse morphology, physiology and behavior across vertebrates. For example, although lampreys are vertebrates, they lack neural crest derivatives including jaws and sympathetic chain ganglia, and do not have myelinating glial cells (McCauley et al., 2015; Shimeld and Donoghue, 2012). Clearly, some important changes in

the neural crest GRN must account for such profound differences between agnathans and gnathostomes. We suggest that a model proposing widespread conservation across the entire neural crest GRN—a complex molecular circuitry of signaling inputs, positive and negative feedback loops and specification/inductive events—fails to account for important changes within individual regulatory modules (e.g., neural crest migration, EMT) that may have effected evolutionarily relevant modifications to developmental programs. Our results highlight this point, and add to current evidence suggesting that the mechanisms controlling neural crest migration in gnathostomes do not necessarily approximate ancestral vertebrate mechanisms. For example, lamprey premigratory and migratory neural crest cells do not express key gnathostome EMT regulators such as *Ets* and *Twist*, despite having multiple paralogs of each (Sauka-Spengler and Bronner-Fraser, 2006, 2008b; Sauka-Spengler et al., 2007). Collectively, these results point to a scenario in which many of the mechanisms that characterize EMT and neural crest migration in gnathostomes may represent a relatively derived condition that has diverged substantially from ancestral states that are likely to be better approximated in basal vertebrates such as lamprey. Further gene expression and functional studies, as well as mapping and comparative analysis of *cis*-regulatory element control of the neural crest migration module between agnathans and gnathostomes will be informative in this regard.

The lack of an obvious change of cadherin expression suggests the presence of unique regulatory mechanisms controlling the onset of neural crest migration in lamprey. This may be due to the fact that lamprey appears to have only a single type I and a single type II cadherin. In the canonical model of neural crest EMT in gnathostomes, the transcription factors Snail, Sip1, Zeb1, E12/47 and many others are direct transcriptional repressors (Barrallo-Gimeno and Nieto,

2005; Comijn et al., 2001; Nieto, 2002; Sánchez-Tilló et al., 2010; Vandewalle et al., 2005) of genes promoting epithelial fate, thereby indirectly enabling the expression of type II cadherins and allowing neural crest cells to dissociate from the underlying neuroepithelium. Although this model provides a heuristic framework for studying neural crest EMT and migration in gnathostomes, it may not operate in the same fashion in lamprey embryos, where a shift in classical cadherin expression is never observed during the onset of neural crest formation or migration. What we do observe is that the domain of expression of the mesenchymal cadherin *CadIIA* in lamprey neural crest cells overlaps with *Snail* expression, and our functional analyses reveal that *Snail* activity may be necessary for proper activation of *CadIIA* in premigratory and migratory crest. Based on an apparent functional requirement for *Snail* to activate *CadIIA* expression, it is possible that *Snail* functions in agnathans as a transcriptional activator in a context-dependent manner, via the differential use of cofactors that mediate switching between repressor or activator functions during embryonic development. This prediction has been confirmed in *C. elegans*, where the *Snail* homolog CES-1, known to act as a repressor, can function conditionally as an activator (Reece-Hoyes et al., 2009). A recent *in vitro* study in cancer cell lines similarly showed that *Zeb1*, which usually acts as a transcriptional repressor of epithelial gene batteries during EMT, is converted into an activator of other target genes when bound to certain cofactors (Lehmann et al., 2016). It would therefore be interesting to identify potential cofactors for lamprey *Snail* to determine if it is able to function as a context-dependent transcriptional activator of *CadIIA* during the onset of neural crest migration.

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Gene Analyzed (ISH, IHC)	Embryonic Stage	Embryos with Putative Mutant Phenotypes (%)	Embryos with Mutant Genotypes (%)
CadIIA (ISH)	T22	30/31 (94%)	7/7 (100%)
SoxE2 (ISH)	T22	11/11 (100%)	6/6 (100%)
CadIIA (ISH)	T26	14/17 (82%)	6/6 (100%)
Hu (IHC)	T26	15/17 (88%)	6/6 (100%)
SoxE1 (ISH)	T26	23/24 (96%)	6/6 (100%)
Caspase3 (IHC)	T22	10/13 (77%)	6/6 (100%)

Table 1. Summary of CRISPR-Cas9 experiments targeting lamprey Snail. Putative Snail mutant embryos were first analyzed for changes in gene expression by *in situ* hybridization (ISH) or immunohistochemistry (IHC) at Tahara stage 22 or 26 (T22, T26). Of the embryos having phenotypes (i.e., loss or significant reduction in expression) in each category, six or seven of these had genomic DNA sequenced to verify a mutant genotype (n=37 total embryos). Representative embryos having a genotype linked to a specific phenotype are shown in Fig. 5 (panel C, I, D, N, Q, T, X). Mutant genotypes from all 37 embryos analyzed are found in Supplementary Fig. S2.

FIGURE LEGENDS

Figure 1. (A-D) Canonical model for neural crest development and cadherin expression during EMT, illustrated as cross sections through a generalized vertebrate embryo, and (E) phylogeny of chordate type I (blue) and type II (red) classical Cadherins. After gastrulation there are three established dorsal cellular domains (A): medial neural plate (blue), lateral epidermal ectoderm (purple) and neural plate border (presumptive neural crest cells, NCCs) in between (red). All three domains express type I cadherins. As neurulation is completed (B) specified neural crest cells are brought dorsally and begin to delaminate from the underlying neuroepithelium by differentially regulating expression of type I and type II cadherins, whereas the neuroepithelium maintains strong expression of only type I cadherins (B). Because cadherins tend to bind in a homophilic fashion, cell populations that express different cadherin proteins at their surface no longer retain strong intercellular adhesion to their neighbors, resulting in detachment of premigratory neural crest cells (pmNCCs) from the surrounding neuroepithelium. By contrast, neuroepithelial cells remain attached to one another as a result of sustained type I cadherin expression. Sustained, differential expression of type I and type II cadherins, concomitant with expression of other pro-mesenchymal genes leads to NCC migration from the dorsal neural tube (C) to various regions in the embryo where many subsequently undergo a mesenchymal to epithelium transition and differentiate into a variety of cell types (D). (E) Lamprey has only a single copy of the type I and type II cadherin genes compared to the gnathostome species analyzed (see Materials and Methods).

Figure 2. Expression (via *in situ* hybridization) of lamprey *CadIA* and *CadIIA* during early neural crest development. There is no detectable expression of the type I cadherin orthologue (*CadIA*) in early neurula stages (A), or in premigratory (asterisks in B) or migratory (asterisks in C) neural crest cells. Similarly, the neural epithelial marker, *NCAM*, is not expressed at the onset of neural crest migration (asterisks in D). A type II cadherin gene (*CadIIA*) is expressed in involuting mesenchymal cells at late gastrula/early neurula-stage (T17) embryos (arrow in E). *CadIIA* localizes to premigratory (PMNC) (arrowhead in F) and early migratory (MNC) (arrowheads in C, D) crest cells, and is expressed in three migratory neural crest streams (maxillary, Mx; mandibular, Mn; branchial, B). *Pax3/7*, a canonical marker of premigratory neural crest in vertebrates is expressed in the dorsal neural tube (I, J), and overlaps in the dorsal neural tube with *CadIIA* expression (K, area between dotted lines in the neural tube indicates overlapping expression in L). The faint purple hue in B and C is overstaining as a result of overdeveloping the NBT/BCIP color reaction to determine possible *CadIA* localization. Black bars denote planes of section corresponding to the inset images in panels B, C, D, F, G, and L. Dorsal view is presented with anterior oriented up for panels A, E and H; Dorsal is up and anterior is oriented toward the left in B–D, F–I, K. NP, neural plate; NPB, neural plate border. NT, neural tube; N, notochord; S, somite. Dashed lines beneath embryo surface trace the ventral surface of the epidermal ectoderm and dorsal and lateral surface of the neural tube.

Figure 3. Lamprey *CadIA*, *CadIIA* and *NCAM* expression is similar to that of gnathostome cognates during later embryonic stages. (A) Beginning at T24, *CadIA* transcripts localize to the developing pharyngeal arches (PA) and oral mesenchyme (OM). (B) At T26 additional sites of

CadIA expression are found in the heart (H) and notochord (N). (C) *CadIIA* expression is observed in neural crest-derived cranial ganglia such as the trigeminal (TG), facial (FG) and lateral line (LLG) branches at T24. (D) *CadIIA* expression domains are retained at T26 with *CadIIA* transcripts becoming enriched in epibranchial (EG) and dorsal root ganglia (DRG). *NCAM* is expressed prominently in the central nervous system (asterisks) at both T24 (E) and T26 (F), and expression is also observed in the epibranchial ganglia at T26.

Figure 4. Expression of transcriptional regulators promoting mesenchymal state overlaps with *CadIIA* in premigratory and migratory neural crest cells. *CadIIA* is expressed in premigratory (arrowheads in A, G, M) and early migratory (asterisks in G) neural crest, and throughout the neural tube. Zinc-finger transcriptional regulators of neural crest EMT, including *Sip1* (B, H, N), *Zeb1* (C, I, O) and *Snail* (D, J, P), are expressed in premigratory neural crest (arrowheads) similar to that of *CadIIA* during the onset of neural crest migration. *Snail* expression extends dorsally in the neural tube, overlapping with *Pax3/7* (E, K, bottom two dashed lines in Q) and *CadIIA* (F, L, bottom two dashed lines in R) expression in the dorsal neural tube where premigratory neural crest cells (arrowheads) reside. The top-most dashed line in panels M–R traces the ventral surface of the epidermal ectoderm and the dorsal and lateral surface of the neural tube. NT, neural tube; N, notochord.

Figure 5. CRISPR-Cas9-mediated knockout of lamprey *Snail* results in loss of migratory neural crest and neural crest derivatives. In wildtype (WT) embryos, *CadIIA* and *SoxE2* are expressed in premigratory (PMNC) and migratory neural crest (MNC), respectively (arrowheads in A, B,

arrowhead in inset panel shows the dorsal view). *CadIIA* is expressed in neural crest derivatives (M), including cranial (TG, trigeminal ganglion; FG, facial ganglion; LLG, lateral line ganglion) and dorsal root ganglia (DRG), largely similar to that of the pan-neural protein marker, Hu (P); *SoxE1* marks neural crest-derived ectomesenchyme of the pharyngeal arches (arrow head in M). In wildtype embryos, there is no Caspase3 protein staining in the neural crest (V, arrowheads in W). Mutagenesis of *Snail* results in complete loss of *CadIIA* expression in premigratory neural crest (arrows in C and inset in C), whereas embryos with hypomorph phenotypes show weak expression of *CadIIA* in premigratory neural crest (arrows in I and inset in I). *SoxE2* expression is lost in neural crest of the midbrain (asterisks in D, arrowhead in J) and there are fewer *SoxE2*-positive migratory cells overall (arrowhead, D). Loss of premigratory and migratory neural crest cells in *Snail* CRISPR KO embryos correlates with a corresponding loss of *CadIIA* and Hu expression in neural crest-derived cranial and dorsal root ganglia (arrowheads in N, Q), and epibranchial ganglia (asterisks in N, Q). Similarly, functional perturbation of *Snail* activity resulted in complete loss of *SoxE1*-positive prechondrocytes in the pharynx (asterisks in T). There are more Caspase3-positive cells in the dorsal neural tube of *Snail* CRISPR mutants (arrowheads in X, Y) where premigratory neural crest cells form (arrowhead in Y). Negative control experiments involving CRISPR-Cas9-mediated knockout of Control CRISPR mutants (E,F,K,L,O,R,U,Z,AA) targeting deletions in the *NKX2.2* gene sequence (see "Materials and Methods") did not result in loss of expression of *CadIIA* (E, K, O), *SoxE2* (F, L), Hu (R), or *SoxE1* (U), and did not result in increased cell death in premigratory neural crest (Z, AA). (BB), Genomic DNA from the individual *Snail* and negative control (*NKX2.2*) CRISPR-injected embryos presented in Fig. 5 was sequenced to confirm mutagenesis at the targeted loci after *in*

situ hybridization or immunostaining. The guide RNA target and protospacer adjacent motif (PAM) sequences are indicated by blue and red text, respectively. Numbers to the right of each mutant sequence indicate the number of base pairs deleted relative to the wildtype sequence. Dashed lines in panels showing cross-sections delineate the interface of the ventral surface of the epidermal ectoderm and the dorsal and lateral surface of the neural tube.

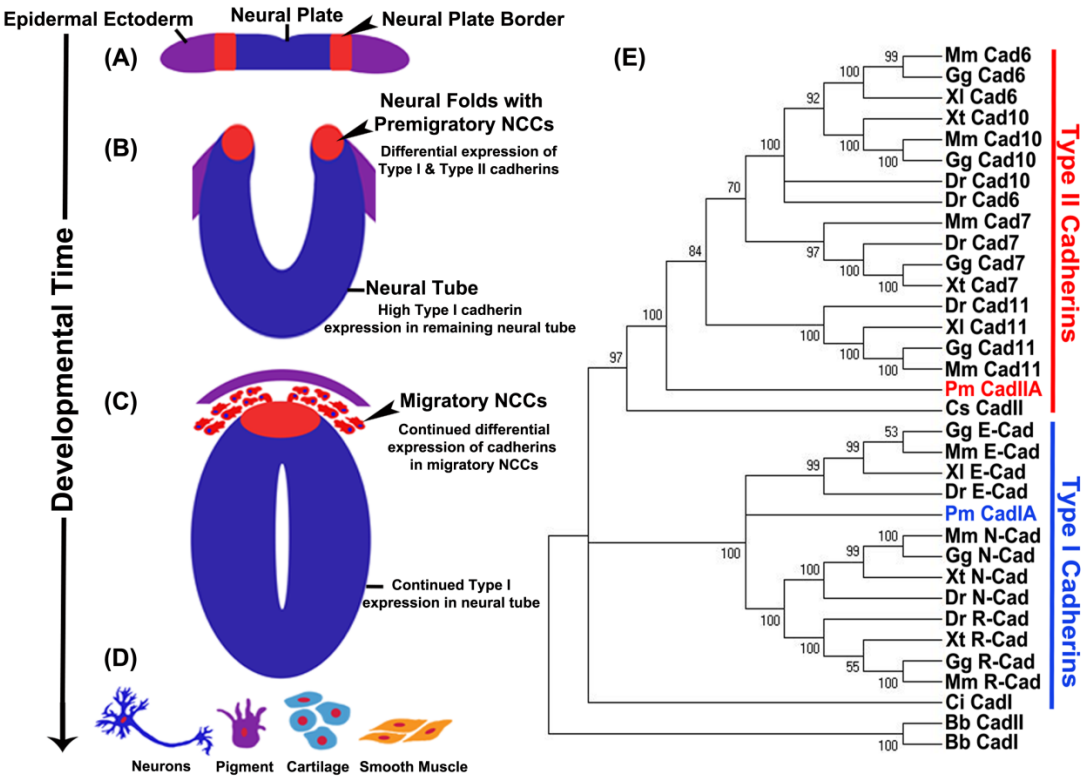
Figure 6. Model for the evolution of cadherin expression-mediated EMT in vertebrate neural crest cells. Representative animals in the chordate phylogeny include from left to right: amphioxus (basal chordate), tunicate (vertebrate sister-group), lamprey (agnathan vertebrate), and zebrafish (gnathostome vertebrate). The last common chordate ancestor (node A) likely possessed a single orthologue of the type I and type II classical cadherins that functioned in epithelial maintenance and tissue morphogenesis, respectively. This functional state was probably maintained in basal vertebrates, as seen in lamprey (node B), but also likely involved co-option of type II cadherins into the new neural crest domain to facilitate their migration. The neural crest evolved as a multipotent and mesenchymal cell population that was maintained in later stages by retention of gene regulatory programs controlling early embryonic development ("cellular neoteny"), obviating the requirement for a shift in expression of classical cadherins to control the onset of neural crest migration. With the evolution of gnathostomes (C), early expression of type I cadherins in the early embryonic neural tube created a barrier to neural crest migration that was overcome by a secondary EMT, mediated by changes in expression of classical cadherins. Additional genome duplications facilitated diversification of cadherin function by sub- and neofunctionalization of cadherin paralogues.

Figure S1. cDNA sequences of lamprey cadherins (*CadIA*, *CadIIA*) and *NCAM* used to generate RNA probes.

Figure S2. Sequences from individual lamprey embryos showing mutations at the *Snail* locus after phenotypic analysis by *in situ* hybridization (ISH) and immunohistochemistry (IHC). Six or seven putative *Snail* CRISPR mutants from each gene expression analysis category in Fig. 5 (*CadIIA*, T22; *SoxE2*, T22; *CadIIA*, T26; Hu, T26; *SoxE1*, T26; Caspase3, T22) were randomly sampled after ISH or IHC for isolation and sequencing of genomic DNA to estimate efficiency of CRISPR-induced mutagenesis (see "Materials and Methods"). Note that every individual embryo sampled contains a mutation within the CRISPR target site (blue = gRNA sequence; red = PAM site). Numbers to the right of each sequence indicate the number of base pairs deleted. Embryos that are represented with images in Fig. 5 are indicated to the left with their corresponding panel IDs.

Figure S3. Effects of CRISPR-induced *Snail* mutations on *Snail* mRNA production. In wildtype embryos, *Snail* is expressed early in premigratory neural crest (A, black arrowhead), and somatic mesoderm (asterisks). During later stages, *Snail* mRNA is enriched in the pharyngeal arches (red arrowhead). In *Snail* CRISPR mutants, *Snail* expression is similar compared to wildtype embryos at both early (C, E) and late (D, F) stages of embryogenesis. Isolation and sequencing of these embryos revealed that they are true mutants (G).

Figure 1


Epidermal Ectoderm
Neural Plate
Neural Plate Border
(A)
Neural Folds with Premigratory NCCs
(B)
Differential expression of Type I & Type II cadherins
Neural Tube
High Type I cadherin expression in remaining neural tube
(C)
Migratory NCCs
Continued differential expression of cadherins in migratory NCCs
Continued Type I expression in neural tube
(D)
Neurons
Pigment
Cartilage
Smooth Muscle
Developmental Time
(E)
Mm Cad6
Gg Cad6
Xl Cad6
Xt Cad10
Mm Cad10
Gg Cad10
Dr Cad10
Dr Cad6
Mm Cad7
Dr Cad7
Gg Cad7
Xt Cad7
Dr Cad11
Xl Cad11
Gg Cad11
Mm Cad11
Pm CadIIA
Cs CadII
Gg E-Cad
Mm E-Cad
Xl E-Cad
Dr E-Cad
Pm CadIA
Mm N-Cad
Gg N-Cad
Xt N-Cad
Dr N-Cad
Dr R-Cad
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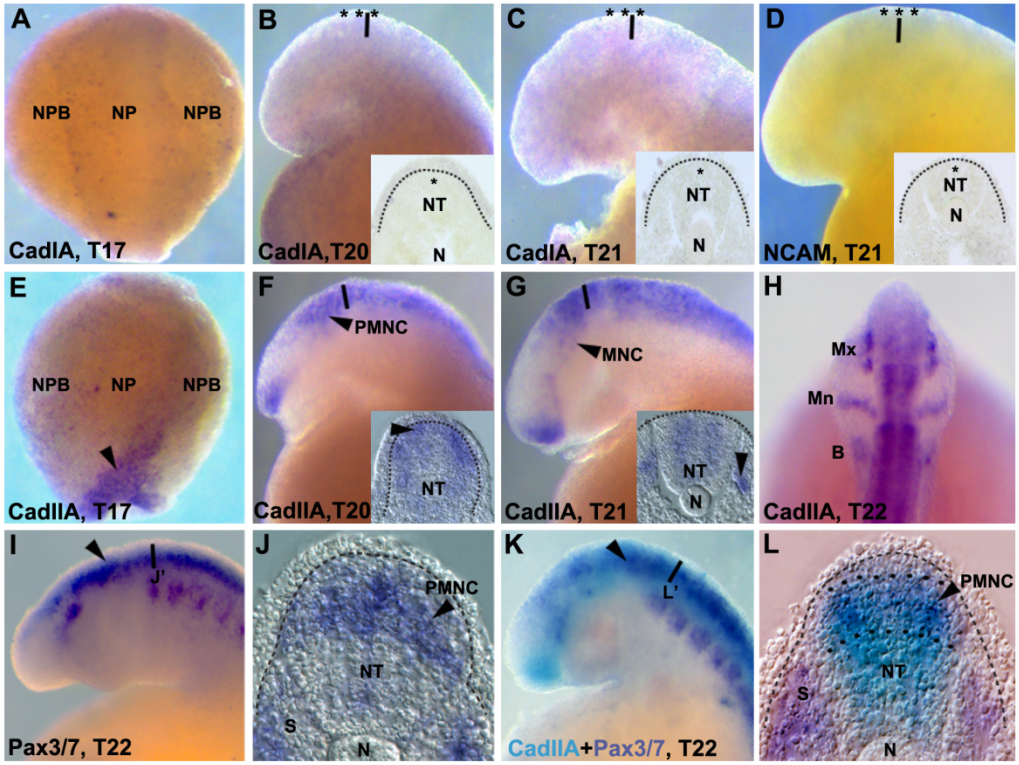
Mm R-Cad
Ci CadI
Bb CadII
Bb CadI
Type II Cadherins
Type I Cadherins

Figure 2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NPB
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NT
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CadIA,T20
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NCAM, T21
E
NPB
NP
NPB
CadIIA, T17
F
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NT
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CadIIA, T21
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Mx
Mn
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CadIIA, T22
I
J'
Pax3/7, T22
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K
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CadIIA+Pax3/7, T22
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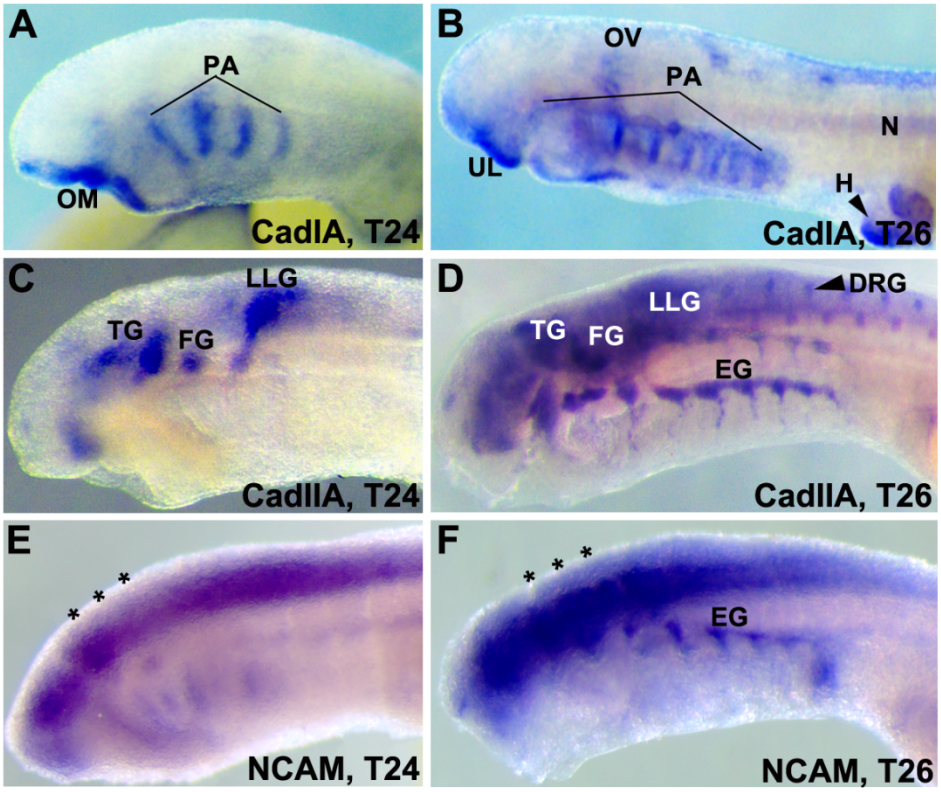
NT
S
N

Figure 3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B
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UL
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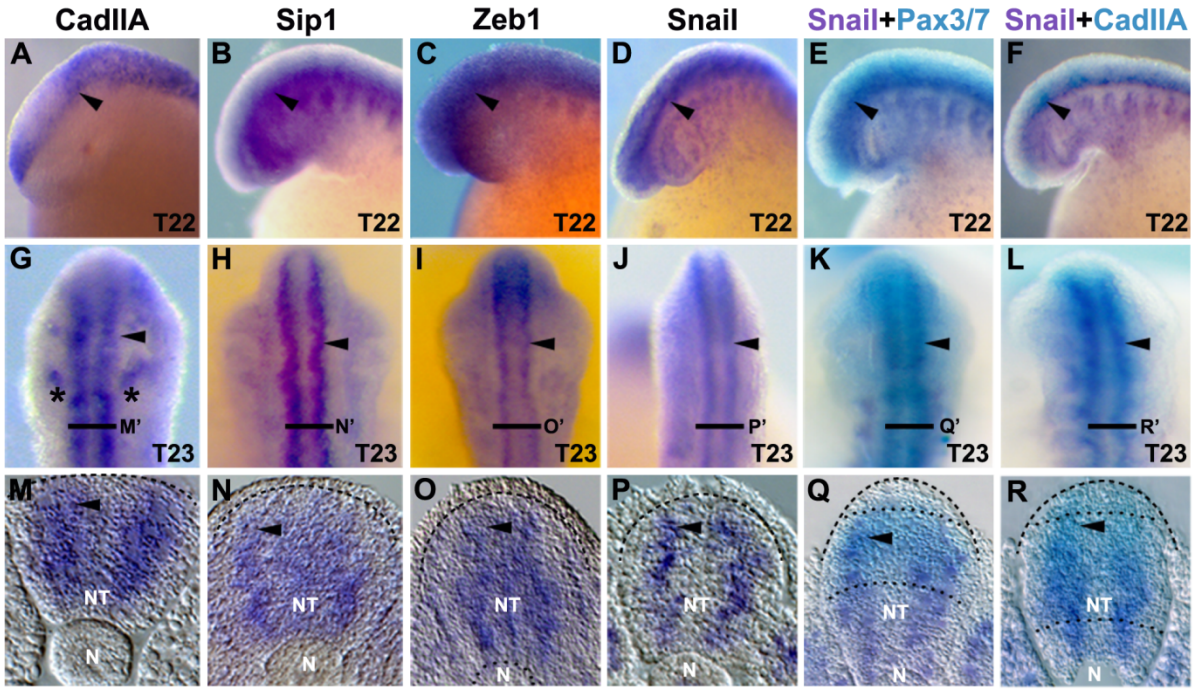
TG
FG
EG
CadIIA, T26
E
NCAM, T24
F
EG
NCAM, T26

Figure 4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Zeb1
Snail
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Snail+CadIIA
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C
D
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H
I
J
K
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T23
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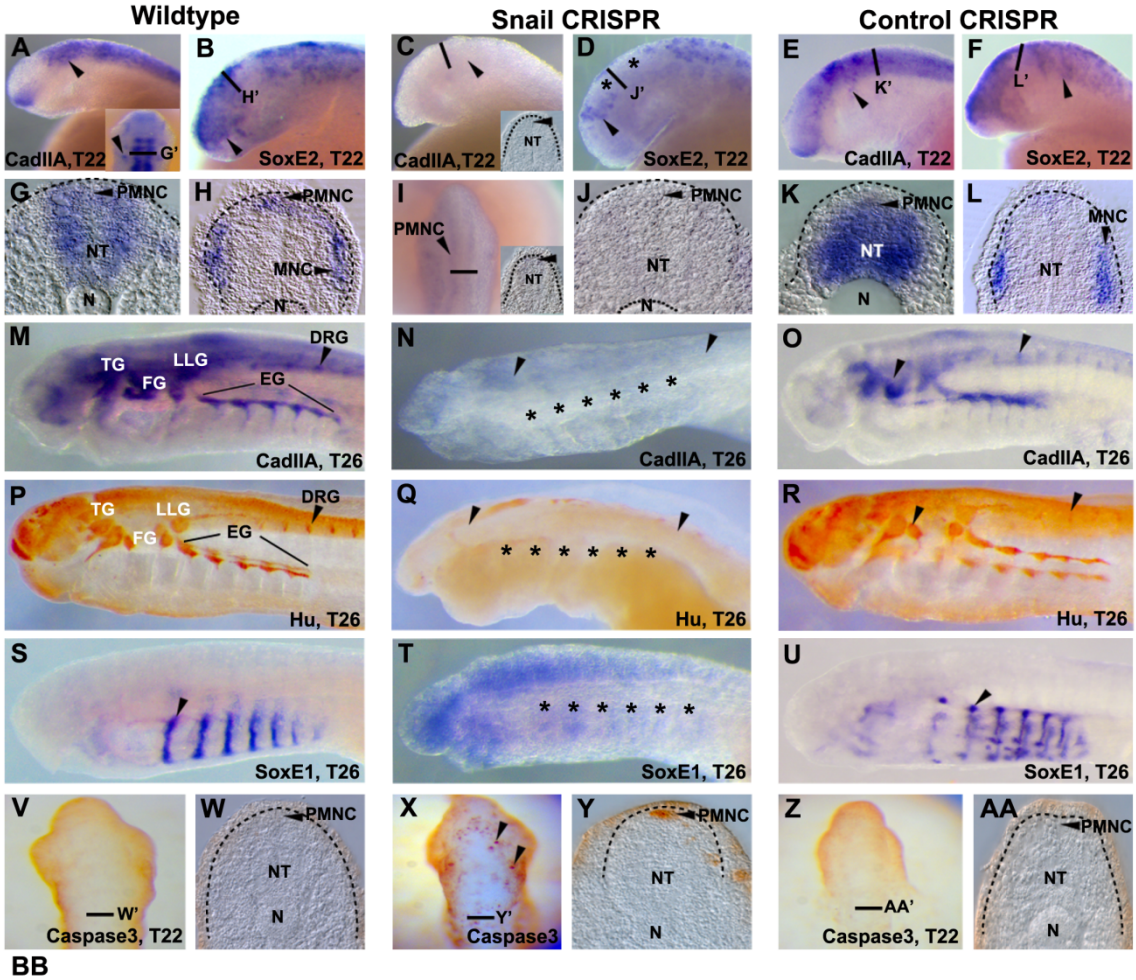
O
P
Q
R
NT
N

Figure 5



BB

Snail	Wildtype	ACGTCCAACCGAGGGTCCAGTGCACAAGGTGCGGGACTCGTTCCTG	
	Panel C	ACGTCCAACCG----------TTCCTG	-30
	Panel I	ACGTCCAACCGAGGGTCCAGTG----------TCCTGG	-19
	Panel D	ACGTCCAACCGAGGGT----------CTCGTTCCTG	-21
	Panel N	ACGTCCAACCGAGGGTCCAGTGCACAAGGT--GGGGACTCGTTCCTG	-2
	Panel Q	ACGTCCAACCGAGGG-----TGCACAAGGTGCGGGGACTCGTTCCTG	-5
	Panel T	ACGTCCAACCG----------TTCCTG	-30
	Panel X	ACGTCCAACCGAGGGTCCAGT----------GGGGACTCGTTCCTG	-11
Control	Wildtype	AAAGCCGTGCGGCGCCTCGACGGGAATCTTAGGAGGCGGAGGACTTG	
	Panel E	AAAGCCGTGCGGCGCCT----------GGAGGCGGAGGACTTG	-15
	Panel F	AAAGCCGTGCGGCGCCTCGA----------TTG	-24
	Panel O	AAAG----------GGACTTG	-36
	Panel R	AAAGCCGTGCGGCGCCTCGAC----------ACTTG	-21
	Panel U	AAAGCCGTGCGGCGCCTCGACGGGAA----------GAGGACTTG	-12
	Panel Z	A----------TAGGAGGCGGAGGACTTG	-27

Figure 6

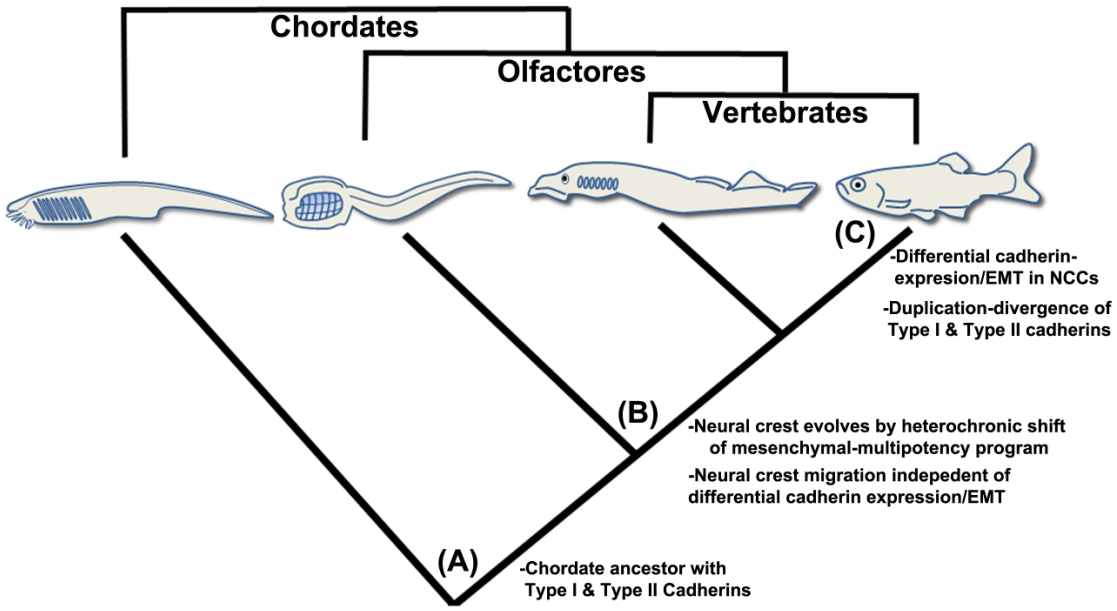

Chordates
Olfactores
Vertebrates
(C)
-Differential cadherin-expresion/EMT in NCCs
-Duplication-divergence of Type I & Type II cadherins
(B)
-Neural crest evolves by heterochronic shift of mesenchymal-multipotency program
-Neural crest migration indepedent of differential cadherin expression/EMT
(A)
-Chordate ancestor with Type I & Type II Cadherins

Figure S1

Pm NCAM

GCCAACCTCACCAACGTGATCGTGCACAGCTTCCCCGGGTTCAGCGTGCTGGAGATTACACCGG
AGTCGCAGTCTGACTTTGGAAGCTACAACTGCGTCGCCGTCAACAACATTGCGAGCGAGTCCAA
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Pm CadIA

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AACGACAATCCGCCCATTTTCGACCCGATCACCGGAACCGGATGTAGCAAGAAAGCTCGTACTG
ACGAGTCAATCACAGACATTACAACCGCTGCAGAATC

Pm CadIIA

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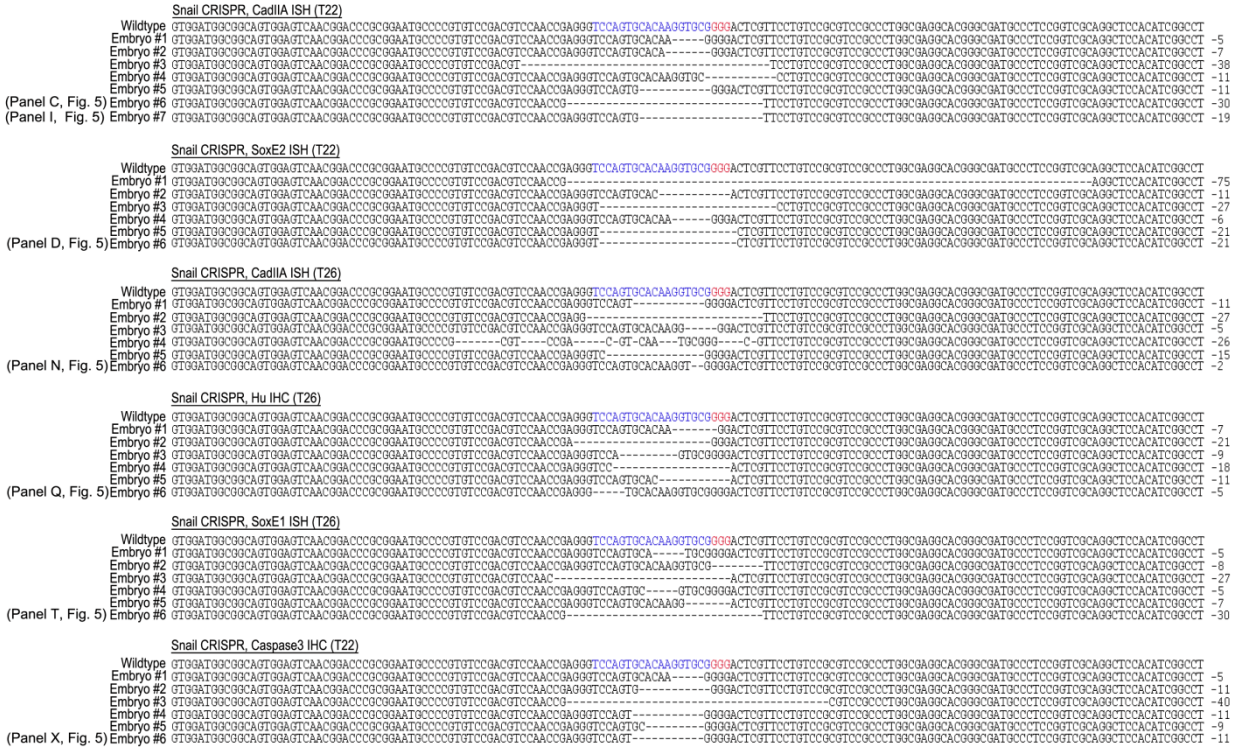
Figure S2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Snail CRISPR, SoxE2 ISH (T22)
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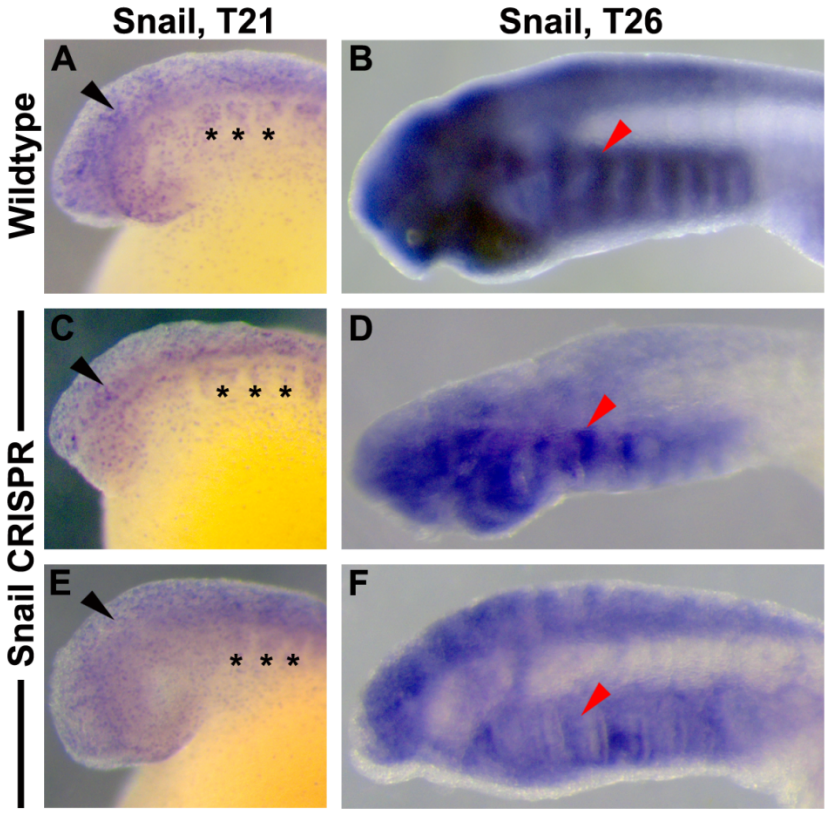
(Panel X, Fig. 5) Embryo #6 GTGGATGGCGGCAGTGGAGTCAACGGACCCGCGGAATGCCCCGTGTCCGACGTCCAACCGAGGGTCCAGT----------GGGGACTCGTTCCTGTCCGCGTCCGCCCTGGCGAGGCACGGGCGATGCCCTCCGGTCGCAGGCTCCACATCGGCCT -11

Figure S3



G

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Wildtype ACGTCCAACCGAGGGTCCAGTGCACAAGGT----GGACTCGTTCCTG
Panel C  ACGTCCAACCGA------------------------------CTG
Panel D  AC-------------------------GTGCGGGGACTCGTTCCTG
Panel E  ACGTCCAACCGAGGGTCCAGTGCACAAGGTGC---TT-TCGTTCCTG
Panel F  ACGTCCAACCGAGGGT------CACAAGGTGCGGGGACTCGTTCCTG
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CHAPTER 4: AN ANCESTRAL ROLE FOR SEMAPHORIN3F-NEUROPILIN SIGNALING IN PATTERNING NEURAL CREST WITHIN THE NEW VERTEBRATE HEAD

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ABSTRACT

The origin of the vertebrate head is one of the great unresolved issues in vertebrate evolutionary-developmental biology. Although many of the novelties in the vertebrate head and pharynx are derived from the neural crest, it is still unknown how early vertebrates patterned the neural crest within the ancestral body plan they inherited from invertebrate chordates. Here, using a basal vertebrate, the sea lamprey, we show that homologues of Semaphorin3F ligand and its Neuropilin receptors show complementary and dynamic patterns of expression that correlate with key periods of neural crest development (migration, patterning of cranial neural crest-derived structures). Using CRISPR/Cas9-mediated mutagenesis, we demonstrate that lamprey Sema3F is essential for patterning of neural crest-derived melanocytes, cranial ganglia and the head skeleton, but is not required for neural crest migration or patterning of trunk neural crest derivatives. Based on comparisons with jawed vertebrates, our results suggest that the deployment of Nrp-Sema3F signalling, along with other intercellular guidance cues, was pivotal in allowing early vertebrates to organize and pattern cranial neural crest cells into many of the hallmark structures that define the vertebrate head.

INTRODUCTION

A key event in early vertebrate evolution was the transition from a sessile, filter-feeding lifestyle to one of active predation (Gans and Northcutt, 1983). This event was driven in large part by a transformation of the ancestral chordate pharynx, resulting in the vertebrate "new head" (Forey and Janvier, 1994; Gans and Northcutt, 1983; Northcutt, 2005; Northcutt and Gans, 1983). The new vertebrate pharynx was muscularized and buttressed by a robust endoskeleton made of cellular cartilage, which in turn provided support and protection for a complex central nervous system and paired sensory organs, thereby facilitating a more active, predatory lifestyle (Donoghue and Keating, 2014; Graham, 2001; Square et al., 2016b). It was the integration and coordination of these traits that distinguished the first vertebrates morphologically and behaviorally from their closest relatives—the invertebrate chordates (Donoghue and Keating, 2014; Gans and Northcutt, 1983).

Much of the head skeleton and sensory organ systems of vertebrates are formed during embryonic development from the neural crest, a migratory and multipotent cell population unique to the vertebrate lineage (Donoghue et al., 2008; Hall, 2008; His, 1868; Le Douarin, 1999; Muñoz and Trainor, 2015; Santagati and Rijli, 2003). At the molecular level, neural crest cell development is controlled by a complex, integrated gene regulatory network (GRN) that progressively refines the developmental state of this cell type from early induction and specification to terminal differentiation (Betancur et al., 2010; Meulemans and Bronner-Fraser, 2004; Sauka-Spengler and Bronner-Fraser, 2008; Simões-Costa and Bronner, 2015). Under the control of this GRN, neural crest cells are specified in the dorsal-most part of the embryonic central nervous system, from which they detach and then migrate throughout the head and trunk

(Bronner, 2012; Clay and Halloran, 2010; Duband et al., 1995). After arriving at their destinations, neural crest cells differentiate into a wide array of cell types, including melanocytes, smooth muscle, neurons and glia of the peripheral sensory nervous system, as well as cartilage and bone that comprise the head and pharyngeal skeleton (Green et al., 2015). Although neural crest cells are a vertebrate innovation, the pharyngeal apparatus—where the neural crest builds much of the head skeleton and sensory systems—is not. The pharynx is in fact a general feature of deuterostome embryos, as is the gene regulatory network that orchestrates pharyngeal development (Gillis et al., 2012; Ou et al., 2012; Rychel et al., 2005; Veitch et al., 1999). Thus, the formation of the new vertebrate head required not just the origin of new cell types and gene regulatory networks, but also the integration and coordination of ancestral (pharynx development) and derived (neural crest development) developmental-genetic programs (Graham and Richardson, 2012; Veitch et al., 1999). Despite the significance of this event in early vertebrate evolution, the molecular mechanisms that coupled these two developmental processes are unknown.

In jawed (gnathostome) vertebrates, the migratory routes of neural crest cells and patterning of neural crest-derived structures in the head and pharynx are controlled in part by signalling interactions between receptors on neural crest cells and their corresponding ligands secreted from other cells into the extracellular environment (Gammill et al., 2007; Gammill et al., 2006; Krull et al., 1997b; Minoux and Rijli, 2010). Examples of such signalling systems include Robo-Slit, Eph-Ephrin, CXCR4-Sdf, and Neuropilin/Plexin-Semaphorin, each of which patterns neural crest cells by attraction, repulsion, or a combination of these two mechanisms (Theveneau and Mayor, 2012; Theveneau and Mayor, 2014). Although many of these signalling

systems are evolutionarily conserved between vertebrates and invertebrates, one particular group—the class III family of Semaphorin ligands (Sema3) and their Neuropilin receptors (Nrp1, Nrp2)—emerged and were duplicated within the vertebrate lineage (Yazdani and Terman, 2006). Thus, the deployment of Nrp/Sema3 signalling in vertebrates correlates with the appearance of neural crest cells and the new vertebrate head, suggesting a possible link between Nrp/Sema3-mediated neural crest patterning and the origin of vertebrate novelties, such as the head skeleton and sensory organ systems. This hypothesis is supported by the fact that, in jawed vertebrates, Sema3 (Sema3D, Sema3F) and Nrp (Nrp1, Nrp2) protein activity is necessary to organize migratory neural crest and pattern neural crest-derived cranial sensory ganglia and elements of the head and pharyngeal skeleton (Berndt and Halloran, 2006; Gammill et al., 2007; Gammill et al., 2006; Kulesa and Gammill, 2010; Yu and Moens, 2005). However, it is unknown if a key Nrp/Sema3 patterning function for neural crest is unique to the jawed vertebrate clade, or is instead a deeply conserved feature of neural crest biology that was also present in the first jawless (agnathan) vertebrates over 500 million years ago.

To distinguish between these possibilities, we examined the expression patterns of the Nrp/Sema3F signalling system, and the functional roles of Sema3F protein during neural crest development in a basal vertebrate, the sea lamprey (Petromyzon marinus). Lampreys are a group of jawless vertebrates that, along with hagfish, constitute the only extant members of the cyclostome ("agnathan") clade, which includes diverse fish-like forms that first appeared in the Paleozoic era (McCauley et al., 2015) and are the sister taxa of all other living vertebrates. Because they occupy the most basal phylogenetic position among extant vertebrates, and readily produce embryos that are amenable to experimental analysis, lampreys are ideal evolutionary

and developmental models to study the origin of vertebrate-specific traits (Green and Bronner, 2014; McCauley et al., 2015) through comparisons with developmental mechanisms in jawed vertebrates. Lampreys, like their jawed vertebrate relatives, have neural crest cells that migrate into the head and pharynx, following stereotyped routes (McCauley and Bronner-Fraser, 2003; York et al., 2017). Once at their targeted destinations, these neural crest cells are patterned into a pharyngeal and head skeleton made of cellular cartilage, as well as cranial sensory ganglia (Jandzik et al., 2014; Lakiza et al., 2011; McCauley and Bronner-Fraser, 2003; McCauley and Bronner-Fraser, 2006; Square et al., 2016b). Although these structures are presumed to be homologous to cranial cartilage and ganglia in jawed vertebrates (McCauley and Bronner-Fraser, 2006; Modrell et al., 2014), the molecular, cellular, and genetic mechanisms responsible for their patterning in jawless vertebrates are unknown.

Our results, together with the prior identification of lamprey Nrp and Sema3 genes (Sauka-Spengler et al., 2007; Shifman and Selzer, 2006; Shifman and Selzer, 2007) reveal that the lamprey genome, like that of many other vertebrates, encodes homologues of both Nrp1/2 and class III Semaphorins. Focusing on Nrp/Sema3F signalling, we show that Sema3F and its Nrp receptors (lamprey NrpA, NrpB, NrpC) show dynamic and complementary patterns of expression that correlate with key steps of neural crest development (migration; early and late patterning of pigment, cranial sensory neurons, head skeleton), similar to jawed vertebrates. Using CRISPR/Cas9-mediated genome editing we demonstrate that Sema3F signalling is not required for the segregation of migratory neural crest streams, but is essential for patterning of pigment, cranial sensory neurons and elements of the head and pharyngeal skeleton at multiple stages of development. Taken together, our results suggest that the deployment of Nrp receptors

and class III Semaphorins—a vertebrate-specific signalling system—allowed stem vertebrates to coordinate neural crest migration and differentiation programs, by acting as cellular guidance cues to pattern the cranial neural crest into vertebrate-specific novelties.

RESULTS

Molecular phylogenetics of vertebrate class III Semaphorins and Neuropilins

Previous research identified a Class III and Class IV Semaphorin, as well as Semaphorin receptors (Nrps) in lamprey (Shifman and Selzer, 2006; Shifman and Selzer, 2007). To investigate Nrp/Sema3 signalling activity during neural crest development in lamprey, we PCR amplified and cloned a 553 bp sequence of the gene previously identified as Semaphorin3. Our analysis of the sea lamprey genome uncovered additional Class III Semaphorins, (Fig. S1A). Phylogenetic analysis confirmed with strong support that the Sema3 sequence first identified (Shifman and Selzer, 2006; Shifman and Selzer, 2007) is a member of the Sema3F clade (Fig. S1A) and also confirms that another previously identified Sema3 in lamprey is likely a member of the Sema3D clade (Fig. S1A) (Sauka-Spengler et al., 2007). Our genomic searches also uncovered three lamprey homologues of vertebrate Nrp1 and Nrp2 receptors. Our phylogenetic analysis suggests that, similar to jawed vertebrates, lampreys have two Nrp paralogy groups (Fig. S1B). However, our analysis was unable to resolve strict paralogy of lamprey Nrps with either jawed vertebrate Nrp1 or Nrp2. We therefore named these groups NrpA and NrpB (Fig. S1B), with the two NrpA copies (NrpA1, NrpA2) likely originating from a lamprey-specific gene duplication event (Fig. S1B).

Lamprey Nrp-Sema3F expression correlates with patterning of cranial neural crest

As a first step toward understanding the contribution of Nrp/Sema3F signalling to neural crest development in basal vertebrates, we characterized their expression patterns throughout lamprey embryogenesis. In jawed vertebrates, early Sema3F expression occurs during neural crest migration, often localizing to neural crest-free zones of the forebrain, hindbrain and pharynx, with migratory neural crest showing complementary expression of Nrp1/2 receptors (Gammill et al., 2007). We found comparable expression patterns of Sema3F/Nrps during lamprey neural crest development (Figs. 1–7). In Tahara stage 22 (T22) lamprey embryos, neural crest cells are migrating (Sauka-Spengler et al., 2007; Square et al., 2016a; Tahara, 1988; York et al., 2017). At this time, Sema3F transcripts are enriched in forebrain and hindbrain, with expression appearing in the ectoderm and evaginating endodermal pouch near pharyngeal arch (pa) three (3, Fig. 1A; Fig. 1B). By T23, Sema3F expression weakens in the forebrain concomitant with expansion into pa three and four (3, 4 in Fig. 1C), with expression in the nascent endodermal pouch of pa4 (Fig. 1C; Fig. 1D). At early T24, post-migratory crest cells have colonized the pharynx and by late T24 gradually become restricted within each of the differentiating pharyngeal arches (McCauley and Bronner-Fraser, 2003). Our expression analysis at these stages shows that expanded Sema3F expression accompanies caudal differentiation of pharyngeal arches three through six, with expression gradually increasing within the pharyngeal ectoderm along the anteroposterior axis (arches 3–5, Fig. 1E, F, and arches 3–6 in Fig. 1G, H).

During T25/26 the lamprey pharynx elongates and neural crest cells within each pa gradually coalesce into dorsal-ventral stacks of prechondrocytes that prefigure the larval cartilage bars within pa3–9 (Martin et al., 2009; McCauley and Bronner-Fraser, 2003; McCauley

and Bronner-Fraser, 2006). Sema3F shows dynamic expression during these early patterning events of the head skeleton (Fig. 2). Continuing from pharyngeal ectodermal expression at late T24 (Fig. 1G, H), Sema3F mRNA by T25 expands into pharyngeal arches one through seven (1–7 in Fig. 2A), with expression in the pharyngeal ectoderm (Fig. 2B) and epithelium lining the oral cavity (Fig. 2C). The relatively broad ectodermal Sema3F expression accompanying the pharyngeal arches at T25 gives way to sharp expression boundaries within arches one through nine by T26 (1–9 in Fig. 2D). Sectioning of the T26 embryo in Fig. 2D revealed upregulated Sema3F expression in the rostral endoderm of each pharyngeal pouch, with expression weakening in the ectoderm (arrowheads and asterisk, Fig. 2E). This Sema3F expression pattern occurs just as neural crest cells in pa3–9 are patterned into SoxE1-positive dorsoventrally stacked rods of pharyngeal prechondrocytes (Cattell et al., 2011; McCauley and Bronner-Fraser, 2006). Double in situ hybridization for Sema3F and SoxE1 revealed Sema3F mRNA adjacent to SoxE1-positive prechondrocytes in the pharyngeal arches, suggesting that Sema3F expression may pattern the early lamprey head skeleton (Fig. 2F).

Downregulation of Sema3F in pharyngeal arches 3–9 by T27 (Fig. 2G), is followed by expression around the ventral mucocartilage—an elastin-like cartilage made of mesenchymal chondrocytes embedded in a loose extracellular matrix (mc, Fig. 2G). Mucocartilage is specific to jawless vertebrates and is the primary cartilage type in the velum (pa1+2), upper and lower lips, and floor of the pharynx (Johnels, 1948; Martin et al., 2009; Yao et al., 2011). From T28–late T30 Sema3F expression expands throughout the mucocartilage of the pharyngeal floor, pa1+2 and upper/lower lips (Fig. 2H–N).

After determining Sema3F expression in the lamprey head, we next characterized expression of the corresponding Nrp receptors to identify cell types that may respond to Sema3F signalling. Our expression analysis reveals that lamprey, similar to jawed vertebrates, expresses Nrp orthologues in cranial neural crest throughout head development in a pattern complementary to that of Sema3F expression (Figs. 3–7). At T22, NrpA1 and NrpA2 paralogues are expressed in migratory cranial neural crest colonizing the mouth and pharyngeal arches one through three, and in the cranial ectoderm (Fig. 3A–D). Similarly, from T23 through late T24, late-migrating and post-migratory neural crest cells expressing NrpA1 and NrpA2 have filled the lateral margin within the mesenchymal core of each of the differentiating pharyngeal arches (Fig. 3E–P). NrpB expression is similar to that of NrpA1 and NrpA2 in migratory cranial neural crest entering the anterior oropharynx and more posterior pharyngeal arches from T22 to early T24 (Fig. 4A–F), eventually occupying the mesenchymal core of each arch by late T24 (Fig. 4G, H). Continuing from late T24, NrpA1-positive neural crest at T25 still occupied the lateral mesenchymal core of each of pharyngeal arch (Fig. 5A, B) and the oral mesenchyme (Fig. 5C). By T26, a some NrpA1-expressing neural crest in the lateral mesenchymal core began to coalescing into the characteristic circular shape of pharyngeal prechondrocytes (Fig. 5D, E; compare Fig. 2F), and was expressed strongly in presumptive mucocartilage occupying the first pharyngeal arch and the lateral velar skeleton (Fig. 5F). From T27–T30, NrpA1 expression in the head skeleton is weakened with upregulation in other pharyngeal structures, including gill epithelium (Figs. 5G–N). Compared to NrpA1, the NrpA2 paralogue showed comparable patterns of expression in post-migratory neural crest throughout the head and pharynx at T25/26 (Fig. 6A–F), but was also expressed in the mesoderm within each arch (Fig. 6B, E). From T27–

T30, NrpA2 mRNA was lost from pharyngeal prechondrocytes (Fig. 6G–J), concomitant with upregulation in the ventral somitic and epibranchial mesoderm (Fig. 6H–J), as well as the neural crest-derived hypobranchial bars (Fig. 6I, J) and larval gills (Fig. 6K–N). Lamprey NrpB expression during later stages of head development was similar to that of NrpA1, with transcripts localizing to post-migratory crest cells in the mouth and lateral margin of the core of each pharyngeal arch at T25 (Fig. 7A–C), with a subset of these cells in each arch apparently contributing to nascent pharyngeal prechondrocytes at T26 (Fig. 7D, E), as well as elements of the velar skeleton, pa1 and pa2 (Fig. 7F). In late embryos and early larvae (T27–late T30), NrpB expression is maintained in the oropharynx and upper lip (Fig. 7G, H, K, N), whereas expression in the neural crest-derived pharyngeal cartilage bars in the posterior pharynx from earlier stages is replaced with mesodermal expression in the ventral somites (Fig. 7G–J) as well as epibranchial mesodermal mesenchyme that contributes to pharyngeal muscle fibers (Fig. 7K–N). In summary, our expression analysis of lamprey Sema3F and Nrps shows that lamprey Nrp receptors occur on migratory cranial neural crest cells colonizing the early embryonic head (T22–T24) and maintain expression in post-migratory neural crest that will be patterned into elements of the head skeleton (T25–T26). At the same time, complementary expression of Sema3F ligand emanates from the adjacent pharyngeal ectoderm and endoderm, in patterns that are spatially and temporally dynamic. From late embryonic stages (T27–T28) into early larval development (T28–T30), Nrp/Sema3F expression is gradually lost from much of the neural crest-derived pharyngeal cartilage bars and is upregulated in cranial mesoderm and mucocartilage.

Lamprey Sema3F is not required for migration of cranial neural crest

Our results show that pharyngeal expression of Nrps and Sema3F commences during neural crest migration and early colonization of the pharynx (~T23–24, Figs. 1, 3, 4). This is followed by pharyngeal arch expression that is suggestive of a role in patterning cartilage bars of the head skeleton in pa3–9 (~T25–26, Fig. 2A–F, Fig. 5A–F, Fig. 6A–F, Fig. 7A–F). Finally, Sema3F and Nrp expression occurs in mucocartilage, (i.e., pa1+2, floor of the pharynx) and the hypobranchial bars of the pharynx (~T27–30, Fig. 2G–N, Fig. 6H–J). Since spatiotemporal differences in Sema3F expression parallel early colonization of the pharynx by Nrp-positive neural crest, formation of cartilage bars in pa3–9, and formation of mucocartilage in pa1+2, we asked if Sema3F signalling is required for each of these processes. To test the functional role of Sema3F during neural crest development, we used CRISPR/Cas9-mediated genome editing, as described in lamprey (Square et al., 2015; York et al., 2017; Zu et al., 2016).

In jawed vertebrates, migratory neural crest cells express transcription factors such as Sox10 and nMyc, among others, and are segregated into three migratory streams (Sauka-Spengler and Bronner-Fraser, 2006; Sauka-Spengler and Bronner-Fraser, 2008; Wakamatsu et al., 1997). The division of these streams is enforced in part by repellent Sema3F signalling in neural crest-free zones of the head, and functional loss of Sema3F activity results in their inappropriate mixing, which can lead to abnormal patterning of neural crest-derived structures (Gammill et al., 2007; Gammill et al., 2006; Kulesa et al., 2010; Kulesa and Gammill, 2010). Similar to jawed vertebrates, lamprey cranial neural crest cells migrate in three streams and express homologues of Sox10 (lamprey SoxE2) and n-Myc (Lakiza et al., 2011; McCauley and Bronner-Fraser, 2003; Sauka-Spengler et al., 2007), raising the possibility that Sema3F may also

function in lamprey to segregate migratory cranial neural crest. However, in contrast to jawed vertebrates, we found that in Sema3F mutant embryos (n=20/20), nMyc+ and SoxE2+ neural crest cells still migrated in three distinct streams (Fig. 8B, D). These migratory patterns were similar to that in negative control embryos (Fig. 8A, C), suggesting the lack of a prominent role for Sema3F in regulating neural crest migration during early development. See Fig. S3 for individual Sema3F mutant genotype sequences.

Sema3F signalling is essential for early patterning of cranial neural crest derivatives

Despite unperturbed patterning of neural crest migration at stage T22/T23 in Sema3F CRISPR mutants (Fig. 8B, D), we observed inappropriate patterning of cranial neural crest derivatives in older mutant embryos (Figs. 9, 10; see Fig. S3 for mutant genotypes). At T26, we observed differentiated neural crest-derived melanocytes in mutants, but these embryos (n=8/10) failed to properly position melanocytes in the anterior head and along the dorsal pharynx (Fig. 9A, B). Mutant embryos (n=7/10) also had severely disorganized cranial ganglia, with apparent fusion and/or uncondensed ganglionic neurons compared to controls (Fig. 9C, D). Vertebrate cranial sensory ganglia, including those of lamprey, are thought to be derived from both neural crest and ectodermal placode cell populations (Modrell et al., 2014; Schlosser, 2005). Because of this, and based on ectodermal expression of Sema3F and Nrps in lamprey (Figs. 1–7), we investigated the gene expression profiles of the placode-specific cranial ganglion markers Pax3/7 and Six1/2, which mark the ophthalmic portion of the trigeminal ganglion (OpV; Pax3/7) and petrosal and posterior lateral line ganglia (pet, pLGG; Six1/2), respectively (Modrell et al., 2014; Schlosser, 2005; Zou et al., 2004). Our results show that OpV ganglia in Sema3F mutants appeared smaller,

with reduced expression of Pax3/7 (Fig. S2A, B). Moreover, Six1/2-positive pLLG and pet ganglia could not be discriminated from each other in mutants, suggesting they may have formed as a single fused ganglion (Fig. S2C, D). These results suggest that proper patterning of non-ectomesenchymal cranial neural crest derivatives, as well as cranial sensory placodes, may be dependent on Sema3F/Nrp signalling.

Next, we focused on the possible patterning functions of Sema3F/Nrp signalling in neural crest cells during early head skeleton development in lamprey. The SoxE and Twist families of transcription factors are widely recognized for controlling specification of neural crest cells, but are also known to govern patterning of neural crest-derived elements of the head skeleton in vertebrate embryos (Carl et al., 1999; Cattell et al., 2011; Cheung and Briscoe, 2003; McCauley, 2008; Soo et al., 2002). Similar to jawed vertebrates, lamprey homologs of these genes (SoxE1, SoxE3, TwistA) are also expressed during early development of the head skeleton and are required for development of cellular cartilage (T25/26) (Lakiza et al., 2011; McCauley and Bronner-Fraser, 2006; Sauka-Spengler et al., 2007). In particular, SoxE1 expression largely occurs in pharyngeal prechondrocytes in pa3–9 (Cattell et al., 2011; McCauley and Bronner-Fraser, 2006), whereas SoxE3 and TwistA transcripts mark prechondrocytes in all PAs, including mucocartilage elements in pa1+2 and upper and lower lips (McCauley and Bronner-Fraser, 2006; Sauka-Spengler et al., 2007; Zhang et al., 2006) (see also Fig. 10A–F). As expected from our results above (Fig. 8), we found that CRISPR targeting of Sema3F did not prevent SoxE1+, SoxE3+ or TwistA+ cells from migrating into the pharynx (Fig. 10). However, the cells expressing these genes did not become organized into serially repeating stacks of prechondrocytes in pa3–9 compared to control embryos (SoxE1, n=18/20, Fig. 10A, G; SoxE3,

n=16/20, Fig. 10B, H; TwistA, n=15/20; Fig. 10C, I). Sectioning of these Sema3F CRISPR mutants revealed that, in some cases, the pharyngeal endoderm had failed to evaginate properly and did not contact the ectoderm laterally (asterisks in Fig. 10J–L, compare to Fig. 10D–F), leading to SoxE1, SoxE3 and Twist-expressing cells apparently able to cross pharyngeal arch boundaries along the anteropoterior axis (arrowheads, Fig. 10J–L), a result similar to that of previous work in lamprey suggesting that proper chondrogenesis and patterning of cartilage precursors may be dependent on proper pharyngeal pouch formation (Jandzik et al., 2014). However, even in pharyngeal arches that had outpocketed completely or nearly so, we still observed post-migratory neural crest cells that had failed to completely condense into pharyngeal prechondrocytes with sharp boundaries (arrows in Fig. 10J, K, L), suggesting that proper patterning of pharyngeal prechondrocytes in Sema3F mutants may involve an indirect effect on pharyngeal pouch morphogenesis, but also a specific neural crest patterning function upon proper pouch formation. We also observed that Sema3F mutant embryos had patterning defects in pa1+2, which will form the mucocartilage-based elements of the mouth and velar skeleton. We observed failure of proper mouth development in embryos, including improperly patterned SoxE3+ and TwistA+ cartilage elements in pa1+2 (compare pa1+2 outline in Fig. 10A–C with asterisks in Fig. 10 G–I). Taken together, these results highlight an important role for Sema3F signalling in patterning the neural crest-derived head skeleton in lamprey embryos.

Sema3F signalling is essential for long-term patterning of pigment, cranial sensory ganglia and cartilage elements of the head skeleton

Finally, given that we observed early defects in the patterning of cranial neural crest-derived melanocytes, cranial sensory ganglia and prechondrocytes during earlier embryonic stages in Sema3F CRISPR mutants (T26, Figs. 9, 10), we asked if these phenotypes persisted into ammocoete larval stages (T30), or were perhaps corrected later in development by a compensatory mechanism. We found that, compared to control larvae, CRISPR/Cas9-mediated knockout of Sema3F did not prevent differentiation of melanocytes, yet most mutant embryos (n=9/10) had melanocytes that appeared to be scattered randomly throughout the head and failed to become properly patterned into a segmental organization in register with each of the pharyngeal arches (compare Fig. 11A, F; see Fig. S3 for mutant genotypes). Moreover, in two embryos, in addition to the apparent random location of melanocytes, these cells were small with a stellate appearance and lacked the dendritic appearance of melanocytes in control embryos (compare Fig. 11B, G).

Sema3F mutant larvae also possessed mature cranial sensory ganglia, yet the ganglia in most larvae (n=11/12) were malformed compared to controls (compare Fig. 11C, D with H, I) as we also observed earlier at stage T26 (Fig. 9). These patterning defects included misshapen ganglia (e.g., posterior lateral line ganglion (pllg), Fig. 11I), and unidentifiable ganglionic protrusions (? in Fig. 11I), to apparent fusion of multiple ganglionic clusters (e.g., maxillomandibular (mmV) + geniculate (g) + vestibuloacoustic (va), Fig. 11I). In some cases, we noted individual ganglia appeared reduced in size (e.g., petrosal ganglion (pet), Fig. 11I), but these effects varied among mutant embryos. In contrast to defects in cranial sensory ganglia, however, these embryos (n=12/12) had normally patterned trunk neural crest-derived dorsal root ganglia and enteric neurons (compare Fig. 11E, J; see Fig. S3 for mutant genotypes).

Among approximately 30 Sema3F mutants that survived to stage 30, larvae developed cellular cartilage of the head and pharyngeal skeleton, including the "stack-of-coin" cartilage bars in pharyngeal arches three through nine, as well as mucocartilage elements in arches one and two and the upper and lower lips (Fig. 12). However, there were consistent and moderate to severe patterning defects in each of these skeletal elements. In all larvae examined (n=15/15 analyzed), the mucocartilage of pharyngeal arches one and two failed to properly condense into a velum, the oral skeletal element that functions in agnathan respiration (compare Fig. 12A control with Sema3F mutants shown in D and G). Rather, these larvae had loosely arranged mucocartilage and alcian blue-positive cellular debris scattered throughout the head and mouth (arrowheads in Fig. 12D, G; see Fig. S3 for mutant genotypes). There were abnormalities also in the cartilage bars in pharyngeal arches three through nine, with the most frequently observed phenotype consisting of severely bent or disjointed cartilage bars (n=13/15) (compare Fig. 12B, E, H, and insets C, F, I). These larvae also had ectopic clusters of fused cartilage nodules from adjacent bars (arrowheads in Fig. 12F, I), or disconnected bars that were not fused together (asterisks in Fig. 12F, I).

Although our gene expression analyses suggest that early patterning of the head skeleton (T25/26) appears to be severely disrupted in Sema3F CRISPR mutants (Fig. 10G–L), the larvae we examined at T30 still displayed some evidence of identifiable dorsal-ventral and anteroposterior patterning of cartilage bars in pa3–9 that allowed us to putatively assign some of their identities (Fig. 12E, F, H, I). We attribute this difference in the severity of early versus late stage phenotypes to lethality effects we observed shortly after T26: approximately 98% of 5000 injected embryos survived to T26 (15 days post fertilization), yet only approximately 30 larvae

(0.6%) had survived to T30 (30 days post fertilization). We speculate that this sharp increase in mortality may be attributed to the fact that embryos with the most severely disrupted pharyngeal development during early head skeleton patterning were unable to properly pattern their oropharyngeal skeleton, which is required for ventilation and survival at later larval stages. Thus, larvae that survived to T30 were those that showed only moderate disruption of Sema3F function and pharyngeal development, which allowed for their subsequent examination.

DISCUSSION

The new head hypothesis proposes that the origin of vertebrates was catalyzed by a series of evolutionary modifications to the chordate head region (Gans and Northcutt, 1983). These modifications included a muscularized, pumping pharynx that was supported by a rigid cellular skeleton, as well as a peripheral nervous system containing elaborate, paired sensory organs (Donoghue and Keating, 2014; Gans and Northcutt, 1983). Each of these innovations was made possible by the acquisition of the neural crest, and therefore one of the primary aims in vertebrate evolutionary-developmental biology has been to dissect the molecular, cellular, and genetic origins of neural crest cells. However, since the neural crest and its underlying gene regulatory network first evolved, it has remained unclear how this new cell population became integrated developmentally into the ancestral chordate body plan and acquired the ability to construct the novelties that define the vertebrate head and pharynx.

Our gene expression and CRISPR/Cas9 functional analyses together suggest that the ability of neural crest cells to become integrated and assembled into distinct structures organized along the anteroposterior axis (e.g., pharyngeal skeleton, cranial ganglia) within the head of

lamprey embryos is driven in part by the deployment of Nrp-Sema3F signalling, an intercellular signalling system that originated prior to the divergence of jawless and jawed vertebrates over 500 million years ago. In jawed vertebrates, Sema3 proteins, especially Sema3F, function to repel migratory and post-migratory neural crest cells that express complementary Nrp1 and Nrp2 receptors, thereby positioning groups of neural crest to differentiate into specific derivatives along the embryonic anteroposterior axis (Gammill et al., 2007). Our results in lamprey, a basal jawless vertebrate, suggest that these complementary patterns of Nrp and Sema3F expression are strikingly similar to jawed vertebrates and correlate with key stages of cranial neural crest patterning events. Moreover, our CRISPR/Cas9 functional experiments revealed a critical role for Nrp/Sema3F-mediated patterning of multiple neural crest-derived structures in the vertebrate head, including the craniofacial skeleton and sensory ganglia, and suggest that Nrp-Sema3F signalling is a deeply conserved function that ancestral vertebrates used to pattern cranial neural crest cells. In light of our findings in lamprey and comparisons with jawed vertebrates, we propose that deployment of intercellular guidance cues such as Class III Semaphorins, along with their corresponding Neuropilin receptors, was instrumental in organizing neural crest cells for the first time into derivatives in the vertebrate head by assuming a role in patterning events inserted temporally between earlier (neural crest specification) and later (neural crest differentiation) steps of neural crest development.

Once the core structural components of the neural crest (head skeleton, cranial sensory ganglia) were fixed in early vertebrates, changes in the spatial and temporal patterns of intercellular signalling and patterning systems via Nrp/Sema3F and others could have enabled further modifications to these and other neural crest-derived structures in the vertebrate head. For

example, the anterior-most pharyngeal arches in early jawless vertebrates and modern lampreys are differentiated into a velum—a cartilaginous skeletal element in the agnathan oropharynx that functions in respiration (Forey, 1995; Mallatt, 1984; Mallatt, 1997; Miyashita, 2016; Square et al., 2016b; Yasui and Kaji, 2008). Our CRISPR/Cas9-mediated knockout results show that condensation and patterning of the lamprey velum requires proper Nrp/Sema3F signalling, suggesting that modifications to the early vertebrate oropharyngeal skeleton may have been mediated at least in part by Nrp/Sema3F signalling. In higher jawed vertebrates, the ancestrally homonomous series of neural crest-derived pharyngeal cartilage bars was gradually transformed into a series of individuated structures, including jaws and hyoid as well as elements of the inner ear and facial skeleton (Gegenbaur, 1878; Kardong, 2002; Kuratani, 2004; Mallatt, 2008; McCauley and Bronner-Fraser, 2006; Romer, 1950; Shigetani et al., 2005). These changes were driven primarily by spatial repositioning of the ancestral pharyngeal structure and new cell-mesenchyme interactions, rather than the origin of new cell types (Dupret et al., 2014; Gegenbaur, 1878; Gillis et al., 2013; Kardong, 2002; Shigetani et al., 2002). Similarly, our functional results in lamprey suggest that rearrangement and novel patterning of the pharyngeal and head skeleton, along with other novelties throughout vertebrate evolution (Noguchi et al., 2017), could have been achieved in part by altering the spatial and temporal activity of a combinatorial repulsion-guidance code of signalling molecules involving Sema3F, among others, for neural crest cells in the head of vertebrate embryos.

Although our findings in lamprey implicate an ancient role for Nrp/Sema3F signalling in patterning neural crest-derived structures in the vertebrate head (cartilage, sensory neurons, pigment), we also observed important differences compared to jawed vertebrates. In mouse and

chicken embryos, for example, Sema3F functions early in neural crest development to enforce the segregation of the three primary cranial neural crest streams, which express Nrp1/2 receptors (Gammill et al., 2007; Kulesa et al., 2010). Functional perturbation of Sema3F activity results in intermingling or complete fusion of these streams, leading to inappropriate patterning of cranial ganglia and the pharyngeal skeleton (Gammill et al., 2007; Gammill et al., 2006; Roffers-Agarwal and Gammill, 2009). In contrast, lamprey does not appear to use Nrp/Sema3F signalling to pattern or segregate migratory crest, and it instead functions primarily in the structural organization of neural crest derivatives, especially craniofacial cartilage and sensory ganglia. This suggests a possible alternative signalling mechanism that mediates segregation of cranial neural crest streams in jawless vertebrates, although the exact guidance cues are unknown. Alternatively, the lack of a Sema3F mutant phenotype for neural crest migration may reflect the relaxation of migratory constraints in the lamprey head as previously described (McCauley and Bronner-Fraser, 2003). In addition to early patterning of migratory crest, Nrp/Sema3F signalling in jawed vertebrates is also critical for patterning trunk neural crest derivatives such as dorsal root ganglia (DRG) (Gammill et al., 2007; Gammill et al., 2006). Like jawed vertebrates, lampreys also have dorsal root ganglia arranged along the trunk in a segmental pattern, and recent work shows that lamprey has a population of trunk neural crest-derived enteric neurons (Green et al., 2017). Both DRGs and enteric neurons form in stereotypical positions in the lamprey trunk and therefore are presumably patterned using intercellular signalling cues (Green et al., 2017). However, Nrp/Sema3F signalling appears to be dispensable for patterning of the trunk neural crest subpopulation in lamprey. This suggests the operation of patterning cues for neural crest in the lamprey trunk that are distinct from those in the head, a situation that also

occurs in jawed vertebrates (Krull et al., 1997a; Kulesa et al., 2010; Robinson et al., 1997). A comprehensive comparative analysis of a wider repertoire of neural crest patterning mechanisms in lamprey and hagfish, another agnathan group, may help address whether ancestral vertebrates patterned cranial versus trunk neural crest subpopulations using distinct or overlapping intercellular signalling mechanisms.

Although bona fide migratory neural crest cells and the structures that they form are vertebrate innovations, there is compelling evidence that the closest extant relatives of vertebrates—the invertebrate chordates—possess "proto-neural crest cells" that have a similar gene regulatory profile and can give rise to similar cell types such as sensory neurons and melanocytes (Abitua et al., 2012; Stolfi et al., 2015). Although some of these cells can migrate endogenously over a short distance, long range migration is only possible when neural crest transcription factors such as Twist are forcibly expressed (Abitua et al., 2012). These migratory cells colonize the pharynx as ectomesenchyme (Abitua et al., 2012), but there has been no gene expression or functional analysis of the contribution of receptor-ligand guidance cues in these cells. We hypothesize that the molecular deployment of Nrp/Sema3 signalling in the head of early vertebrates, in conjunction with co-option of other guidance and repulsion cues into a combinatorial receptor-ligand patterning "code", was an important step that allowed stem vertebrates to organize neural crest cells for the first time into many of the hallmark traits that define the new vertebrate head. It would therefore be interesting to determine if and how migratory neural crest-like cells in invertebrate chordates deploy intercellular patterning systems.

MATERIALS AND METHODS

Embryo collection

To collect embryos, gravid adult sea lampreys (Petromyzon marinus) were obtained from the Hammond Bay Biological Station, Millersburg, MI, and shipped to the University of Oklahoma. Adults were housed at 14° C in a recirculating water system. Eggs were stripped manually from gravid females into a beaker of water (~200ml) and mixed with sperm expressed from a male directly onto the eggs. Embryos were reared in small Pyrex dishes under constant flow in deionized water supplemented with 0.05X Marc's Modified Ringers solution (MMR) chilled to 18°C. All procedures involving adult lampreys were performed with approval from the University of Oklahoma Institutional Animal Care and Use Committee (IACUC, R15-027).

Molecular Phylogenetics

To determine sequence orthology of lamprey group 3 Semaphorins (Sema) and Neuropilin (Nrp) receptors, we constructed neighbor joining phylogenetic trees, using gnathostome group 7 Semaphorin and Neuropilin and Tolloid-Like (NETO) genes as outgroups, respectively. Untrimmed sequences were aligned in MEGA version 7.0 using MUSCLE, and a JTT+G model for protein evolution was chosen for phylogeny reconstruction (Kumar et al., 2016). Results were obtained after 1000 parametric bootstrap replicates. Gene sequences analyzed and corresponding accession numbers (in parentheses) included: Dr, Danio rerio (Sema3B: NP_001121818.1; Sema3C: XP_017210807.2; Sema3D: AAI62510.1; Sema3Fa: AAI63764.1; Sema3Fb: AAW56082.1; Nrp1a: AAI63888.1; Nrp2: NP_998130.1); Gg, Gallus, gallus (Sema3A: NP_990308.2; Sema3C: NP_989574.1; Sema3D: NP_990704.1; Sema3E: NP_989573.1;

Sema3F: NP_989589.1; Sema3G: XP_015148335; Sema7A: NP_001186678.1; Nrp1: NP_990113.1; Nrp2: NP_989615.1); Mm, Mus musculus (Sema3A: AAH90844.1; Sema3B: AAH90669.1; Sema3C: NP_038685.3; Sema3D: NP_083158.3; Sema3E: NP_035478.2; Sema3F: AAH10976.1; Sema3G: NP_001020550.1; Nrp1: AAH51447.1; NETO1: EDL09346.1); Pm, Petromyzon marinus (Sema3F: AAU94360.1 ; all other putative lamprey Semaphorin and Neuropilin sequences were obtained from manual searches of the 2010 version of the sea lamprey genome assembly); Rn, Rattus norvegicus (Nrp2: NP_110496.1); Xt, Xenopus tropicalis (Sema3A: AAK38166.1; Nrp2: AAI36102.1); Xl, Xenopus laevis (Sema3B: AAI66183.1; Sema3D: NP_001087589.1; Sema3F: NP_001011157.1; Nrp1: NP_001081380.1; NETO2: NP_001072912.1).

Gene cloning, in situ hybridization, immunostaining and alcian blue staining

Partial clones for Sema3F (553bp), NrpA1 (670 bp), NrpA2 (550 bp), NrpB (584 bp), Six1/2 (706 bp) were isolated by direct amplification from a sea lamprey cDNA library (primers: Sema3F forward: 5′-CCACGGAATCTGGCAACCAGAA-3′; Sema3F reverse: 5′-GCGATGCGCGTGAACTTGTA-3′; NrpA1 forward: 5′-CTGAGATTGTCCTGCGATTCCAC-3', NrpA1 reverse: 5′-CGCACGAACCGCGTCAGCAC-3'; NrpA2 forward: 5′-ATGCTCGCACATGTTCACAGC-3', NrpA2 reverse: 5'-CGGATCATCTCTGCTGGGCG-3' ; NrpB forward: 5′-GGATCCTCTCGCTCTCCTTC-3', NrpB reverse: 5′-GGAGATGTGACAGCCGTAGA-3' ; Six1/2 forward: 5′-TCCACAAGAACGAGAGCGTG-3' , Six1/2 reverse: 5′-TGCTGAGACATGTGGCTCTG-3') (kindly provided by J. Langeland), ligated into a pGEM-T-easy vector and sequenced. All other clones (SoxE1, SoxE2, SoxE3,

nMyc, TwistA, Pax3/7) were isolated from previous cDNA library screenings (McCauley and Bronner-Fraser, 2006; Sauka-Spengler et al., 2007). Clones were then used to generate antisense riboprobes for single or double chromogenic in situ hybridization as previously described (York et al., 2017). To visualize differentiated cellular cartilage, alcian blue staining was performed as previously described (Martin et al., 2009). For Hu immunostaining, the primary antibody (Hu C/D, mouse IgG2b; Invitrogen) was diluted (1:300) in 10% sheep serum, and detected using either goat anti-mouse IgG conjugated to horseradish peroxidase followed by DAB staining or Alexa544-conjugated goat anti-rabbit IgG (1:300).

CRISPR/Cas9 Experiments

For all CRISPR experiments, lamprey zygotes were microinjected prior to first cleavage (~5 nl) with 1 ng-ul-1 Cas9 protein (PNA Bio), 500 pg guideRNA (gRNA) and 10% fluorescein dextran tracer in nuclease-free water. Approximately 5000 injected embryos were screened by fluorescence four days after injection and those lacking fluorescence were discarded. Injected embryos were reared to desired stages, fixed in 4% MEMFA, dehydrated and stored at -20ºC in 100% methanol.

a) Sema3F CRISPR experiments

To disrupt lamprey Sema3F function, we microinjected a guide RNA (gRNA) that efficiently and specifically (see Figs. S3–6) targets Sema3F genomic coding sequence (Sema3FgRNA1: 5′-GGAGCACCTTCCTGAAGGCCCGG-3′; protospacer adjacent motif (PAM) sequence is underlined). This gRNA construct was carefully selected to recognize only a single region of

Sema3F and to avoid off-target cleavage effects based on the following stringency criteria described previously for lamprey (Square et al., 2015; York et al., 2017): 50–80% GC content; targeted regions as close as possible to the presumptive start codon (or 5′ end of available genomic sequence); no potential non-specific/off-target hits to the known P. marinus genome that had greater than 80% similarity by BLAST analysis.

b) Control CRISPR experiments

To rule out the possibility that Sema3F mutant phenotypes result from a general artifact of Sema3F gRNA1 construct injection and/or toxicity, we performed negative control experiments in which we microinjected a gRNA with a "scrambled" sequence of nucleotides (5'-AATAAGTTGGGGTTTCCA-3') into zygotes from the same batch of eggs for which we performed our Sema3F CRISPR injections.

c) Genotyping of Sema3F Individual CRISPR Mutants

After immunostaining or in situ hybridization of putative Sema3F CRISPR mutants, we genotyped individual embryos from Figs. 8–12 and Fig. S2 (see Results) in order to directly link mutant genotypes to phenotypes. To ensure that tissue fixation or damage to genomic DNA during the in situ hybridization or immunostaining protocols did not generate "false positive" mutations in our sequencing reactions, we compared the sequences of putative mutant embryos to negative control CRISPR embryos that were also fixed and then assayed by in situ hybridization or immunostaining. To this end, following gene expression analyses and imaging, embryos were incubated 24–48h with 0.1 mg ml-1 proteinase K prior to extraction of genomic

DNA (Sive et al., 2000). Oligonucleotides (Sigma) flanking the Sema3F (forward: 5′-TCAATGTCACGAGTTGCAAG-3′; reverse: 5′- TTAATCGAATCGCTAGCTAG-3′) genomic CRISPR target site were used to PCR amplify and sequence 742 bp of the Sema3F genomic locus. For each individual embryo in which we performed this protocol, we sequenced four different clones to verify mutagenesis.

d) Efficiency of mutagenesis at the Sema3F locus

We estimated the general efficiency of CRISPR-Cas9-mediated mutagenesis of Sema3 gRNA1 at the Sema3F locus by pooling 5 randomly selected Sema3F gRNA1 CRISPR-injected embryos at ~Tahara stage 26 (~embryonic day 15), isolating genomic DNA per standard methods, PCR amplifying the targeted genomic locus (oligonucleotide sequences listed above in section "c"), and then sequencing 50 clones. Efficiency (%) of mutagenesis at the Sema3F target locus was then calculated by dividing the number of mutant genotypes by the total number of clones sequenced. We found that our Sema3F gRNA1 construct was in general highly efficient at inducing mutations at the targeted Sema3F locus in randomly selected embryos, with an estimated mutagenesis efficiency of 98% (Figs. S4). Graphical representation as a box and whisker plot (Fig. S5) of mutant genotypes obtained from individual and pooled embryos (Figs. S3, S4) was prepared in R (R Core Team, 2013) using the package 'ggplot2' (Wickham, 2010).

e) Genomic analysis of off-target CRISPR sites

Although our Sema3F gRNA construct was designed explicitly to minimize the potential for off-target cleavage (see "Sema3F CRISPR experiments" above), we nonetheless sought to verify

that our Sema3F mutant phenotypes are specific to cleavage at the Sema3F locus and are therefore not likely to be attributable to mutagenesis of other loci ("off-target" effects) by Sema3F gRNA1. To this end, we performed an in silico analysis in which we conducted BLAST searches of the Sema3F gRNA1 sequence against the 2010 version of the sea lamprey genome (https://genome.ucsc.edu/cgi-bin/hgGateway) (see similarly (Servetnick et al., 2017)). From our searches, the top five potential off-target genomic sites that had an intact PAM cleavage sequence (NGG) still had two or more mismatches in the 13 bp "seed sequence" proximal to the PAM site (see Table S1). It has been shown previously that two or more mismatches within the gRNA seed sequence are sufficient to inhibit Cas9-mediated cleavage at off-target sites (Hsu et al., 2013; Pattanayak et al., 2013). Therefore, these top potential off-target loci are not likely to be cleaved by Sema3F gRNA1. Nonetheless, to ensure that Sema3 gRNA1 did not cleave potential off-target sites, we isolated genomic DNA from the same five pooled Sema3F CRISPR injected embryos (Tahara stage 26) from which we calculated on-target mutagenesis efficiency (see "Efficiency of mutagenesis at the Sema3F locus" above) and sequenced 10 clones from the top three off-target regions (Hox3, g-variable lymphocyte receptor (gVLR), ABCB7, see Table S1). Our results suggested that our Sema3F gRNA was highly specific, with no evidence of a tendency to induce mutations at the top three potential off-target loci (Fig. S6). The following primers were used to amplify potential off-target regions: Hox3, forward: 5′-AGCAGGGTGCCTACAACATC-3′, reverse: 5′-GCTGTCCACGTATCCTCCTC-3′; gVLR, forward: 5′-CCGCTCACTACCAAACCATT-3′, reverse: 5′-AACATACGTTTTGGGGCAAG-3′; ABCD7, forward: 5'-GAGAGAGACGCAAGGAAGG-3', reverse: 5'-GGCTGAGTAGACCCAACTCG-3'.

Sectioning and Imaging

Whole mount embryos stained by in situ hybridization and immunohistochemistry were mounted in 75% glycerol and photographed on a Zeiss Discovery V12 stereomicroscope. For sectioning, selected embryos were embedded in 5% agarose and Vibratome sectioned (20 μm). Sections were mounted in 75% glycerol on a coverslipped glass slide and photographed on a Zeiss Axioimager Z1 compound microscope using Zeiss Axiovision software (v 4.7). Fluorescent visualization of alcian blue-stained lamprey cellular cartilage bars is described elsewhere (Martin et al., 2009). Image stacks of fluorescent lamprey cartilage and high magnification Hu immunostaining of cranial sensory ganglia were rendered as maximum intensity projections using the Inside 4D module of the Axiovision software package. All figures were assembled using Adobe Photoshop CS5.5.

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Off-target gene ID	Sema3F gRNA1 seed sequence (PAM)	Off-target accession#	Off-target seed sequence match (PAM)
Petromyzon marinus transcription factor Hox 3 (Hox3) gene, complete cds	CTTCCTGAAGGCC(NGG)	JQ706315.1	TCCTGAAGGCC(CGG)
Petromyzon marinus clone gVLR_Contig variable lymphocyte receptor (VLR) gene, partial sequence	CTTCCTGAAGGCC(NGG)	AY577941.1	TGAAGGCC(AGG)
Petromyzon marinus ABCB7 mRNA, complete cds	CTTCCTGAAGGCC(NGG)	KM232922.1	TGAAGGCC(CGG)
Petromyzon marinus ABCD2 mRNA, complete cds	CTTCCTGAAGGCC(NGG)	KM232938.1	TGAAGGCC(GGG)
Petromyzon marinus ABCC2 mRNA, complete cds	CTTCCTGAAGGCC(NGG)	KM232929.1	AGGCC(CGG)

Table S1. Summary of top 5 potential Sema3F gRNA1 CRISPR off-target sites obtained by BLAST analysis of the sea lamprey genome.

FIGURE LEGENDS

Fig 1. Early cranial expression of lamprey Sema3F. (A, B), At T22, Sema3F transcripts localize to the forebrain (fb), hindbrain (hb), and pharyngeal arch three (labeled "3") in the evaginating endoderm (arrowhead, B) and ectoderm (ect, B). This pattern appears again at T23 (C, D) in arches three and four (numbered 3, 4), as forebrain expression weakens (asterisk, C). (E–H), Throughout T24, as its expression still weakens in the forebrain (compare fb in A with asterisks in C, E, G), Sema3F expression expands throughout the pharyngeal ectoderm (ect, F, H) to more posterior pharyngeal arches (numbered 3–6 in E, G). Anterior facing left for all panels. Scale bars = 100 μm.

Fig 2. Cranial expression of Sema3F during head skeleton patterning. (A), At T25, Sema3F expression is segmental in the ectoderm of pharyngeal arches one through seven (1–7, A; ect, B) and in the stomadeal epithelium (st in C). By T26, Sema3F expression in the arches (1–9, D) resolves sharply to the anterior pharyngeal endoderm (arrowheads, E), with weakened ectodermal expression (asterisk, E), a pattern adjacent to neural crest-derived pharyngeal prechondrocytes expressing SoxE1 (arrows, F). (G), By T27, Sema3F expression is upregulated ventrally in mucocartilage (mc, arrow). (H–N), At T28, Sema3F expression remains in mucocartilage (mc, H, J), is lost from the pharyngeal cartilage bars (cb, J) and appears in mesenchyme around mucocartilage of arches one and two (pa1+2) and upper (ul) and lower lips (ll), a pattern continuing through T30 (K–N). Except for cross-sections in I, J, M, and N, all panels are oriented with anterior facing left. da, dorsal aorta. Scale bars = 100 μm.

Fig. 3. Early cranial expression of lamprey NrpA1 and NrpA2. (A–D), At T22, NrpA1 and NrpA2 are expressed in pharyngeal ectoderm (ect) and migratory cranial neural crest colonizing arches one through three (labeled 1–3, neural crest indicated by arrowheads), a pattern that continues into late T24 (E–P) as NrpA1-positive and NrpA2-positive neural crest cells colonize arches one through six (1–6, E–P). By late T24, both NrpA1 and NrpA2 expression occupies pharyngeal ectoderm (ect) and the mesenchymal core of each arch (M–P). All panels are oriented with anterior facing left. Scale bars = 100 μm.

Fig. 4. Early cranial expression of lamprey NrpB. (A), Migratory cranial neural crest expressing NrpB enters the pharynx in pharyngeal arches one through three (1–3, A), colonizing the mesenchymal core of nascent pharyngeal arches (arrowheads, B). NrpB expression also occurs in pharyngeal ectoderm (ect, B). This pattern of NrpB-positive neural crest migration continues in pharyngeal arches one through six from T23 to late T24 (arches labeled 1–6, neural crest indicated by arrowheads), but with weakening ectodermal expression (ect). All panels oriented with anterior facing left. Scale bars = 100 μm.

Fig. 5. Cranial expression of NrpA1 during head skeleton patterning. (A), At T25, NrpA1 expression is in pharyngeal arches one through seven (1–7, A) in the lateral mesenchymal cores (arrowhead, B), and in upper lip (ul) mesenchyme around the stomadeum (st, C). T26 NrpA1 expression remains in the lateral mesenchymal cores of arches one through nine (1–9, D; arrowhead, E), with some cells coalescing into pharyngeal prechondrocyte bars (arrows, E), as well as the skeleton of pharyngeal arch 1 (pa1, F) and lateral velar skeleton (vs, F). (G–J), At

T27, T28 NrpA1 pharyngeal expresion (arrowheads) weakens in the cartilage bars (cb, outlined in J), and is upregulated in the gills (g in J), a pattern maintained into late T30 (K–N). Except for cross-sections in I, J, M, and N, all panels are oriented with anterior facing left, and dorsal up. nt, neural tube; s, somite; Scale bars = 100 μm.

Fig. 6. Cranial expression of NrpA2 during head skeleton patterning. (A), At T25, NrpA2 is expressed in post-migratory neural crest in pharyngeal arches one through seven (1–7, A) in the lateral mesenchymal cores (arrowhead, B) and mesoderm (mes in B), and also in upper lip (ul) mesenchyme around the stomadeum (st, C). T26 NrpA2 transcripts occur throughout the mesenchymal cores of arches one through nine (1–9 in D; arrowhead, D) in the neural crest (arrowhead, E) and mesoderm (mes, E), and in prechondrocytes in pharyngeal arches one and two (pa1, pa2 in F), but is absent from the velar skeleton (vs, F). (G–J), At T27, T28 NrpA2 expression is in the upper lip (ul), but in the pharynx (arrowheads), expression weakens in the pharyngeal cartilage bars (cb, outlined in J), and is upregulated in the somitic and epibranchial mesoderm, and the hypobranchial bar (ebm, hbb in J). (K–N), Throughout T30, upper lip (ul) expression is maintained, with pharyngeal expression in the gills (g in N). Except for cross-sections in I, J, M, and N, all panels are oriented with anterior facing left, and dorsal up. Scale bars = 100 μm.

Fig. 7. Cranial expression of NrpB during head skeleton patterning. (A), NrpB at T25 is expressed in post-migratory neural crest in pharyngeal arches one through seven (1–7, A) within the lateral compartment of the mesenchymal cores (arrowhead, B) and in the upper lip (ul)

mesenchyme circumscribing the stomadeum (st in C). T26 NrpB expression remains in the lateral mesenchymal cores of arches one through nine (1–9 in D; arrowhead, E), with some cells coalescing into the circular shape of pharyngeal prechondrocyte bars (arrows, E), skeleton of pharyngeal arches one and two (pa1, pa2 in F), and the velar skeleton (vs, F). (G–J), T27 and T28 NrpB expression remains in the upper lip (ul), is gradually lost in the pharynx (arrowheads), from pharyngeal cartilage bars (cb in J), but is upregulated in ventral somites (s in J), epibranchial mesoderm (ebm in J), and pharyngeal arch muscle (pam in J). (K–N), Throughout T30, NrpB expression is in the upper lip (ul) and ventral somite-derived epibranchial and pharyngeal arch musle (ebm, pam in M). nt, neural tube. Except for cross-sections in I, J, M, and N, all panels are with anterior facing left, and dorsal up. Scale bars = 100 μm.

Fig 8. CRISPR/Cas9 knockout of Sema3F does not impair neural crest migration. (A, C), Control CRISPR embryos (ContCR) showing SoxE2 (A, T22) and nMyc (C, T23) expression in migratory neural crest streams (black arrowheads) separated by thin, crest-free zones (black asterisks). (B, D), Sema3F CRISPR mutants showing migratory neural crest streams (black arrowheads) expressing SoxE2 (B, T22) and nMyc (D, T23) segregated (black asterisks) similar to that of controls. Anterior facing left and dorsal up in all panels. Scale bars = 100 μm.

Fig. 9. CRISPR/Cas9 knockout of Sema3F results in early mispatterning melanocytes and cranial ganglia. (A), T26 Control (ContCR) embryo showing normal patterning of melanocytes linearly over the pharynx (arrows) and melanocyte migration into the anterior head (arrowhead) and upper lip (ul). (B), Sema3F CRISPR (Sema3FCR) mutants have mispatterned melanocytes at

T26, including a lack of melanocyte migration into the upper lip (asterisk, B) and dispersed melanocytes over the pharynx (arrows). (C), T26 control embryo immunostained for Hu in cranial sensory neurons. (D), Sema3F CRISPR mutants showed defects in patterning of cranial sensory neurons, including a lack of condensation of the opV ganglion, apparent fusion of ganglionic clusters (g+pet+epg?), and splitting of interconnected ganglia (asterisk, epg). epg, epibranchial ganglion; g, geniculate ganglion; mmV, maxillomandibular branch of the trigeminal ganglion; opV, ophthalmic branch of the trigeminal ganglion; pet, petrosal ganglion; pllg, posterior lateral line ganglion. Scale bars = 100 μm. Anterior facing left and dorsal up in all panels.

Fig 10. CRISPR/Cas9 knockout of Sema3F causes patterning defects during early head skeleton development. (A–F), Control CRISPR embryos (ContCR) at T25, T26. (A), T26 embryo with SoxE1 expression in prechondrocyes of pharyngeal arches three through nine (3–9), with weak expression in presumptive mucocartilage of arches one and two (1+2, velum outlined) and upper and lower lips (ul, ll). (B), T26 embryo with SoxE3 perichondrial expression in the presumptive velum in arches one and two (1+2, velum in dashed lines), upper/lower lips (ul, ll), and arches three through nine (3–9). (C), T25 embryo with TwistA expression in post-migratory neural crest in arches one and two (dashed line), upper and lower lips (ul, ll), and arches three through nine (3–9). (D–F), Horizontal sections through the pharynx of embryos in A–C showing pharyngeal pouches (endoderm outlined), and properly patterned prechondrocytes (arrowheads, D, E) or post-migratory crest (arrowheads in F) surrounding the mesoderm (mes in F). (G–L), Sema3F CRISPR mutant embryos (Sema3FCR) at T25, T26. (G), T26 mutants have disorganized SoxE1-

postive neural crest in arches three through nine (arrowhead) and an abnormally shaped oropharynx (asterisk). (H), Sema3F T26 mutants have disorganized SoxE3-positive neural crest in arches one/two and upper/lower lips (asterisk) and arches three through nine (arrowhead). (I), T25 mutants have TwistA-positive cells scattered in arches one/two and upper/lower lips (yellow asterisk), and arches three through nine (arrowhead). (J–L), Horizontal sections through the pharynx of mutants in G–I showing disorganized prechondrocytes and post-migratory crest in formed or partly formed pharyngeal arches (arrows, endoderm outlined) and neural crest cells spanning the boundary (arrowheads) between pharyngeal arches in which the endoderm failed to evaginate (asterisks). Anterior facing left in all panels. Scale bars = 100 μm.

Fig 11. CRISPR/Cas9 knockout of Sema3F results in disorganized melanocytes and cranial ganglia. (A–E), Control CRISPR larvae (ContCR) at T30. (A), control larvae had melanocytes arranged in segmentally in register with pharyngeal arches (numbered 1–9). (B), High-magnification of melanocytes in control embryo with typical size and stellate appearance. (C), Fluorescent Hu immunostaining showing properly patterned cranial ganglia in control larva. (D), higher magnification image of inset in (C) showing individual cranial ganglia. (E), Fluorescent Hu immunostaining in the trunk of control larva showing segmental organization of dorsal root ganglia (drg, arrowheads) and enteric neurons (en, arrow). (F–J), Sema3F mutant larvae (Sema3FCR) at T30. (F), Mutants had disorganized melanocytes (arrowheads), and (G) melanocytes were smaller and lacked the stellate branches (arrowheads, G). (H), Sema3F mutants had mispatterned cranial ganglia (inset, I), but dorsal root ganglia (drg, arrowheads in J) and enteric neurons (en, arrows in J) appeared unaffected. Key: epg, epibranchial ganglion; e,

eye; g, geniculate ganglion; mmV, maxillomandibular branch of trigeminal trigeminal ganglion; opV, ophthalmic branch of trigeminal ganglion; pet, petrosal ganglion; pllg, posterior lateral line ganglion; va, vestibuloacoustic ganglion. mmV+g+va, fusion of geniculate and vestibuloacoustic ganglia; ?, unidentifiable ganglionic protrusion from part of the mmV ganglion. Anterior facing left and dorsal oriented up in all panels. Scale bars = 100 μm.

Fig 12. CRISPR/Cas9 knockout of Sema3F results in a disorganized head skeleton. (A) Control CRISPR larva (ContCR) with alcian blue staining of the head skeleton. Mucocartilage elements include upper/lower lips (ul, ll), medial velar skeleton (mvs) and pharyngeal arches one and two (1, 2, outlined). Cartilage bars of arches three through nine are indicated (3–9). (B) Same larva in panel (A) but with fluorescence in arches three through nine (Martin et al., 2009). (C), higher magnification image of inset in (B) showing cartilage bar morphology (top arrowhead) and ventral fusion of the branchial basket (bottom arrowhead). (D–F), Sema3F CRISPR mutant larva (Sema3FCR). (D), Alcian staining reveals disorganized mucocartilage of arches one and two (arrowheads). (E) Fluorescence (E, inset in F) of the same larva shows disarticulation of cartilage bars four through six (asterisks, F) whereas the middle of these cartilage bars are clustered and disjointed (arrowhead, F). (G–I), Another Sema3FCR larva. (G), Mucocartilage cells are scattered throughout the oral skeleton that never condensed into the velum in arches one and two (arrowheads). (H), fluorescent imaging (H, inset in I) of the same larva shows disarticulated (asterisk in I, arch 6) and fused and bent cartilage bars in arches three through nine (arrowhead, I, arch 8). Anterior facing left and dorsal oriented up in all panels. Scale bars = 100 μm.

Fig. S1. Molecular phylogeny (neighbor joining analysis) of vertebrate class III Semaphorin ligands and Neuropilin receptors. Sea lamprey (*Petromyzon marinus*, Pm) orthologues are in purple text with their gnathostome cognates in black text. Bootstrap values for groupings are at node points. The previously identified Sema3 gene in Sauka-Spenger et al., 2007 groups in the Sema3D clade (Pm Sema3D-1).

Fig. S2. Lamprey Sema3F CRISPR mutants show defects in patterning of placode-derived portions of cranial sensory ganglia. (A) Control ($Cont^{CR}$) T25 embryo showing *Pax3/7* expression in the ophthalmic portion of the trigeminal ganglion (opV), whereas Sema3F mutants ($Sema3F^{CR}$) (B) have smaller opV ganglia with reduced *Pax3/7* expression. (C) *Six1/2* placode expression in control T25 embryos occurs in the petrosal (pet) and posterior lateral line (pllg) cranial sensory ganglia; expression is also observed in the upper lip mesenchyme (ul) and pharyngeal arches (pa). (D) Sema3F CRISPR mutants have no discernible spatial separation between the Six1/2-positive pet and pllg (arrowhead and arrow); mutants also show scattered *Six1/2* expression throughout the mouth and pharynx, consistent with defects in oropharyngeal patterning observed in Fig 10. Scale bars = 100 μm.

Fig. S3. Sequences of individual Sema3F mutant lamprey embryos in Figs. 8–12 and Fig. S2. For each embryo/larva, the lamprey Sema3F wildtype sequence (Pm Sema3F) is at the top with the gRNA1 target site in red and PAM site underlined. Four clones from each individual were sequenced to confirm mutagenesis, with the number of base pairs deleted (-) or inserted (+) listed to the right of each sequence. Deletions are indicated by dashed lines; insertions are colored blue;

transition/transversion substitutions are colored green. All individuals had 100% mutagenesis efficiency (4/4 mutant alleles each).

Fig. S4. Sequencing results from five randomly pooled Sema3F gRNA1-injected T26 lamprey embryos to estimate mutagenesis efficiency. Lamprey Sema3F wildtype sequence (Pm Sema3F) is at the top with the gRNA1 target site in red and PAM site underlined. Fifty clones sequenced from the genomic DNA of the pooled embryos are listed below the wildtype sequence, with the number of base pairs deleted (-), inserted (+) or unchanged (WT) listed to the right of each sequence. Deletions are indicated by dashed lines; insertions are colored blue; transition/transversion substitutions are colored green. Mutagenesis efficiency is 98% (49/50 mutant alleles), with 78% (39/50) out-of-frame mutations.

Fig. S5. Box-whisker plot summarizing the distribution of mutations induced at the Sema3F target locus by Sema3F gRNA1 for individual mutants and pooled mutant embryos. The bottom and top of each blue box indicates the first and third quartiles; bold horizontal black lines in each box indicate the median; the top and bottom of each vertical black line are the maximum and minimum values; black dots are outliers.

Fig. S6. Sequencing of top three potential Sema3 gRNA1 off-target sites in the lamprey genome (see also Table S1). (A) Comparison of the lamprey Sema3F gRNA1 target locus and Hox3 potential off-target locus reveals sequence similarity (red base pairs) in the 13 bp proximal to the PAM site (underlined). However, sequencing of 10 Hox3 clones from genomic DNA of Sema3F

mutant embryos reveals no mutations at the Hox3 locus. (B, C) Similarly, although the lamprey Sema3F gRNA1 on-target locus and gVLR and ABC7 potential off-target loci are similar in sequence (red base pairs) near the PAM site (underlined), sequencing of 10 gVLR and 10 ABC7 clones from genomic DNA of Sema3F mutant embryos failed to uncover mutagenesis at either locus.

Figure 1

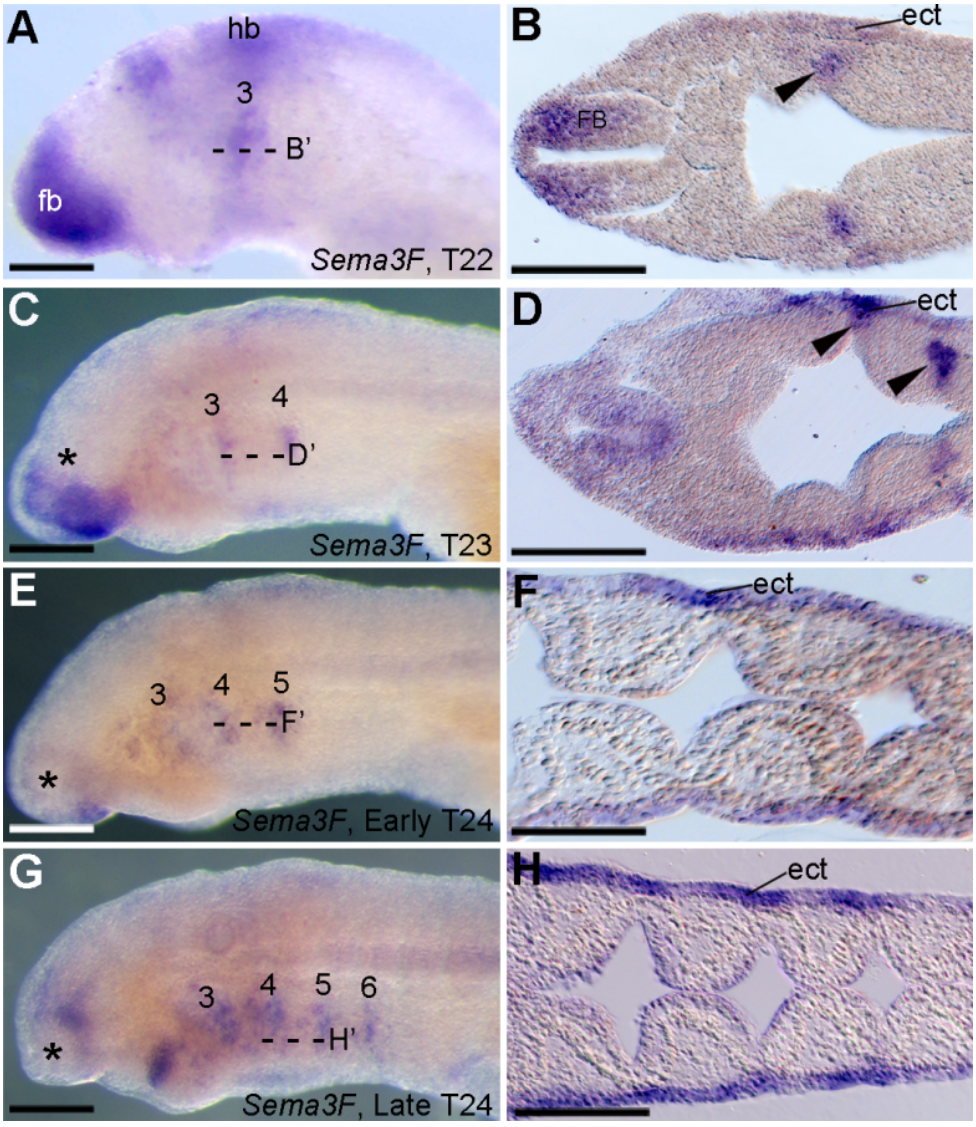
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Figure 2

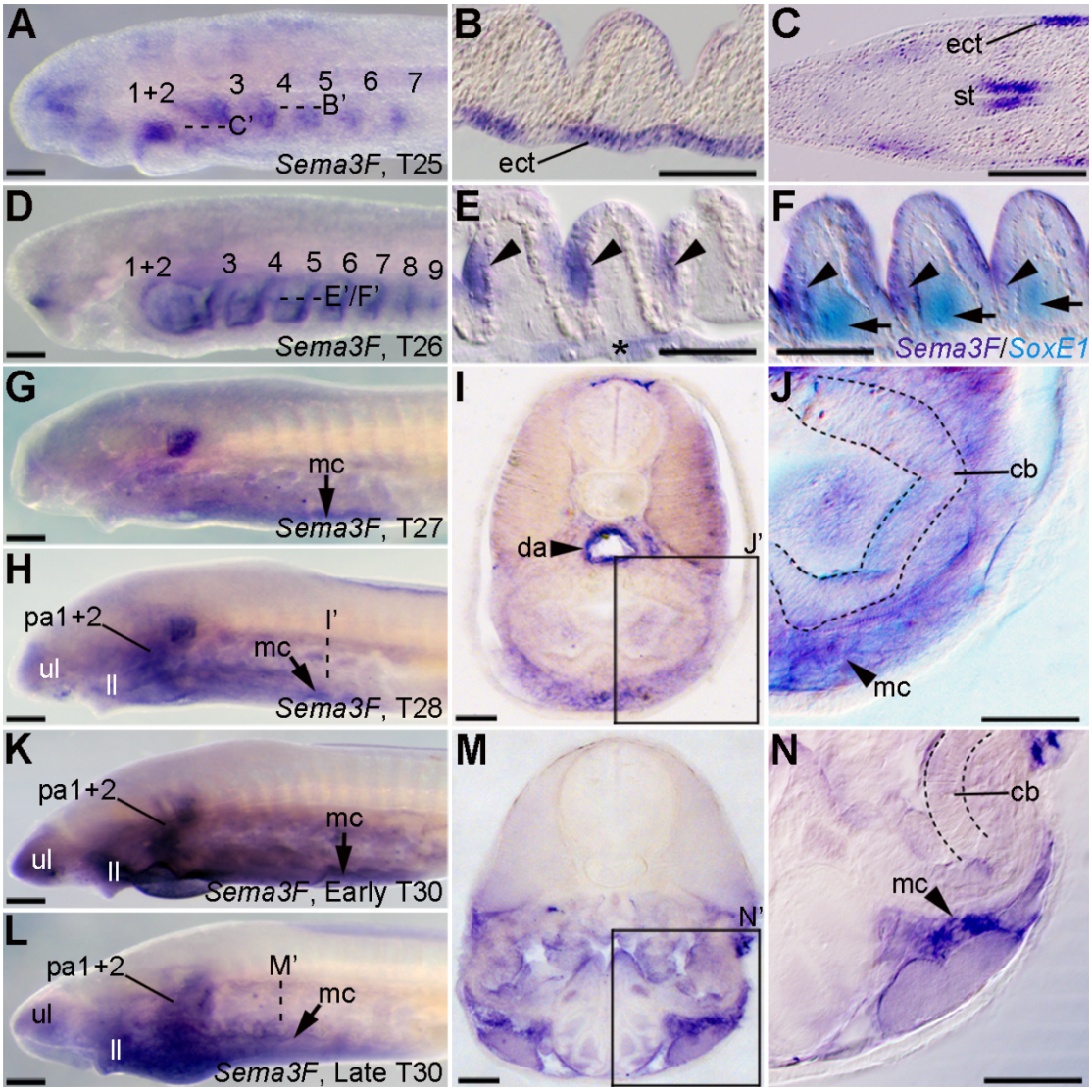
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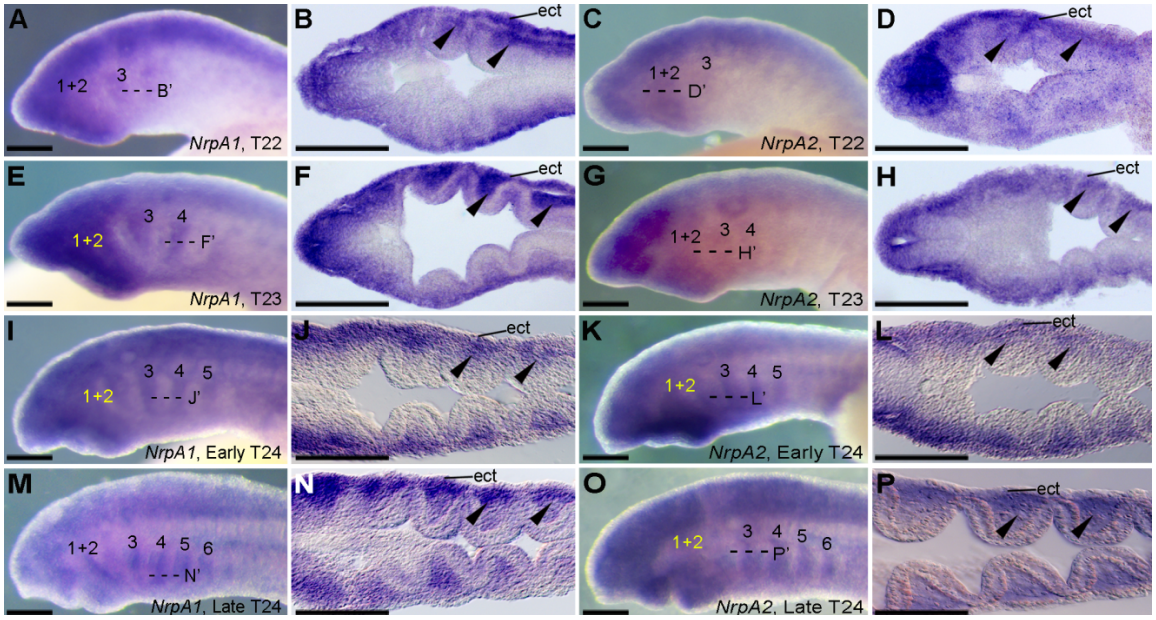
Figure 3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ect
M
1+2
3
4
5
6
N'
NrpA1, Late T24
N
ect
O
1+2
3
4
5
6
P'
NrpA2, Late T24
P
ect

Figure 4

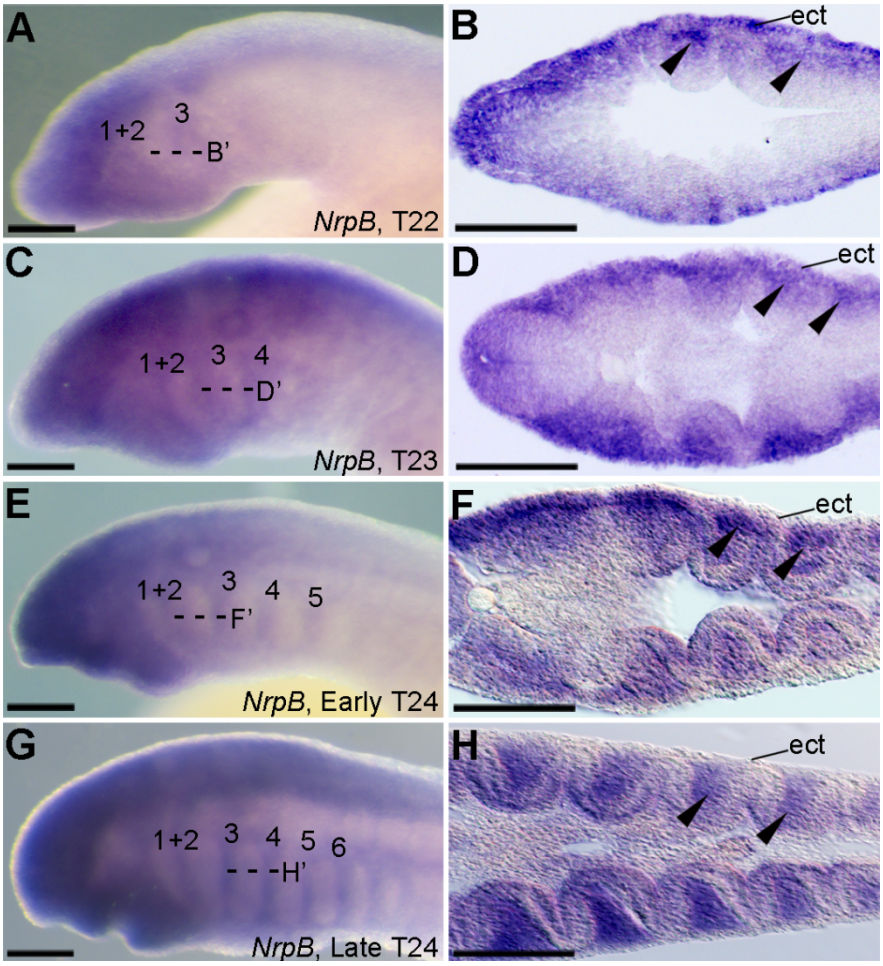

A
1+2
3
B'
NrpB, T22
B
ect
C
1+2
3
4
D'
NrpB, T23
D
ect
E
1+2
3
4
5
F'
NrpB, Early T24
F
ect
G
1+2
3
4
5
6
H'
NrpB, Late T24
H
ect

Figure 5

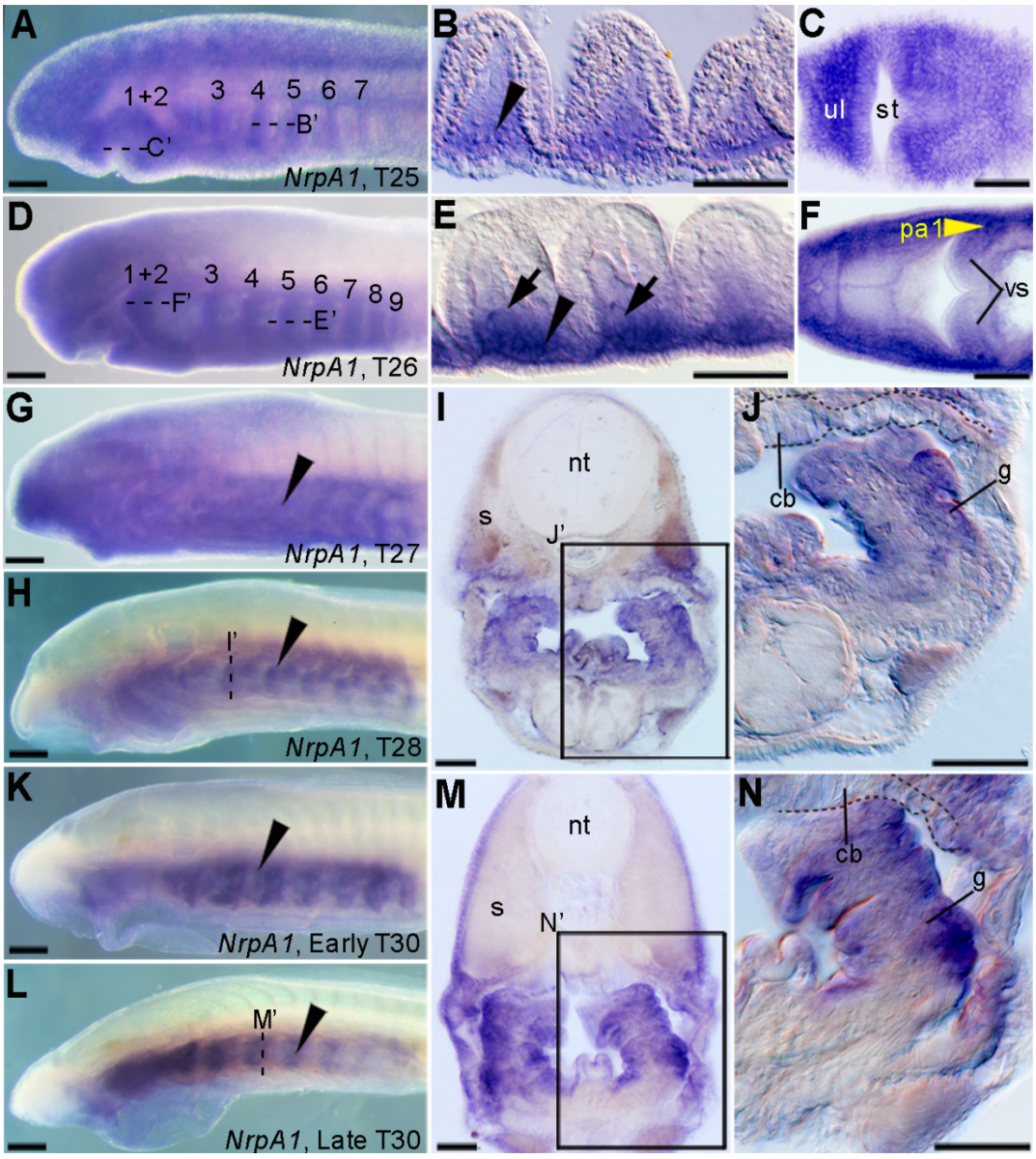

A
1+2
3
4
5
6
7
- - -B'
- - -C'
NrpA1, T25
B
C
ul
st
D
1+2
3
4
5
6
7
8
9
- - -F'
- - -E'
NrpA1, T26
E
F
pa1
vs
G
NrpA1, T27
H
I'
NrpA1, T28
I
nt
s
J'
J
cb
g
K
NrpA1, Early T30
L
M'
NrpA1, Late T30
M
nt
s
N'
N
cb
g

Figure 6

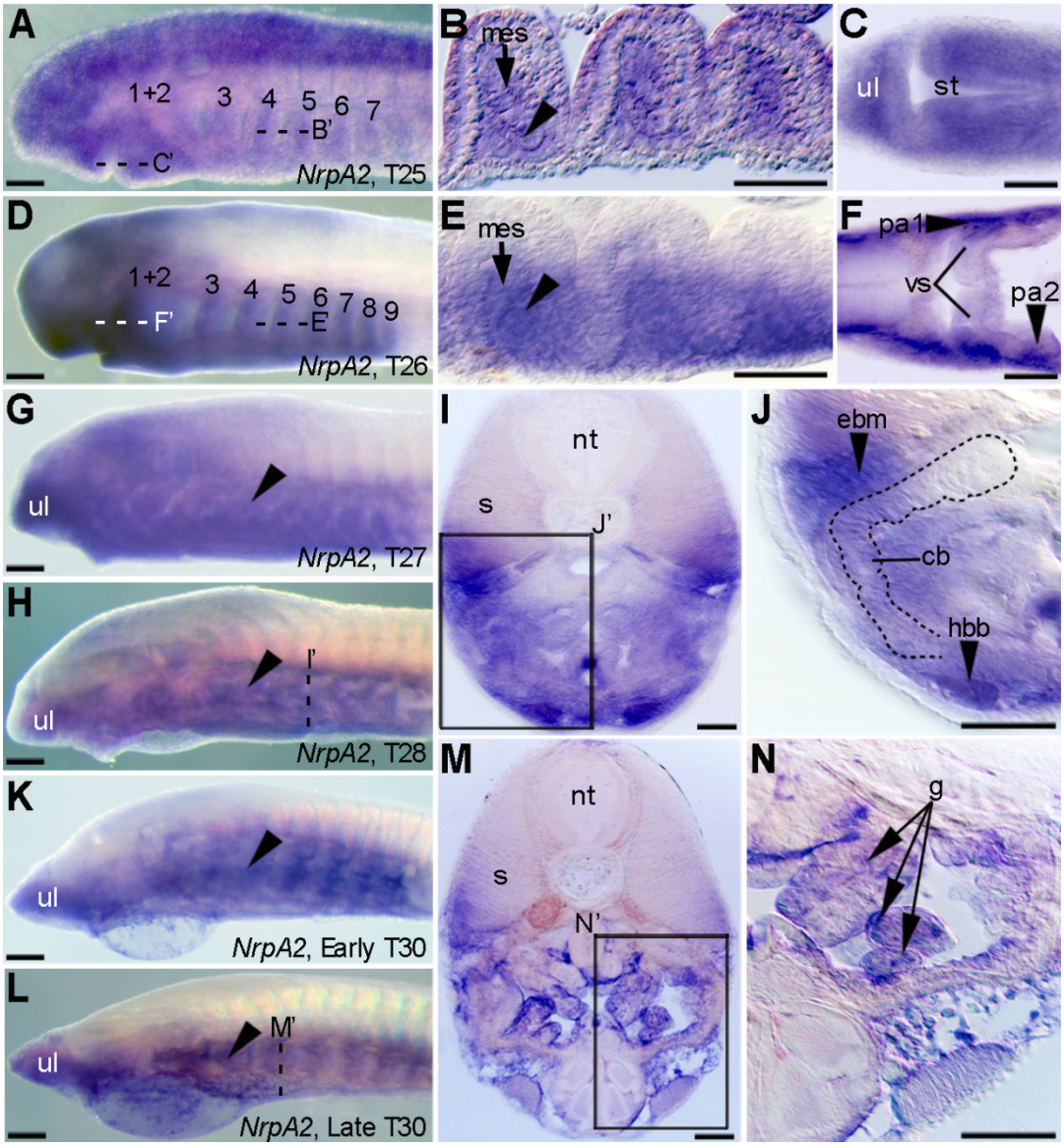

A
1+2
3
4
5
6
7
B'
C'
NrpA2, T25
B
mes
C
ul
st
D
1+2
3
4
5
6
7
8
9
F'
E'
NrpA2, T26
E
mes
F
pa1
vs
pa2
G
ul
NrpA2, T27
I
nt
s
J'
J
ebm
cb
hbb
H
ul
I'
NrpA2, T28
K
ul
NrpA2, Early T30
M
nt
s
N'
N
g
L
ul
M'
NrpA2, Late T30

Figure 7

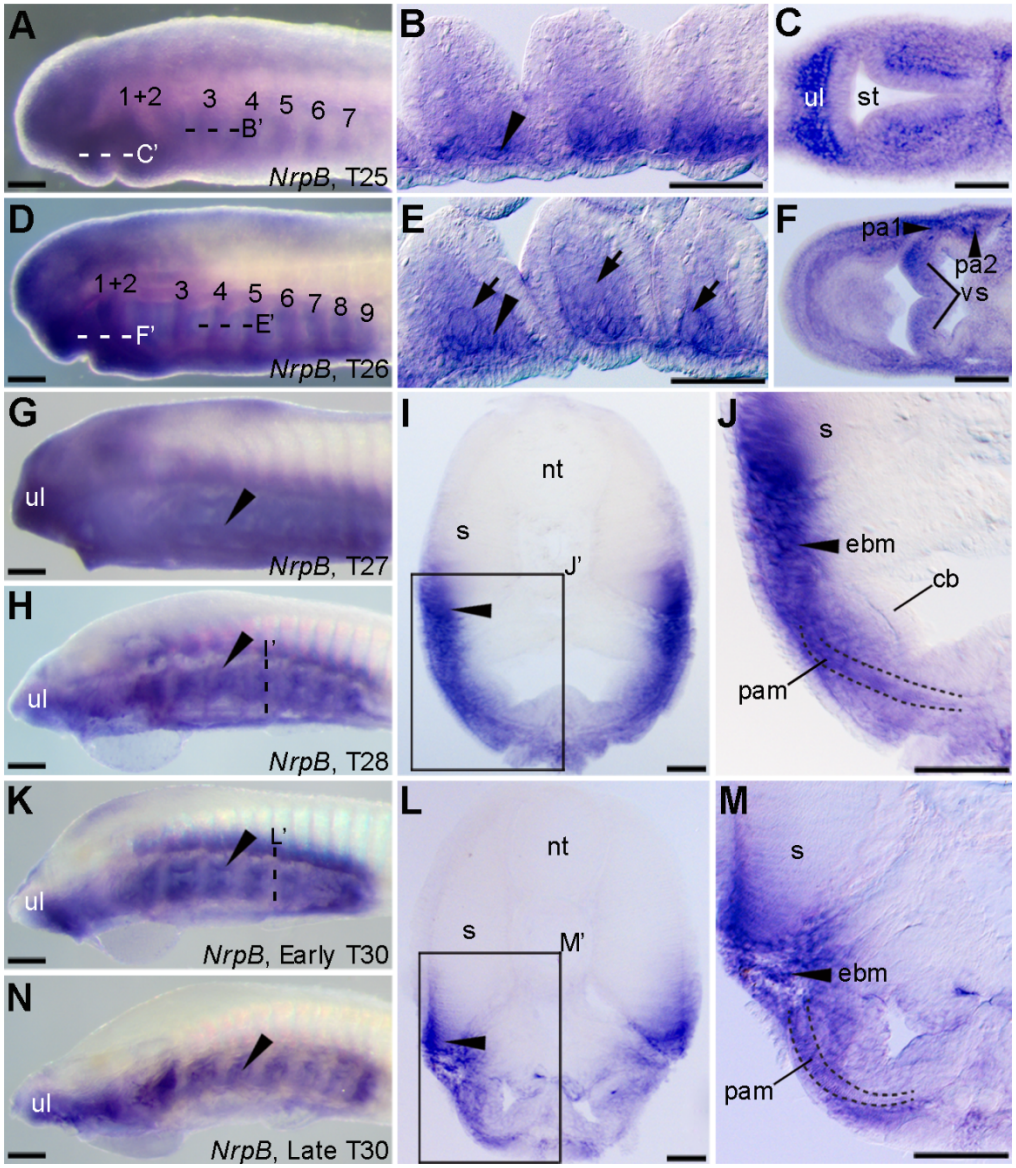

A
1+2
3
4
5
6
7
B'
C'
NrpB, T25
B
C
ul
st
D
1+2
3
4
5
6
7
8
9
E'
F'
NrpB, T26
E
F
pa1
pa2
vs
G
ul
NrpB, T27
I
nt
s
J'
J
s
ebm
cb
pam
H
I'
ul
NrpB, T28
K
L'
ul
NrpB, Early T30
L
nt
s
M'
M
s
ebm
pam
N
ul
NrpB, Late T30

Figure 8

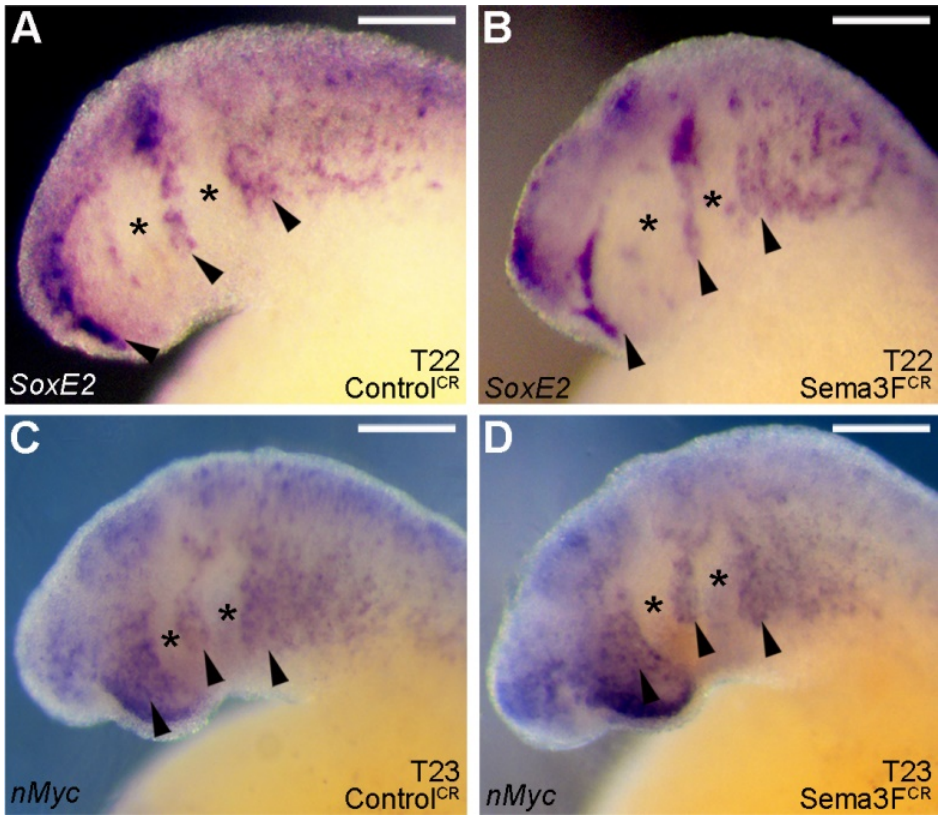

A
SoxE2
T22
Control^CR
B
SoxE2
T22
Sema3F^CR
C
nMyc
T23
Control^CR
D
nMyc
T23
Sema3F^CR

Figure 9

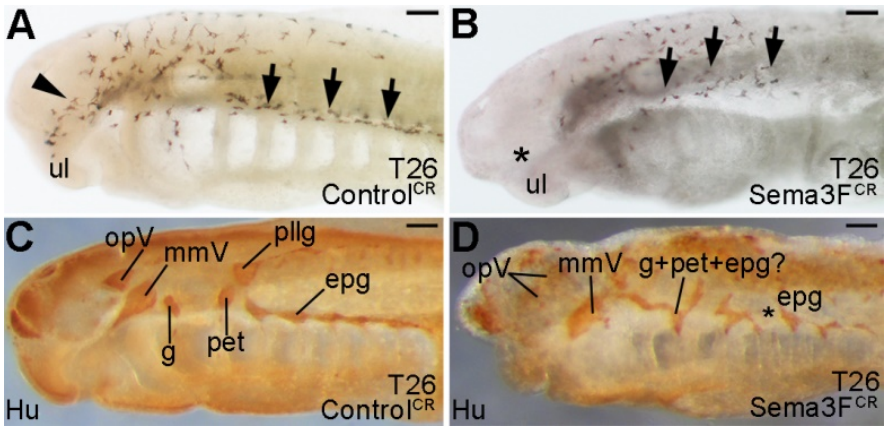

A
ul
T26
Control CR
B
*
ul
T26
Sema3F CR
C
opV
mmV
pllg
epg
g
pet
Hu
T26
Control CR
D
opV
mmV
g+pet+epg?
epg
*
Hu
T26
Sema3F CR

Figure 10

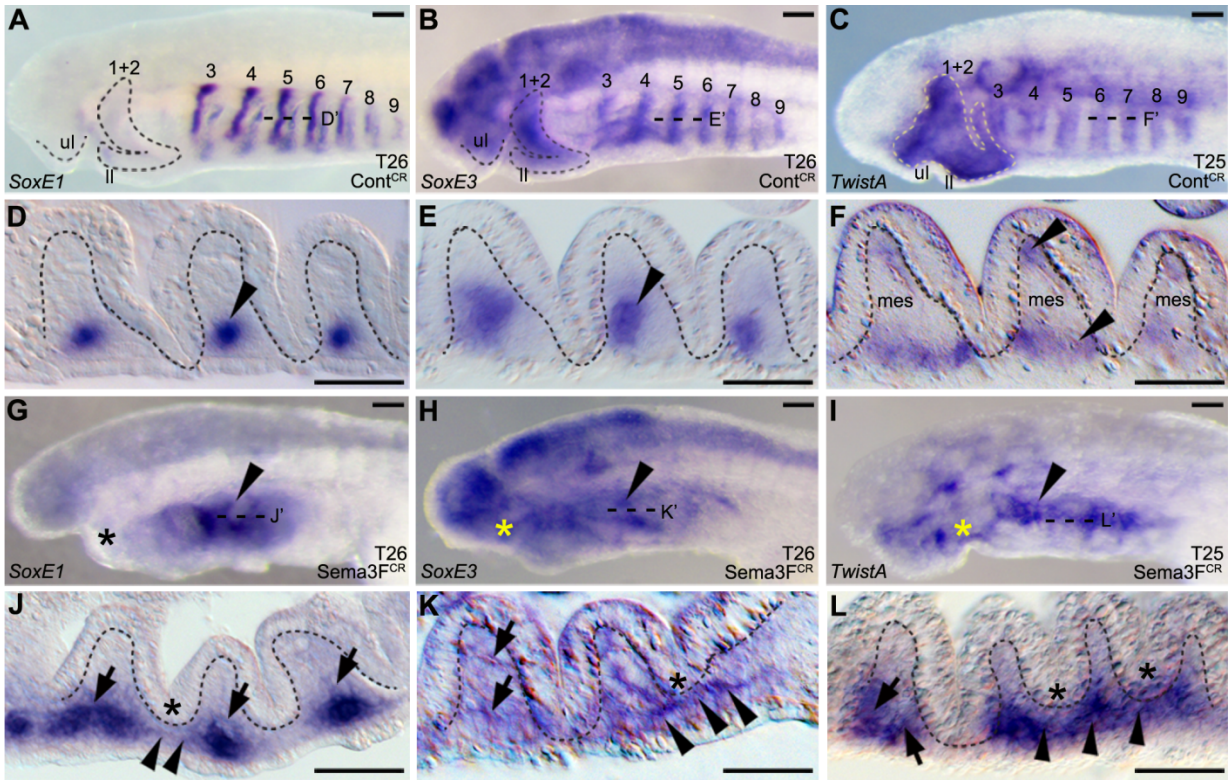

A
1+2
3 4 5 6 7 8 9
D'
ul
ll
SoxE1
T26
Cont^CR
B
1+2
3 4 5 6 7 8 9
E'
ul
ll
SoxE3
T26
Cont^CR
C
1+2
3 4 5 6 7 8 9
F'
ul
ll
TwistA
T25
Cont^CR
D
E
F
mes
mes
mes
G
J'
SoxE1
T26
Sema3F^CR
H
K'
SoxE3
T26
Sema3F^CR
I
L'
TwistA
T25
Sema3F^CR
J
K
L

Figure 11

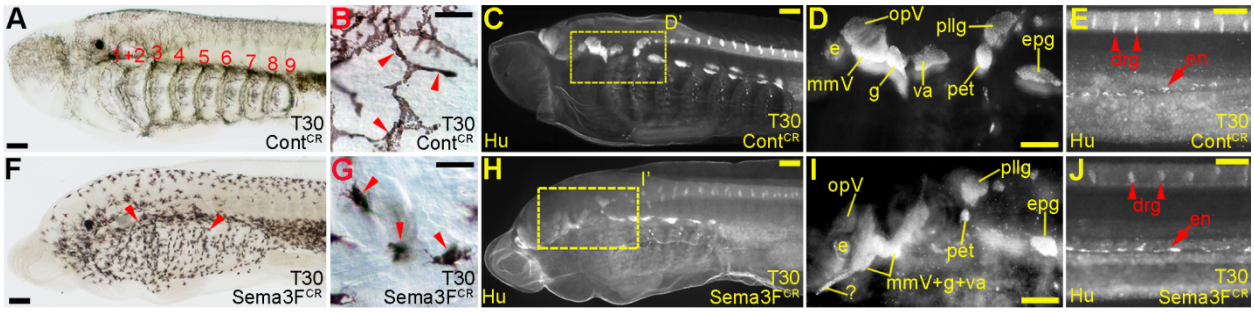

A
1+2 3 4 5 6 7 8 9
T30
Cont^CR
B
T30
Cont^CR
C
D'
Hu
T30
Cont^CR
D
opV
pllg
epg
e
mmV
g
va
pet
E
drg
en
Hu
T30
Cont^CR
F
T30
Sema3F^CR
G
T30
Sema3F^CR
H
I'
Hu
T30
Sema3F^CR
I
opV
pllg
epg
e
pet
mmV+g+va
?
J
drg
en
Hu
T30
Sema3F^CR

Figure 12

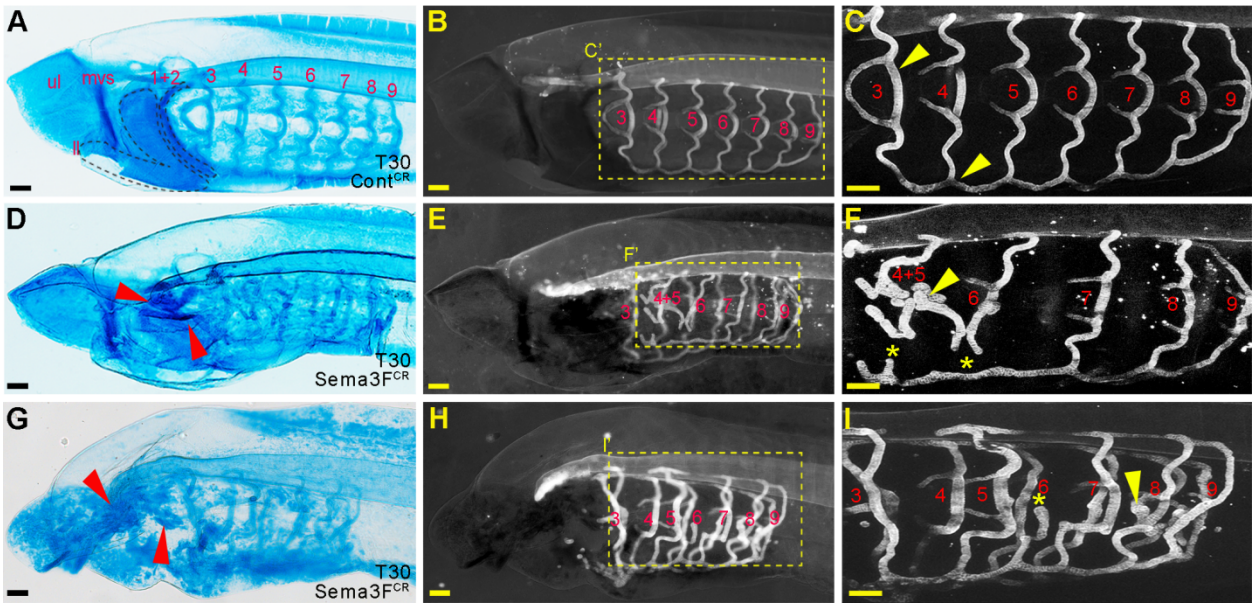

A
ul
mvs
1+2
3
4
5
6
7
8
9
ll
T30
Cont^CR
B
C'
C
D
T30
Sema3F^CR
E
F'
F
4+5
*
G
T30
Sema3F^CR
H
I'
I

Figure S1

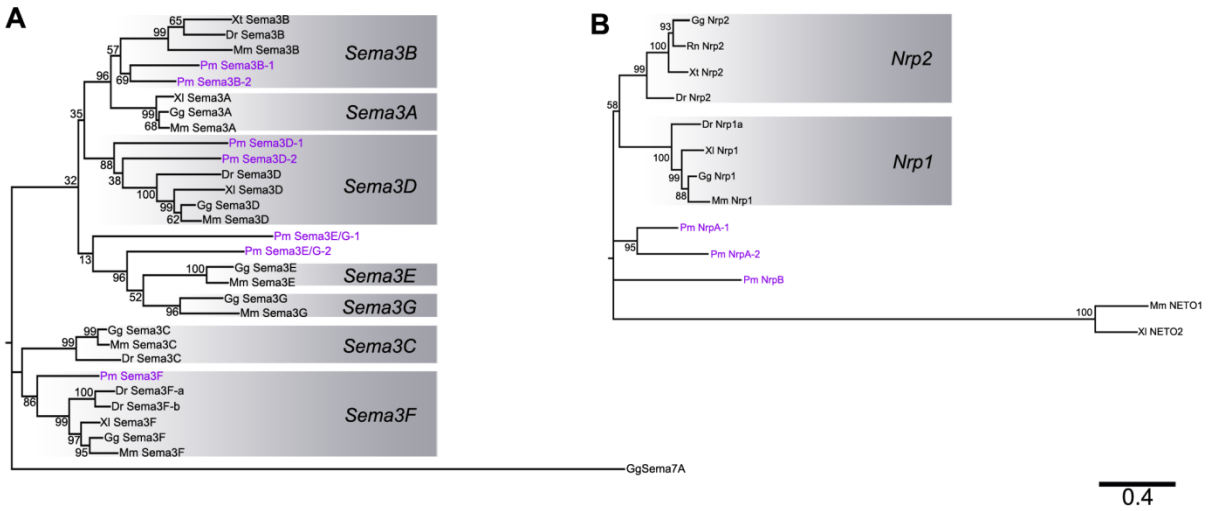

A
Xt Sema3B
Dr Sema3B
Mm Sema3B
Sema3B
Pm Sema3B-1
Pm Sema3B-2
Xl Sema3A
Gg Sema3A
Mm Sema3A
Sema3A
Pm Sema3D-1
Pm Sema3D-2
Dr Sema3D
Xl Sema3D
Gg Sema3D
Mm Sema3D
Sema3D
Pm Sema3E/G-1
Pm Sema3E/G-2
Gg Sema3E
Mm Sema3E
Sema3E
Gg Sema3G
Mm Sema3G
Sema3G
Gg Sema3C
Mm Sema3C
Dr Sema3C
Sema3C
Pm Sema3F
Dr Sema3F-a
Dr Sema3F-b
Xl Sema3F
Gg Sema3F
Mm Sema3F
Sema3F
GgSema7A
B
Gg Nrp2
Rn Nrp2
Xt Nrp2
Dr Nrp2
Nrp2
Dr Nrp1a
Xl Nrp1
Gg Nrp1
Mm Nrp1
Nrp1
Pm NrpA-1
Pm NrpA-2
Pm NrpB
Mm NETO1
Xl NETO2
0.4

Figure S2

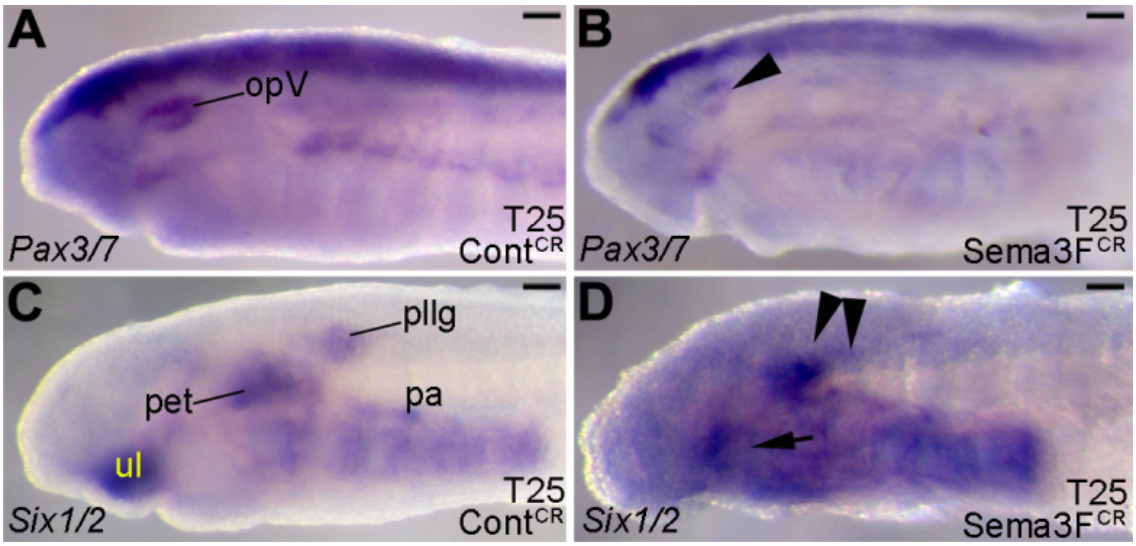

A
opV
Pax3/7
T25
Cont^CR
B
Pax3/7
T25
Sema3F^CR
C
pllg
pet
pa
ul
Six1/2
T25
Cont^CR
D
Six1/2
T25
Sema3F^CR

Figure S3

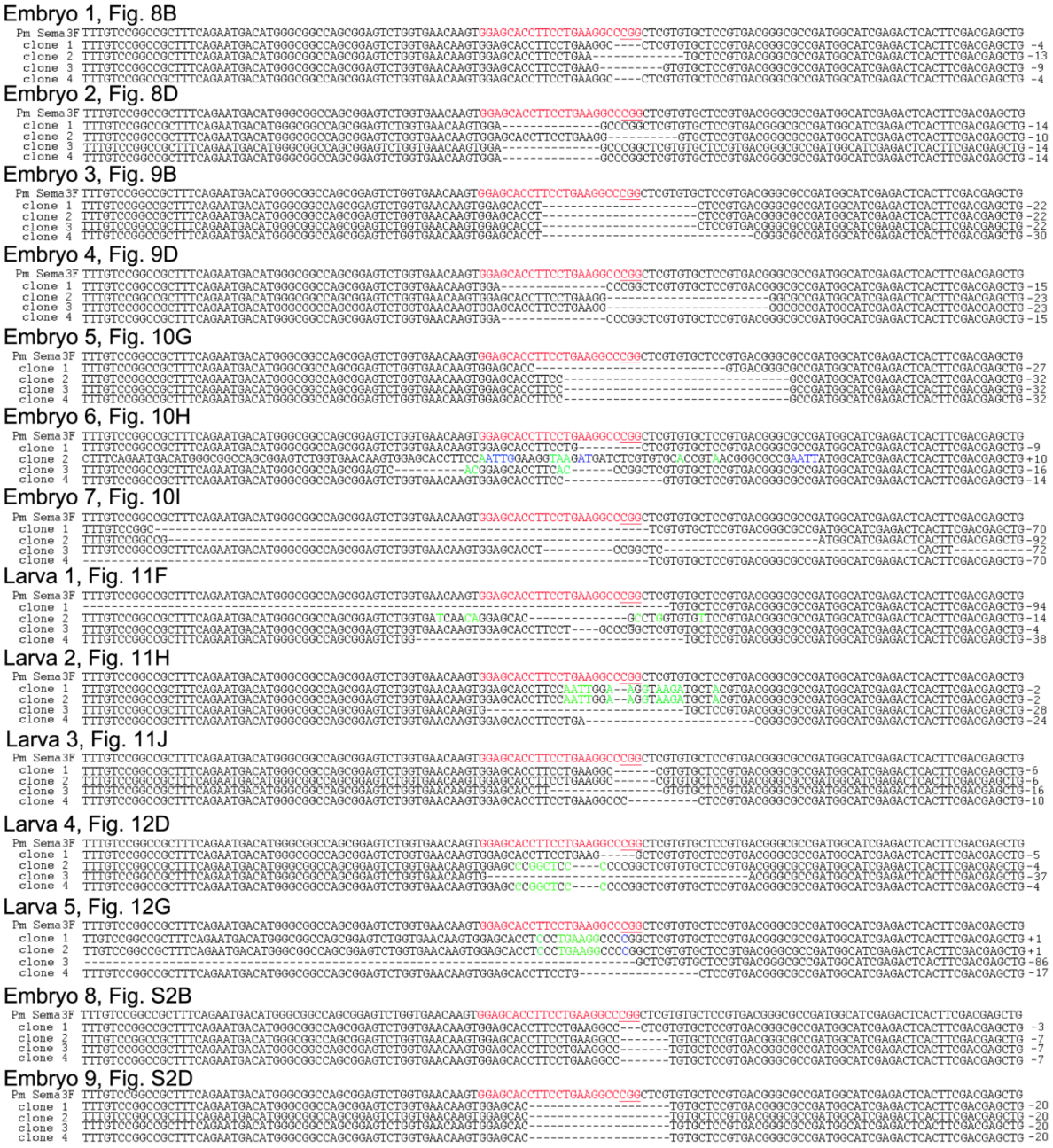

Embryo 1, Fig. 8B
Embryo 2, Fig. 8D
Embryo 3, Fig. 9B
Embryo 4, Fig. 9D
Embryo 5, Fig. 10G
Embryo 6, Fig. 10H
Embryo 7, Fig. 10I
Larva 1, Fig. 11F
Larva 2, Fig. 11H
Larva 3, Fig. 11J
Larva 4, Fig. 12D
Larva 5, Fig. 12G
Embryo 8, Fig. S2B
Embryo 9, Fig. S2D

Figure S4

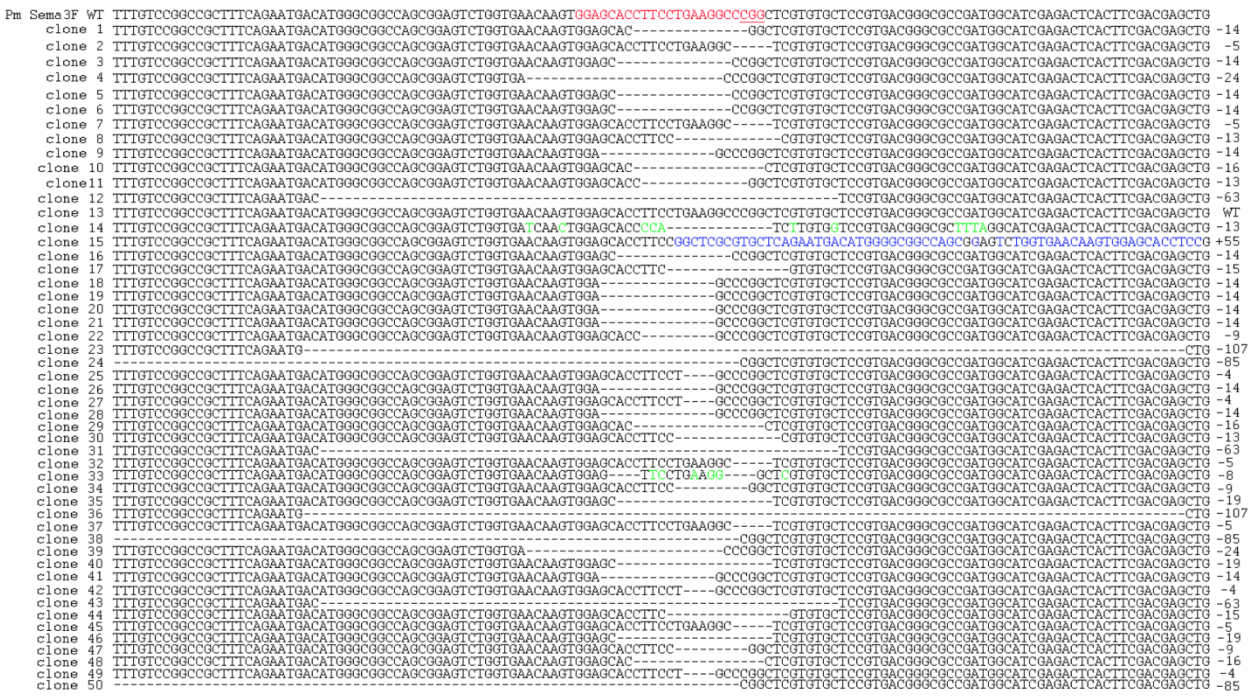

Pm Sema3F WT TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCTGAAGGCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG
clone 1 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCAC-------------GGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 2 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCTGAAGGC-----TCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -5
clone 3 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGC-------------CCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 4 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGA-----------------------CCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -24
clone 5 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGC-------------CCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 6 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGC-------------CCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 7 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCTGAAGGC-----TCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -5
clone 8 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCC-------------CGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -13
clone 9 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGA-------------GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 10 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCAC---------------CTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -16
clone11 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACC-------------GGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -13
clone 12 TTTGTCCGGCCGCTTTCAGAATGAC---TCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -63
clone 13 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCTGAAGGCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG WT
clone 14 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGATCAACTGGAGCACCCCA-------------TCTTGTGGTCCGTGACGGGCGCTTTAGGCATCGAGACTCACTTCGACGAGCTG -13
clone 15 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCGGCTCGCGTGCTCAGAATGACATGGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTCCG +55
clone 16 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGC-------------CCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 17 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTC---------------GTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -15
clone 18 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGA-------------GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 19 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGA-------------GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 20 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGA-------------GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 21 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGA-------------GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 22 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACC---------GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -9
clone 23 TTTGTCCGGCCGCTTTCAGAATG---CTG -107
clone 24 --CGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -85
clone 25 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCT----GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -4
clone 26 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGA-------------GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 27 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCT----GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -4
clone 28 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGA-------------GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 29 TTTGTCCGGCCGCTTTCAGAATGACATGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCAC----------------CTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -16
clone 30 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCC-------------CGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -13
clone 31 TTTGTCCGGCCGCTTTCAGAATGAC---TCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -63
clone 32 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCTGAAGGC-----TCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -5
clone 33 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAG----TTCCTGAAGG----GCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -8
clone 34 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCC---------GGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -9
clone 35 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGC-------------------TCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -19
clone 36 TTTGTCCGGCCGCTTTCAGAATG---CTG -107
clone 37 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCTGAAGGC-----TCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -5
clone 38 --CGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -85
clone 39 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGA-----------------------CCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -24
clone 40 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGC-------------------TCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -19
clone 41 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGA-------------GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -14
clone 42 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCT----GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -4
clone 43 TTTGTCCGGCCGCTTTCAGAATGAC---TCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -63
clone 44 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTC---------------GTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -15
clone 45 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCTGAAGGC-----TCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -5
clone 46 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGC-------------------TCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -19
clone 47 TTTGTCCGGCCGCTTTCAGAATGACATGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCC---------GGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -9
clone 48 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCAC---------------CTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -16
clone 49 TTTGTCCGGCCGCTTTCAGAATGACATGGGCGGCCAGCGGAGTCTGGTGAACAAGTGGAGCACCTTCCT----GCCCGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -4
clone 50 --CGGCTCGTGTGCTCCGTGACGGGCGCCGATGGCATCGAGACTCACTTCGACGAGCTG -85

Figure S5

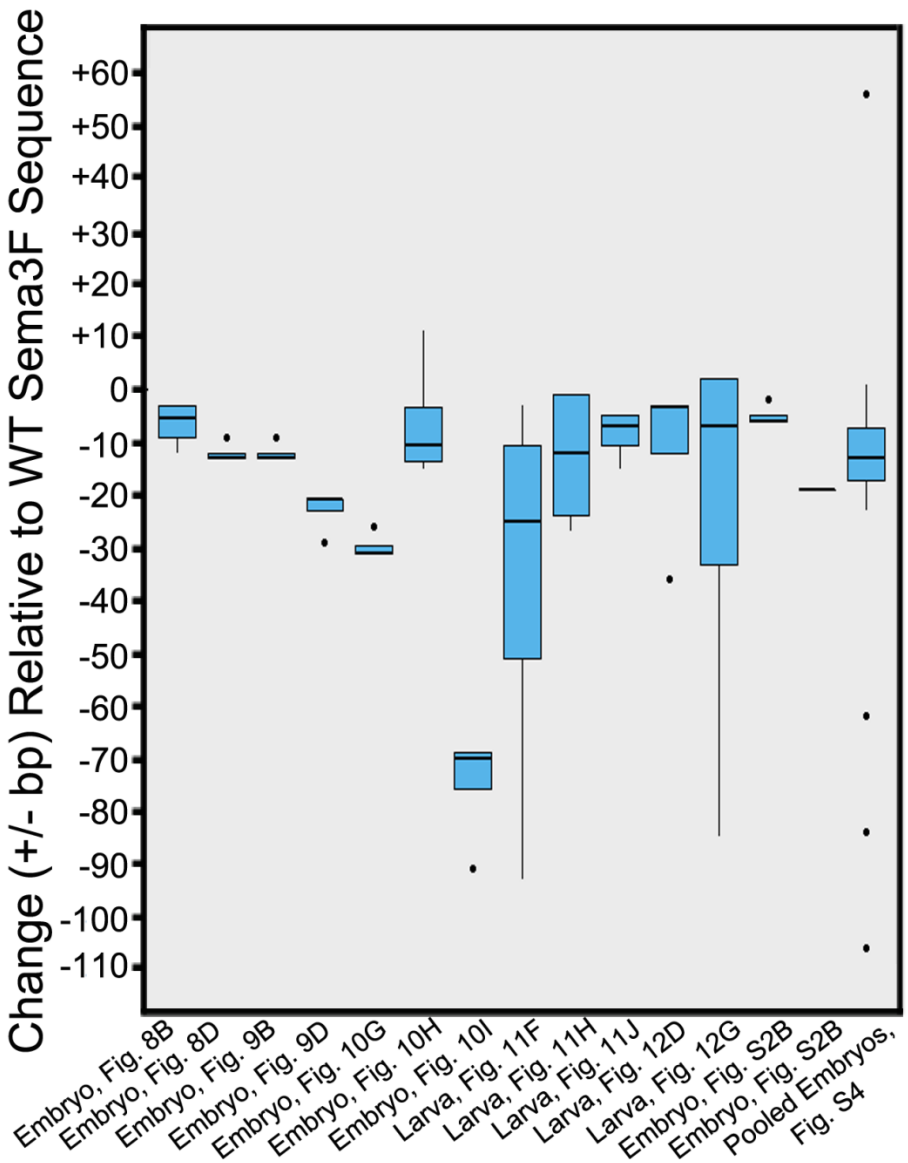
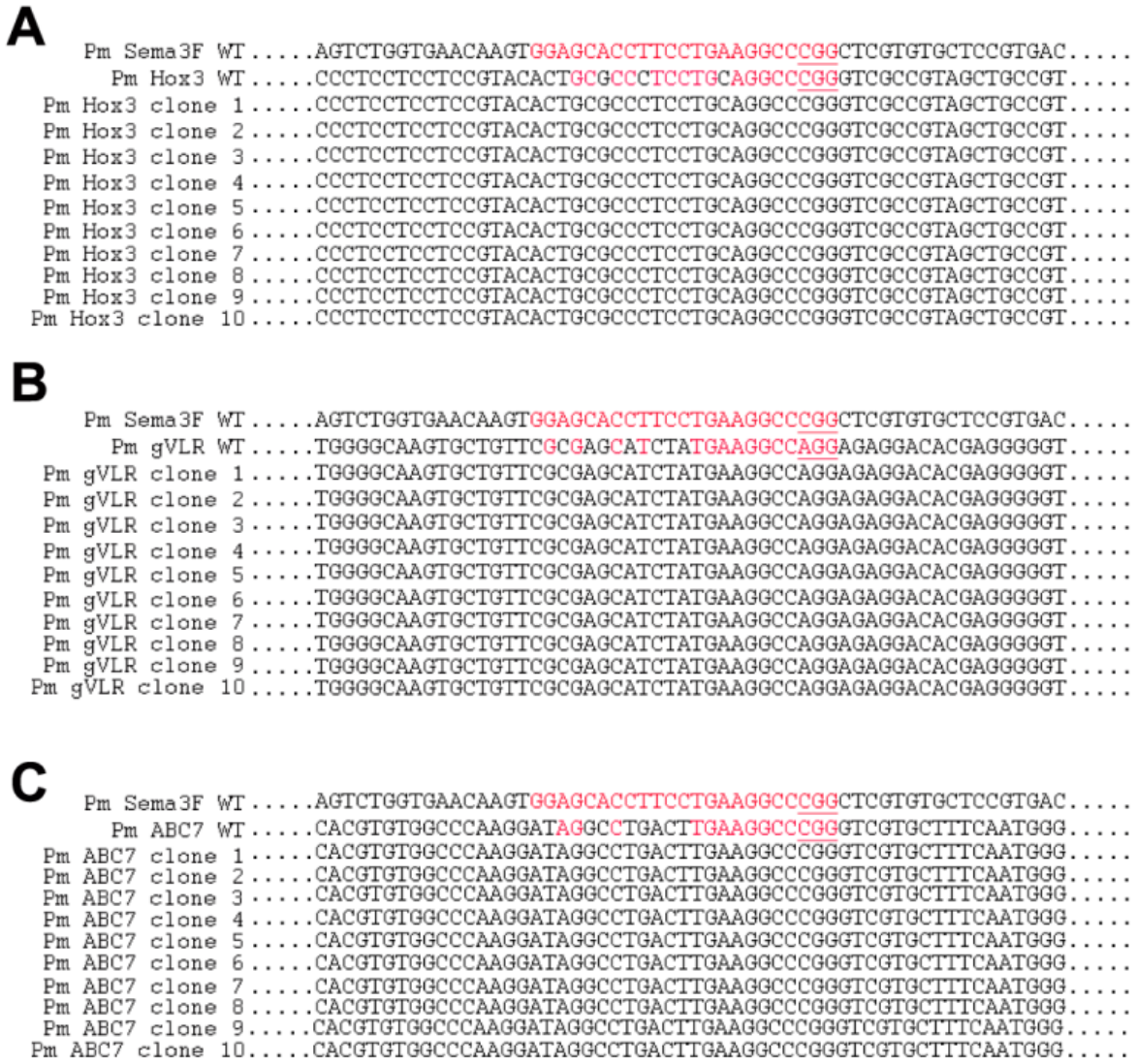

Change (+/- bp) Relative to WT Sema3F Sequence
+60
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
Embryo, Fig. 8B
Embryo, Fig. 8D
Embryo, Fig. 9B
Embryo, Fig. 9D
Embryo, Fig. 10G
Embryo, Fig. 10H
Embryo, Fig. 10I
Larva, Fig. 11F
Larva, Fig. 11H
Larva, Fig. 11J
Larva, Fig. 12D
Larva, Fig. 12G
Embryo, Fig. S2B
Embryo, Fig. S2B
Pooled Embryos, Fig. S4

Figure S6


A
Pm Sema3F WT .....AGTCTGGTGAACAAGTGGAGCACCTTCCTGAAGGCCCGGCTCGTGTGCTCCGTGAC.....
Pm Hox3 WT .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
Pm Hox3 clone 1 .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
Pm Hox3 clone 2 .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
Pm Hox3 clone 3 .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
Pm Hox3 clone 4 .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
Pm Hox3 clone 5 .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
Pm Hox3 clone 6 .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
Pm Hox3 clone 7 .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
Pm Hox3 clone 8 .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
Pm Hox3 clone 9 .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
Pm Hox3 clone 10 .....CCCTCCTCCTCCGTACACTGCGCCCTCCTGCAGGCCCGGGTCGCCGTAGCTGCCGT.....
B
Pm Sema3F WT .....AGTCTGGTGAACAAGTGGAGCACCTTCCTGAAGGCCCGGCTCGTGTGCTCCGTGAC.....
Pm gVLR WT .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
Pm gVLR clone 1 .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
Pm gVLR clone 2 .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
Pm gVLR clone 3 .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
Pm gVLR clone 4 .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
Pm gVLR clone 5 .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
Pm gVLR clone 6 .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
Pm gVLR clone 7 .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
Pm gVLR clone 8 .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
Pm gVLR clone 9 .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
Pm gVLR clone 10 .....TGGGGCAAGTGCTGTTCGCGAGCATCTATGAAGGCCAGGAGAGGACACGAGGGGGT.....
C
Pm Sema3F WT .....AGTCTGGTGAACAAGTGGAGCACCTTCCTGAAGGCCCGGCTCGTGTGCTCCGTGAC.....
Pm ABC7 WT .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....
Pm ABC7 clone 1 .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....
Pm ABC7 clone 2 .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....
Pm ABC7 clone 3 .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....
Pm ABC7 clone 4 .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....
Pm ABC7 clone 5 .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....
Pm ABC7 clone 6 .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....
Pm ABC7 clone 7 .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....
Pm ABC7 clone 8 .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....
Pm ABC7 clone 9 .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....
Pm ABC7 clone 10 .....CACGTGTGGCCCAAGGATAGGCCTGACTTGAAGGCCCGGGTCGTGCTTTCAATGGG.....

CHAPTER 5: EVOLUTION OF SNAIL-MEDIATED REGULATION OF NEURAL CREST AND PLACODES FROM AN ANCIENT ROLE IN BILATERIAN NEUROGENESIS

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ABSTRACT

A major challenge in vertebrate evolution is to identify the gene regulatory mechanisms that facilitated the origin of neural crest cells and placodes from ancestral precursors in invertebrates. Here, we show in lamprey, a primitively jawless vertebrate, that the transcription factor *Snail* is expressed simultaneously throughout the neural plate, neural plate border, and pre-placodal ectoderm in the early embryo and is then upregulated in the CNS throughout neurogenesis. Using CRISPR/Cas9-mediated genome editing, we demonstrate that *Snail* plays functional roles in all of these embryonic domains or their derivatives. We first show that *Snail* patterns the neural plate border by repressing lateral expansion of *Pax3/7* and activating *nMyc* and *ZicA*. We also present evidence that *Snail* is essential for *DlxB*-mediated establishment of the pre-placodal ectoderm but is not required for *SoxB1a* expression during formation of the neural plate proper. At later stages, *Snail* regulates formation of neural crest-derived and placode-derived PNS neurons and controls CNS neural differentiation in part by promoting cell survival. Taken together with established functions of invertebrate *Snail* genes, we identify a pan-bilaterian mechanism that extends to jawless vertebrates for regulating neurogenesis that is dependent on *Snail* transcription factors. We propose that ancestral vertebrates deployed an evolutionarily conserved *Snail* expression domain in the CNS and PNS for neurogenesis and then acquired derived functions in neural crest and placode development by recruitment of regulatory genes downstream of neuroectodermal *Snail* activity. Our results suggest that *Snail* regulatory mechanisms in vertebrate novelties such as the neural crest and placodes may have emerged from neurogenic roles that originated early in bilaterian evolution.

INTRODUCTION

The origin of the vertebrates has been one of the most important and controversial topics in evolutionary biology and natural history for almost 200 years (Gegenbaur, 1878; Geoffroy Saint-Hilaire, 1830; Haeckel, 1860; Romer, 1950). Much of what distinguishes the vertebrates from their invertebrate relatives can be traced to two small cell populations, neural crest and placodes, which appear only transiently in vertebrate embryos (Gans and Northcutt, 1983; Gee, 1996; Green et al., 2015; Horstadius, 1950; Le Douarin and Kalcheim, 1999; Northcutt and Gans, 1983). The neural crest is a migratory, multipotent cell population that forms along the dorsal neural tube, from which it then detaches, migrates and differentiates into a wide array of cell types, including craniofacial cartilage and bone, smooth muscle, pigment, as well as most of the neurons and glia of the peripheral sensory nervous system (Green et al., 2015; Simões-Costa and Bronner, 2015). Placodes, which arise as ectodermal thickenings in the head, also migrate and give rise to cells in special sense organs (e.g., lens, otic, nasal, adenohypophysis, lateral line) as well as many of the sensory neurons in cranial ganglia (Graham and Shimeld, 2013; Schlosser, 2010, 2014, 2015). Both cell populations are tightly regulated during development by evolutionarily conserved gene regulatory networks (GRNs) that involve the activity of numerous signaling molecules and transcription factors (Betancur et al., 2010; Maharana and Schlosser, 2018; Sauka-Spengler and Bronner-Fraser, 2008; Sauka-Spengler et al., 2007; Schlosser, 2006). Together, neural crest and placodes form a wide range of vertebrate novelties and are therefore thought to have driven the origin and diversification of the vertebrate body plan (Gans and Northcutt, 1983; Northcutt, 2005; Northcutt and Gans, 1983; Trainor, 2013).

Despite their importance in vertebrate development, the evolutionary origins of neural crest and placodes, as well as their underlying regulatory networks, have proven enigmatic. An example of this is highlighted by vertebrate *Snail* genes (*Snail1, Snail2*, also known as *Snai1* and *Snai2*), which belong to the *Snail* superfamily, a group of zinc finger transcription factors with deep phylogenetic origins (Barrallo-Gimeno and Nieto, 2005, 2009; Grau et al., 1984; Manzanares et al., 2001; Nieto, 2002; Nieto et al., 1994; Nüsslein-Volhard et al., 1984). *Snail* genes likely duplicated from a single ancestral gene present in the last common ancestor of eumetazoans and have since acquired a diverse repertoire of important functions throughout eumetazoan embryogenesis (Barrallo-Gimeno and Nieto, 2009; Hemavathy et al., 2000; Nieto, 2002; Wu and Zhou, 2010). Among eumetazoans, *Snail1* and *Snail2* are pivotal for mesodermal genesis and patterning (Barrallo-Gimeno and Nieto, 2009; Nieto, 2002). However, they also control development of neural crest and placodes during various stages of vertebrate embryogenesis (Locascio et al., 2002; Manzanares et al., 2001; Nieto, 2001; Nieto, 2002; Taneyhill et al., 2007). This suggests that *Snail*-mediated regulation of neural crest and placodes could have been co-opted from a genetic program for mesoderm development (Langeland et al., 1998; Manzanares et al., 2001; Nieto, 2002). An alternative to co-option from a mesodermal regulatory program is the possibility that neural crest and placode regulation might have been acquired from a program driving neurogenesis. Support for this hypothesis comes from the fact that *Snail* is expressed in, and regulates the development of, central nervous system (CNS) neurons in diverse lophotrochozoans, ecdysozoans, and invertebrate deuterostomes (Ashraf and Ip, 2001; Barrallo-Gimeno and Nieto, 2009; Dill et al., 2007; Hudson et al., 2015; Kerner et al., 2009; Langeland et al., 1998; Lespinet et al., 2002; Manzanares et al., 2001; Nieto, 2002;

Stollewerk, 2016). However, vertebrates do not use *Snail* to pattern their nervous system and invertebrates were thought to lack neural crest and placodes (Gans and Northcutt, 1983; Green et al., 2015; Northcutt and Gans, 1983). Thus, apparent similarities in the use of *Snail* in neural crest and placodes of vertebrates with a CNS-neurogenic function among invertebrates appear superficial and suggest that *Snail* regulatory activity in these different cell populations may have evolved independently.

However, recent work suggests that there may be a closer affinity than previously thought between *Snail* involvement in neurogenesis of invertebrates and in neural crest and placodes among vertebrates. Functional experiments and lineage tracing in tunicates—now recognized as the closest living relatives of vertebrates—suggest that these animals may have embryonic rudiments of both neural crest and placodes, and the regions that generate these rudiments express *Snail* similar to vertebrate embryos (Abitua et al., 2015; Abitua et al., 2012; Delsuc et al., 2006; Horie et al., 2018; Jeffery, 2006; Jeffery et al., 2008; Jeffery et al., 2004; Stolfi et al., 2015). Tunicates also use *Snail* to pattern the neural plate and CNS, a function similar to that among lophotrochozoans and ecdysozoans (Hudson et al., 2018; Hudson et al., 2015). These observations suggest that *Snail* function in deuterostomes such as tunicates shares features with both protostome invertebrates on one hand (CNS, neurogenesis), and vertebrates on the other (neural crest, placodes). However, so far there has been no evidence of a vertebrate *Snail* gene that bridges the regulatory gap linking a CNS-neurogenic domain with neural crest and placodes across the invertebrate-vertebrate divide. In contrast to this, we and others have demonstrated that *Snail* is expressed throughout much of the developing CNS in embryos of the sea lamprey (*Petromyzon marinus*) (Rahimi et al., 2009; Sauka-Spengler et al., 2007; York et al., 2017).

Lampreys are members of a primitively jawless group of vertebrates (cyclostomes) that are the sister group to all other (jawed, or gnathostome) vertebrates and therefore occupy a critical phylogenetic position to understand the earliest events in vertebrate history, including the origins of neural crest and placode regulation (Green and Bronner, 2014; McCauley et al., 2015; Rahimi et al., 2009; York et al., 2019; York et al., 2017). We previously showed that *Snail* expression and function in lampreys shares similarities with jawed vertebrates, including roles in neural crest migration and differentiation (Barrallo-Gimeno and Nieto, 2005; Dill et al., 2007; Lespinet et al., 2002; Nieto, 2002; Rahimi et al., 2009; Weller and Tautz, 2003; York et al., 2017). Despite these similarities, the broad neural expression in the CNS of lamprey *Snail* is unlike that of any other vertebrate but is similar to that in the developing nervous systems of protostome invertebrates, as well as invertebrate deuterostomes such as tunicates and amphioxus. However, the phylogenetic significance of these observations has not yet been explored.

Here, we address this issue by focusing on *Snail* expression and function in the CNS, neural crest, and placodes to gain insight into the potential roles of *Snail* in each of these populations in lamprey embryos. We then cast our results within a broad comparative embryology framework to test if the pre-vertebrate origins of *Snail* function in neurogenesis might be linked to the evolution of *Snail* regulation in neural crest and placodes in vertebrates. To this end, we describe lamprey *Snail* expression broadly in the neuroectoderm, encompassing the neural plate, neural plate border, and pre-placodal ectoderm contiguously, and show that *Snail* maintains robust expression in cells derived from these territories throughout embryogenesis. Using CRISPR/Cas9-mediated genome editing, we find that while *Snail* is not essential for early development of the neural

plate border, in part by activating gene expression, but also by patterning the medial-lateral axis of the neural plate border. We also demonstrate a role for *Snail* in establishment of the pre-placodal ectoderm, and in formation of cranial sensory neurons of both placode and neural crest origin. After confirming a role in early neural crest and placode development, we finally demonstrate that although *Snail* is not essential for CNS neurogenesis, it is required for neurogenic differentiation within the CNS and does so by promoting cell survival. Taken together, our results demonstrate that lamprey *Snail* is expressed in, and appears to be capable of, regulating the development of neural crest, placodes, and CNS neurons simultaneously. This multi-functional role shares similarities with both vertebrates (neural crest, placode development) and invertebrates (CNS and PNS neurogenesis). We propose that these expression domains and functional roles in lamprey reflect an ancestral *Snail*-positive domain for neural crest and placode development in vertebrates that was co-opted from an evolutionarily ancient role in CNS and PNS neurogenesis that is conserved across bilaterians.

MATERIALS AND METHODS

Embryo collection and gene cloning

Gravid sea lampreys were collected from streams and rivers near the Hammond Bay Biological Station, Millersburg, MI, and shipped to the University of Oklahoma. Adult lampreys were maintained at 14°C in a recirculating water system. Eggs were obtained manually from ovulating females and then mixed with sperm expressed from a mature male in a small beaker of water. Embryos were raised in Pyrex dishes under the flow of water (18°C) that was supplemented with 0.05X Marc's Modified Ringers solution. All procedures requiring adult lampreys were approved

by the University of Oklahoma Institutional Animal Care and Use Committee (IACUC, R15-027). Embryos were staged according to Tahara (Tahara, 1988), with those from desired stages for analysis fixed in MEMFA. *PCNA* and *Phox2* partial cDNA fragments were cloned from a sea lamprey cDNA library kindly provided by J. Langeland. Other partial cDNA clones (*nMyc*, *NCAM, ZicA*, *Tfap2a*, *Snail, SoxB1a, Pax3/7, DlxB, Six1/2*) were obtained from previous library screenings or PCR-based isolation as described elsewhere (McCauley and Bronner-Fraser, 2002; McCauley and Bronner-Fraser, 2006; Rahimi et al., 2009; Sauka-Spengler et al., 2007; York et al., 2017).

In situ hybridization and immunohistochemistry

Protocols for *in situ* hybridization were performed as previously described (York et al., 2017). For immunohistochemistry involving Hu (Hu C/D, mouse IgG2b; Invitrogen) and cleaved Caspase3 protein (Anti-ACTIVE Caspase-3 pAb; Promega), antibodies were diluted (1:300) in TBT (Tris-buffered saline with 0.1% Triton X-100) with 10% goat serum and then detected using goat anti-mouse IgG conjugated to horseradish peroxidase and developed as described elsewhere (York et al., 2017; Ch. 3). For double labeling of *Snail* and Hu, *in situ* hybridization for *Snail* was followed by immediate washes in PBST, and then immunohistochemistry for Hu. Sectioning (20 μm) was performed on a Vibratome with embryos embedded in 5% agarose.

CRISPR/Cas9-targeting of lamprey Snail

To disrupt *Snail* function *in vivo* guide RNAs (gRNAs) targeting the *Snail* genomic coding sequence (*Snail* gRNA1: 5′-CCCCGCACCTTGTGCACTGGACC-3′; *Snail* gRNA2: 5'-

CCTGGCGAGGCACGGGCGATG-3'; protospacer adjacent motif (PAM) sequences are underlined) were delivered by microinjection (described below). We used several different, but complementary, methods when selecting gRNAs. First, we used CRISPOR software to computationally identify optimal gRNA target sites within the *Snail* genomic coding sequence obtained from the recently completed sea lamprey germline genome assembly (http://crispor.tefor.net/) (Concordet and Haeussler, 2018; Smith et al., 2018). We then supplemented our computational approach by taking into account features that have been optimized for gRNA selection in lamprey (Square et al., 2015; York et al., 2018; York et al., 2017): 50–80% GC content, with targeted regions as close as possible to the presumptive start codon (or 5′ end of available genomic sequence). All gRNAs were then prepared according to a previously published protocol (Square et al., 2015). Approximately 1hr after fertilization, zygotes were injected (~5 nl) with a cocktail containing 1 ng-ul^{-1} Cas9 protein (PNA Bio), 500pg gRNA and 10% fluorescein dextran tracer, prepared in nuclease-free water. After waiting 10 min for the Cas9-gRNA complex to form, approximately 5000 embryos were microinjected for several hours, with replicates of these injections performed multiple times over the summer breeding season. Injected embryos were screened by fluorescence three or four days later and non-fluorescent embryos were discarded. Successfully injected embryos were raised to appropriate stages, fixed in MEMFA, and then dehydrated and stored at -20ºC in 100% methanol until needed for analysis.

Control CRISPR experiments

To control for potential toxicity of Cas9 protein and gRNAs, as well as other unforeseen effects resulting from microinjection, we microinjected the same concentration of Cas9 protein (1 ng-ul^{-1}) and a "scrambled" negative control gRNA (500pg; 5'-AATAAGTTGGGGTTTCCA-3') into zygotes from each cohort of fertilized eggs that were microinjected with our *Snail* gRNAs. All control embryos analyzed had the same morphological appearance and gene expression patterns as un-injected wildtype embryos.

Genotyping of individual CRISPR mutant embryos

Following immunostaining or *in situ* hybridization, we selected five embryos to link individual gene expression phenotypes to a specific mutant genotype. To control for the possibility that tissue fixation and/or damage to genomic DNA during the *in situ* hybridization or immunostaining protocols did not generate erroneous "mutations" during sequencing, the sequences of putative mutant embryos were compared to negative control embryos (see above) that were fixed and assayed by *in situ* hybridization or immunostaining (see also York et al., 2018). These embryos were incubated 24–48h with 0.1 mg ml^{-1} proteinase K; genomic DNA was extracted using standard methods (Sive et al., 2000). Oligonucleotides (Sigma) surrounding the *Snail* (forward: 5′-GACGGAGCAGCAGAACGATGGT-3′; reverse: 5′-ACCGCTCCCCATAAAACACGC-3′) CRISPR target site were used to PCR amplify and sequence the locus. For each embryo, four different clones were sequenced to document mutagenesis. Our results confirmed that these embryos were actual mutants, thereby effectively

linking *Snail* CRISPR phenotypes with specific mosaic mutant genotypes (Fig. S4, 19/20 mutant alleles, 95% efficiency).

Estimating efficiency of CRISPR/Cas9 mutagenesis

The efficiency of mutagenesis at the *Snail* genomic locus was estimated by pooling 5 randomly selected *Snail* gRNA1 CRISPR-injected embryos at T26. Genomic DNA was isolated per standard methods, the targeted locus was PCR amplified (oligonucleotide sequences listed above), and 40 clones were sequenced. Efficiency (%) was calculated by dividing the number of mutant genotypes by the total number of clones sequenced (see Fig. S5). Injections targeting *Snail* proved to be highly efficient at inducing mutations in these randomly selected embryos, with an estimated mutagenesis efficiency of 90% (36/40 mutant alleles), and 73% (29/40) of these being out-of-frame (Fig. S5).

Evaluation of off-target CRISPR sites

As described above, our *Snail* gRNAs were designed to minimize potential mutagenesis at off-target loci. Nevertheless, we verified that *Snail* gRNA1 mutant phenotypes were specific to cleavage at the *Snail* locus. To do this, we conducted a BLAST search targeting sequences most similar to the *Snail* gRNA1 sequence in the sea lamprey genome (https://genome.ucsc.edu/cgi-bin/hgGateway). This search revealed that the top three potential off-target genomic loci that contained a PAM cleavage sequence (NGG or reverse complement, CCN) had numerous mismatches, with several of these occurring in the 13 bp "seed sequence" proximal to the PAM site (see Table S2). Two or more mismatches within the seed sequence are often sufficient to

prevent off-target mutagenesis (Hsu et al., 2013; Pattanayak et al., 2013). Hence, these potential off-targets are unlikely to be cleaved. To confirm this, genomic DNA isolated from the same five pooled *Snail* CRISPR-injected embryos (Tahara stage 26) that were used to calculate mutagenesis efficiency (see above) was also used to PCR amplify and sequence 10 clones that encompassed these potential off-target regions (see Table S2, Fig. S6) using the following primers: blood plasma apolipoprotein LAL2, forward: 5′-CTTCAGGCCAGTCACCAATG-3′, reverse: 5′-GATGAGGCTTCGATCCATCA-3′; CD45, forward: 5′-TATCACGATCCCTTCAGCTC-3′, reverse: 5′- CACTCAACATAAGCCTGCCA-3′; variable lymphocyte receptor B, forward: 5′-TCGAGAGGCTGCATAGCTAC-3′, reverse: 5′-GTCATGGCAAGCCGTGCGTT-3′. Sequencing revealed no evidence of mutations at these potential off-target sites, which suggests that embryonic CRISPR phenotypes are specific to mutagenesis at the targeted *Snail* locus (Fig. S6).

Measures of spatial gene expression in neural plate border, pre-placodal ectoderm and neural plate

To test if *Snail* regulates medial-lateral gene expression patterning in the neuroectoderm, we quantitatively compared spatial expression patterns of genes that maintained expression in the neural plate border (*Pax3/7*, *Tfap2a*) and neural plate (*SoxB1a*) in *Snail* CRISPR mutants (see Results). To do this, we measured the total width (μm) of the dorsal surface of T17 embryos and then measured (μm) either the total width of expression (for *Pax3/7* and *SoxB1a*) or the width of the non-expressing area between the neural plate borders (for *Tfap2a*) for controls and *Snail* CRISPR mutants. We standardized these values across individual embryos by creating an index

of spatial gene expression that divided expression width by embryonic width. Data were non-normally distributed for measures of *Pax3/7* and *SoxB1a* expression (Shapiro-Wilks, p's < 0.01), so we compared indices using a Wilcoxon Rank Sum test. Because measures for *Tfap2a* were normally distributed (Shapiro-Wilks, $p = 0.91$), we used a *t*-test. All analyses were performed in R (R Development Core Team, 2013). Graphical representation of indices as a box and whisker dot plot was performed in R using the package 'ggplot2' (Wickham, 2016). Measurements are in Supplementary Data File 1.

Character state reconstruction of Snail expression domains

Character state reconstruction analysis for categorical data was performed in Mesquite (Maddison, 2008). Expression patterns of eumetazoan *Snail* homologs in the CNS/PNS, placodes, neural crest, and mesoderm were obtained from the literature and organized into a character matrix (character state present = 1; absent = 0). Default parameters were chosen for the "parsimony ancestral states" analysis option. The data matrix and supporting references are in Table S1.

RESULTS

Molecular phylogenetics of vertebrate Snail genes

As a first step toward exploring a possible developmental link in *Snail* activity among neural crest, placodes, and CNS/PNS neurons in lamprey embryos, we characterized the genomic complement of the *Snail* family in the lamprey genome. In jawed vertebrates, *Snail* family genes include *Snail1*, *Snail2* and *Scratch*, with some of these having undergone independent

duplications in certain lineages (e.g., teleosts, Thisse et al., 1995; Thisse et al., 1993). Our previous work, coupled with BLAST searches, library screenings, and phylogenetic analysis of *Snail* genes from the sea lamprey germline and somatic genomes consistently reveal a single lamprey *Snail* orthologue residing at the base of the vertebrate *Snail1*/*Snail2* clade (Fig. S1), as well as a putative *Scratch* orthologue nested within the *Scratch* clade (Rahimi et al., 2009; Smith et al., 2013; Smith et al., 2018; York et al., 2017). Although lampreys may have lost a second *Snail* gene copy, searches of the published *P. marinus* somatic and germline genomes (Smith et al., 2013; Smith et al., 2018), coupled with our phylogenetic analysis, nonetheless support the notion that lampreys contain a single *Snail* orthologue, a feature also shared with hagfish, the sister group to lampreys (Ota et al., 2007).

Snail regulates neural plate border and pre-placodal ectoderm, but not neural plate

Previous studies have documented expression of *Snail* in early lamprey embryos, with transcripts localizing contiguously in the neural plate, neural plate border, and pre-placodal ectoderm, and maintenance of overlapping CNS-neural crest expression in the neural tube (Rahimi et al., 2009; Sauka-Spengler et al., 2007; York et al., 2017; see also Fig. S2). Based on these overlapping expression patterns, we asked if there might be a *Snail*-mediated functional link among these embryonic territories. To test this, we used CRISPR/Cas9-mediated genome editing to impair *Snail* function and then examined for developmental defects in each cell population.

We previously demonstrated a role for *Snail* during lamprey neural crest migration and differentiation (York et al., 2017). Here, we asked if *Snail* might also be necessary for establishment of the neural plate border GRN module by examining expression of several

neural plate border transcription factors, including *Pax3/7*, *nMyc, ZicA*, and *Tfap2a* (Betancur et al., 2010; Milet and Monsoro-Burq, 2012; Nikitina et al., 2008; Plouhinec et al., 2014; Sauka-Spengler et al., 2007). Our results suggested that *Snail* is not required for establishment of the neural plate border via *Pax3/7* (0/12 loss of expression, 0%, Fig. 1A, G). However, we did find that *Snail* patterns the medial-lateral axis of the neural plate border by repressing lateral expansion of *Pax3/7*. Indeed, *Pax3/7* expression expanded laterally by 34% in *Snail* mutants relative to controls ($z_{2,22}$ = -3.64, p = 0.0003; Fig. 1A, G, M). Our CRISPR knockouts also revealed that *Snail* is required for activation of *nMyc* and *ZicA* in the neural plate border, as evidenced by nearly complete loss of expression of these markers in the embryos analyzed (*nMyc*: 17/18, 94%, Fig. 1B, H; *ZicA*: 14/17, 82%; Fig. 1C, I). By contrast, *Snail* was not required for *Tfap2a* expression (0/11 loss of expression, 0%, Fig. 1D, J), nor for patterning the spatial boundaries of *Tfap2a* expression along the medial-lateral axis of the neural plate border ($t_{2,20}$ = -0.61, p = 0.55, Fig. 1M).

After demonstrating that *Snail* regulates development of the neural plate border, we next asked if *Snail* might be required for development of the pre-placodal ectoderm, given that its expression extends into this area (Fig. S2). In vertebrates, the pre-placodal domain is delineated in the neuroectoderm just anterior and lateral to the neural plate border, which can be marked in part by expression of *Dlx* cognates in gnathostomes and lamprey (*DlxB*) (Betancur et al., 2010; Sauka-Spengler et al., 2007). Our findings suggest that *Snail* helps establish the pre-placodal ectoderm in lamprey as most embryos (23/26, 88%, Fig. 1E, K) showed a near-total loss of *DlxB* expression anteriorly.

Finally, we tested if *Snail* was required for development of the neural plate by examining expression of *SoxB1a*, a lamprey member of the SoxB family of transcription factors that are important regulators of early CNS development across bilaterians (Pevny and Placzek, 2005; Royo et al., 2011). Although *Snail* has a strong mRNA signal throughout the lamprey neural plate (Fig. S2), we found no evidence that *Snail* is functionally required for *SoxB1a*-mediated establishment of this embryonic domain (0/13 loss of expression, 0%, Fig. 1F, L). In jawed vertebrates, loss of neural plate border transcription factors (e.g., *Snail*) can result in compensatory lateral expansion of neural plate markers (e.g., *SoxB*) into the neural plate border (Langer et al., 2008). However, spatial measures of *SoxB1a* gene expression did not reveal significant differences between controls and *Snail* CRISPR mutants ($z_{2,24}$ = -0.52, p = 0.60, Fig. 1M). In summary, we found that lamprey *Snail* is essential for proper development of the neural plate border and pre-placodal ectoderm but is not required for establishment or patterning of the neural plate.

Snail expression prefigures domains of CNS neurogenesis and differentiation

After examining the expression and function of *Snail* in the lamprey neuroectoderm (Fig. 1, Fig. S2), we characterized its expression from the onset of neurogenesis (~T24) into later stages of neural differentiation (~T26) in the CNS and PNS. Following early expression in the neural plate and subsequently closed cranial neural tube (Fig. S2), lamprey *Snail* expression is maintained in the cranial CNS at T24 (Fig. 2A), accumulating in the centrally located ventricular zone (Fig. 2B), where vertebrate CNS neural stem cells arise, and also peripherally in the marginal zone (Fig. 2B) where some of the earliest differentiated neurons first appear, as evidenced by

immunostaining for the neuron-specific differentiation marker, Hu (Fig. 2C, D; Temple, 2001). Indeed, at T24 double labeling for *Snail* mRNA and Hu protein revealed partially overlapping expression within the marginal zone (Fig. 2E). At later stages (T26) when *Snail* expression occupies the entire trunk CNS (Fig. 2F, G), a large area of the trunk CNS marginal zone contains Hu-positive neurons (Fig. 2H, I), and this domain overlaps with *Snail* mRNA localization (Fig. 2J). Taken together, these expression patterns demonstrate maintenance of robust expression of *Snail* throughout stages of neurogenesis into neuronal differentiation.

Snail is essential for early stages of PNS, but not CNS, neurogenesis

Based on *Snail* expression from the onset of neurogenesis through neural differentiation (Fig. 2), we asked if *Snail* activity might be essential for each of these processes in the CNS and PNS. We first tested *Snail* function during early neurogenesis in the CNS, which can be tracked by expression of *SoxB1a* at T24/T25 (Uy et al., 2012). Similar to that in the early neuroectoderm, we also found that *Snail* does not seem to be required for the onset of *SoxB1a*-mediated neurogenic expression in the lamprey CNS, during either early (0/22 loss of expression, 0%, T24, Fig. 3A, B) or relatively later (0/16 loss of expression, 0%, T25, Fig. 3C, D) stages of development. We then tested if *Snail* is required for neurogenesis in the cranial PNS by analyzing expression of *Six1/2*, *Pax3/7*, and *Phox2* in neurons of different cranial sensory ganglia at T25 (McCauley and Bronner-Fraser, 2002; Modrell et al., 2014; York et al., 2018). Our functional results suggested that *Snail* activity is essential for the onset of gene expression patterns that promote PNS neurogenesis, as demonstrated by complete or nearly complete loss of expression of *Six1/2* in epibranchial and posterior lateral line ganglia (9/13, 69%), *Pax3/7* in the

ophthalmic division of the trigeminal nerve (7/10, 70%), and in *Phox2*-positive epibranchial ganglia (12/17, 71%) (Fig. 3E–J). Moreover, in several of these affected T25 embryos (20/28, 68%) we observed variable levels of abnormal head development, including an overall decrease in head size as well as abnormalities in the oropharynx (e.g., Fig. 3F, H, J; see also Fig. 4H). These variable patterns are most likely linked to variation in loss or reduction of cranial neural crest cells (York et al., 2017), a feature that probably stems from generating mosaic CRISPR/Cas9 mutants (Square et al., 2015; Zu et al., 2016).

Snail is required for the differentiation of CNS neurons

Because our results thus far suggested that *Snail* is not required for establishment of the neural plate at T17 (Fig. 1) or CNS neurogenesis at T24/25 (Fig. 3), we asked if *Snail* was required for neuronal differentiation. We showed recently that two markers of neural differentiation, Hu protein and *Neural Cell Adhesion Molecule* (*NCAM*) mRNA, are expressed in the developing lamprey CNS and PNS (York et al., 2017). Here, we show that knockout of *Snail* function resulted in a complete or nearly complete loss of expression of both Hu (*n*=13/16, 81%, Fig. 4A–F) and *NCAM* (*n*=16/16, 100%, Fig. 4G, H) throughout much of the lamprey cranial and trunk CNS. Moreover, consistent with our analysis of *Snail* involvement in PNS neurogenesis (Fig. 3), we also observed loss of Hu (*n*=14/15, 93%) and *NCAM* (*n*=15/15, 100%) expression in PNS sensory neurons in the head and trunk (Fig. 4A–H).

Snail regulates CNS differentiation by promoting cell survival

Snail has been shown to regulate apoptosis and cell proliferation (Metzstein and Horvitz, 1999; Thellmann et al., 2003; Vega et al., 2004). Thus, we tested if increased cell death and/or decreased cell proliferation might account for the apparent loss of CNS neural marker expression in *Snail* CRISPR mutants by examining CNS expression of Caspase3 protein and *Proliferating Cellular Nuclear Antigen* (*PCNA*) mRNA, respectively (Barrallo-Gimeno and Nieto, 2005; Campbell et al., 2018; Lara-Ramirez et al., 2019; Metzstein and Horvitz, 1999; Vega et al., 2004). *Snail* CRISPR mutants had extensive apoptosis in the CNS (*n*=5/8, 63%, Fig. 4I, K), with most apoptotic cells occurring in the marginal zone where neurons are undergoing differentiation. By contrast, there appeared to be no appreciable change in *PCNA* expression in the neural stem cell-producing ventricular zone within the CNS of *Snail* mutants (*n*=0/8, 0%, Fig. 4J, L).

Ancestral state reconstruction reveals an ancient Snail-positive domain in the bilaterian CNS and CNS-neural crest-placode function in early vertebrates

Our combined expression and functional analyses point to an important role for lamprey *Snail* in promoting the development of CNS neurons, in addition to roles in neural crest and placode development at various stages (Figs. 1–4). Interestingly, these multi-functional roles in lamprey share similarities with vertebrates on one hand (*Snail* in neural crest, placodes) and invertebrates on the other (*Snail* in CNS/PNS neurogenesis). We asked if these expression domains reflect phylogenetically conserved states by mapping *Snail* expression patterns (CNS/PNS neurons, neural crest, placodes, mesoderm) onto a consensus eumetazoan phylogeny using character state

reconstruction analysis (Fig. 5, Fig. S3, Table S1). We found that expression in the mesoderm (or endoderm in diploblasts) probably represents the most ancient expression domain of *Snail* (Fig. S3). We also found support for an ancestral *Snail* expression domain in CNS/PNS neurons that likely dates back to the last common ancestor of bilaterians (Fig. 5). Finally, our analysis suggested that a *Snail*-positive CNS-"proto-neural crest"-"proto-placode" domain was likely present in the last common ancestor of tunicates and vertebrates, with *Snail* likely regulating an overlapping CNS-neural crest-placode domain in ancestral vertebrates, but being lost in one or more of these domains in some taxa (Fig. 5).

DISCUSSION

Neural crest cells and placodes are hallmarks of the vertebrate clade (Green et al., 2015; Meulemans and Bronner-Fraser, 2005). Yet, the mechanisms underlying the integration of key genes, such as *Snail*, into neural crest and placode regulatory networks from their pre-vertebrate origins have remained elusive. Our results here highlight key points of conservation, but also divergence, in the deployment of *Snail* not only between lamprey and jawed vertebrates, but also across a diverse range of eumetazoan taxa, with implications for the origin of neural crest and placode regulatory mechanisms.

An ancient role for Snail in patterning bilaterian neuroectoderm and regulating neurogenesis

One of the surprising features of lamprey embryogenesis that we have described here is the persistent expression of *Snail* in neurogenic tissues, particularly in the CNS. *Snail* is expressed in lamprey throughout the neural plate and then maintains robust expression within the neural tube

from neurogenesis to terminal differentiation of neurons (Fig. S2, Fig. 2). Expression of *Snail* in the lamprey neural tube was noted briefly in an earlier study (Rahimi et al., 2009), but the authors did not investigate the significance of this observation. This particular pattern has not, to our knowledge, been described in any jawed vertebrate, but there are striking parallels among several invertebrates. For example, a lamprey-like pattern of *Snail* CNS expression occurs in the invertebrate chordates, amphioxus and *Ciona* (Hudson et al., 2018; Hudson et al., 2015; Langeland et al., 1998). Similarly, *Snail* is expressed throughout the neuroectoderm of invertebrate deuterostomes such as hemichordates (Green et al., 2013). These patterns can also be found in diverse lophotrochozoans and ecdysozoans, including annelids, molluscs, insects, arachnids, crustaceans, and nematodes where *Snail*-positive cells occur in neural precursors and/or ventral nerve cord (Ashraf and Ip, 2001; Dill et al., 2007; Kerner et al., 2009; Kim et al., 2017; Klann and Stollewerk, 2017; Lespinet et al., 2002; Metzstein and Horvitz, 1999; Nieto, 2002; Sommer and Tautz, 1994; Southall and Brand, 2009; Ungerer et al., 2011; Weller and Tautz, 2003). Based on these shared patterns of expression and ancestral state analysis (Fig. 5), we propose that *Snail* was deployed in ancestral bilaterians for regulating CNS and neural development, in addition to mesoderm specification (Fig. S3).

Although we find overall patterns of evolutionary conservation between lamprey and invertebrates for a CNS-neurogenic role for *Snail sensu lato*, there are also lineage-specific differences, particularly in the extent and timing of expression. For example, expression of *Snail* in lamprey is ubiquitous in the neuroectoderm with gradual resolution to more discrete domains within neurogenic tissues (e.g., CNS marginal zone, Figs. S2, Fig. 2). By contrast, *Snail* expression in invertebrate chordates such as amphioxus similarly begins as broadly

neuroectodermal, but then quickly sharpens to the neural plate border, only to be upregulated secondarily throughout the larval CNS in a pattern even broader than that of lamprey (Langeland et al., 1998). In tunicates, *Snail* expression in the early gastrula patterns the neural plate and fates specific cells to the lateral CNS and is then upregulated in the cerebral vesicle of the tadpole larva (Esposito et al., 2017; Hudson et al., 2018; Hudson et al., 2015; Imai et al., 2006; Imai et al., 2009). There is also variation in *Snail* neurogenic expression among lophotrochozoans and ecdysozoans. *Snail* expression occurs in neural precursors and sensory organs in insects, arachnids and crustaceans, but also in differentiating neurons in some of these clades (Dill et al., 2007; Kerner et al., 2009; Lespinet et al., 2002; Weller and Tautz, 2003). In annelids and molluscs, there are typically two *Snail* paralogs, with expression in the ventral nerve cord and in cells fated to become paired ventral ganglia (Dill et al., 2007; Kim et al., 2017; Lespinet et al., 2002; Osborne et al., 2018).

In contrast to these examples of conservation, not all bilaterians have retained *Snail* for a neurogenic capacity. In echinoderms, *Snail* is expressed in mesodermal cells, particularly those that are migrating or ingressing, but there is no evidence that *Snail* is ever expressed during neurogenic patterning or differentiation (Saunders and McClay, 2014; Wu and McClay, 2007). This may be related to the radical reorganization of the bilaterally symmetrical CNS in ancestral echinoderms to one of pentameral symmetry that characterizes extant clades (Dominguez et al., 2002; Gee, 1996, 2018). This dramatic morphological alteration to the echinoderm CNS appears to have been accompanied by commensurate changes in gene expression patterns, as indicated by, for example, the lack of expression of *Hox* genes in the embryonic nervous system (Arenas-Mena et al., 2000; Gee, 2018). Similarly, in hagfish *Snail* does not appear to be expressed in the

CNS, presumably reflecting secondary loss of expression after splitting from the lamprey lineage (Ota et al., 2007), although there is yet to be a comprehensive analysis of *Snail* expression in this cyclostome group. In addition to spatiotemporal variation in expression, there is also evidence for functional variation in *Snail* regulatory activity. In insects, *Snail* regulates neurogenesis by controlling asymmetric cell divisions of daughter cells through *Prospero*, whereas in other invertebrates, *Snail* promotes neuroblast fate and survival (Ashraf and Ip, 2001; Lai et al., 2012; Metzstein and Horvitz, 1999; Thellmann et al., 2003; Weller and Tautz, 2003). Our results here in lamprey suggest that *Snail* may be involved in neural differentiation within the CNS by promoting cell survival rather than by controlling early neurogenesis. Regardless of these heterochronic and heterotopic shifts in *Snail* expression, or variation in function, our analysis nonetheless points to a general domain of evolutionarily conserved *Snail* activity in bilaterian nervous systems.

Snail patterning of neural crest and placode territories in the vertebrate neuroectoderm

We have identified important roles for *Snail* in the establishment, maintenance and patterning of the neuroectoderm in the lamprey embryo that highlight important similarities with jawed vertebrates on one hand and invertebrates on the other. Our results suggest that *Snail* plays at least two important roles early in lamprey neural crest development by regulating the neural plate border. First, *Snail* acts as a transcriptional repressor by setting the boundaries to *Pax3/7* expression laterally, thereby ensuring proper patterning of the medial-lateral axis of the neural plate border. In this regard *Snail* function in the lamprey neuroectoderm is similar to that of *Snail* governing medial-lateral patterning in the neuroectoderm of tunicates and points to a recurring

role for *Snail* in bilaterians as a transcriptional repressor to enforce embryonic territorial boundaries (Fujiwara et al., 1998; Kosman et al., 1991; Leptin, 1991). Second, we find that *Snail* is required for activation of *nMyc* and *ZicA* expression in the neural plate border, a result which places *Snail* relatively high within the neural plate border and neural crest specifier modules in the lamprey neural crest GRN. Although these findings support a general role for *Snail* in regulating the neural plate border in lamprey, it is worth pointing out that in gnathostomes it is *Pax3* and *Zic1* that synergistically activate *Snail2* expression, whereas our results suggest that a single *Snail* ortholog is required for activation of these neural plate border specifiers in lamprey (Sato et al., 2005). Thus, although there is certainly evolutionary conservation of the neural plate border regulatory module across vertebrates in the broad sense (Sauka-Spengler et al., 2007), our results suggest that some of the regulatory "wiring" for early neural crest development may be quite different in lamprey.

In addition to the neural crest, we also found that *Snail* plays a pivotal role in the development of another key vertebrate innovation, cranial ectodermal placodes. Our results suggest that *Snail* is essential for *DlxB*-mediated establishment of the pre-placodal territory in the anterior neuroectoderm. We therefore interpret the loss of cranial sensory ganglia in lamprey *Snail* mutants described here (Figs. 3, 4) and previously (York et al., 2017) as resulting primarily from genetic ablation of *Snail* during the earliest stages of placode development, although we cannot rule out that these phenotypes may be related to a requirement of *Snail* in placode differentiation. To our knowledge there is no evidence that *Snail* is essential for establishment of the pre-placodal territory in jawed vertebrates. Although this result in lamprey is quite different from that in other vertebrates, there are again interesting parallels to be found among tunicates.

For example, in *Ciona*, the *Snail*-positive lateral neural border gives rise to the evolutionary precursors of vertebrate neural crest and placodes, and recent work suggests that these cell populations share a common evolutionary origin (Abitua et al., 2015; Abitua et al., 2012; Horie et al., 2018). If this hypothesis is correct, then there should have been significant regulatory overlap in placode and neural crest development during early vertebrate evolution. In support of this, we find that *Snail* is expressed in the pre-placodal ectoderm and neural plate border in lamprey and is essential for early development of each of these populations simultaneously. We therefore propose that the broad expression of lamprey *Snail* in the early neuroectoderm enables the dual regulation of both neural crest and placodes. Similarities in *Snail* activity among lamprey, jawed vertebrates and invertebrate chordates, supports the idea of a pre-vertebrate regulatory link between neural crest and placodes, coupled in part by *Snail*, that was retained in ancestral vertebrates.

Implications for evolution of vertebrate neural crest and placodes

Although there is no evidence that the invertebrate chordate amphioxus has neural crest or placodes, there is now a strong case to be made that tunicates—the sister group to all vertebrates—do have rudiments of each of these cell populations (Abitua et al., 2015; Abitua et al., 2012; Horie et al., 2018; Stolfi et al., 2015). Intriguingly, the "proto-neural crest" and "proto-placodes" in tunicates derive from a *Snail*-positive neural plate border, with additional *Snail* expression in the tunicate neural plate and neural tube that patterns the medial-lateral axis and specifies CNS lineages (Abitua et al., 2015; Abitua et al., 2012; Hudson et al., 2018; Hudson et al., 2015; Stolfi et al., 2015). These shared functions for *Snail* in the CNS, proto-neural crest, and

proto-placodes are reminiscent of *Snail* activity among invertebrates (CNS, PNS neurons) and jawed vertebrates (neural crest, placodes), but there has been no evidence thus far for a *Snail*-mediated regulatory link between these populations that spans the invertebrate-vertebrate divide.

Our results here describing the expression and multiple functional roles of lamprey *Snail* in the CNS, neural crest and placodes now provide evidence for such a link. Conservation of *Snail* expression and function in the lamprey CNS, neural crest and placodes, coupled with analysis of *Snail* expression across bilaterians, suggests a new hypothesis for the integration of *Snail* into the ancestral neural crest regulatory network and for regulation of placodes. In the last common ancestor of vertebrates and tunicates (i.e., olfactores), we hypothesize that *Snail* simultaneously regulated patterning of the neural plate/CNS as well as precursors of neural crest and placode populations (Fig. 5). During the invertebrate chordate-vertebrate transition (Fig. 5), we propose that the ancestral CNS expression domain of *Snail* was retained in jawless vertebrates and acquired novel functions and transcriptional targets (e.g., *SoxE*, *ZicA*, *nMyc*, *Pax3/7*) in bona fide neural crest and placode development, with secondary functions relating to neural crest migration and differentiation (Fig. 5; York et al., 2017). Ancestral vertebrates (Fig. 5) therefore would have had multiple functional roles for *Snail* including a symplesiomorphic function in CNS neurogenesis, and apomorphic functions in development of neural crest and placodes, with all of these roles being retained in lamprey (Fig. 5). As a corollary, our model predicts that the lack of a CNS-neural crest-placode function for *Snail* in jawed vertebrates may be the result of loss of *Snail* expression in much of the CNS proper, but retention of *Snail* in the dorsal neural tube for neural crest specification (Fig. 5). Similarly, this predicts that hagfish, too, have lost these ancestral domains (Ota et al., 2007), with trait loss being a common occurrence as

a result of their derived life history. Despite these variations, our findings nonetheless identify ancestral jawless vertebrates as occupying a key node intermediate to that of invertebrates (*Snail* in neurogenesis) and higher jawed vertebrates (*Snail* in neural crest), with a multi-functional role for *Snail* in CNS neurogenesis, neural crest and placode development.

We have recently shown that regulators of neural crest epithelial-mesenchymal transition (EMT) and migration in lamprey, including *Sip1*, *Zeb1* and *CadIIA* are co-expressed with *Snail* in a similar pattern throughout the CNS, concomitant with specification and migration of neural crest and CNS neurogenesis (York et al., 2017). These results raise the possibility that, similar to *Snail*, these and possibly other transcriptional regulators of neural crest development may have had their origins in early vertebrate evolution playing roles in both CNS neurogenesis and neural crest/placode development that eventually became partitioned exclusively to neural crest and/or placodes while losing the ancestral CNS neurogenic function in jawed vertebrates.

Finally, it is important to note that *Snail* is a key regulator of mesoderm development, and thus expression of *Snail* in mesodermal tissues points to an alternative hypothesis in which *Snail*-mediated control of neural crest and placode development could have been co-opted from a pre-existing mesodermal program (Fig. S3). Our results here, however, point to a deeply conserved role for *Snail* in the CNS-PNS-neuroectoderm that predated vertebrates and was then coupled to a novel neural crest and placode function early in olfactorean and vertebrate evolution (Fig. 5). Importantly, there is also evidence that *Snail*, along with *FoxD*, *SoxE*, and other neural crest transcriptional regulators are all co-expressed in the neuroectoderm and PNS of invertebrate deuterostomes and protostomes, a pattern not found in the mesoderm of these same groups (Lauri, 2013). Indeed, evidence from annelids suggests that transcriptional regulators and

differentiation markers within the neural crest GRN (e.g., *Prdm1, ColA, Brn3, Msx, Olig*) are all expressed with *Snail* in the larval CNS and/or PNS, rather than mesoderm (Lauri, 2013). Those observations support the notion that *Snail* may have been co-opted for neural crest and placode regulation, possibly from within a larger, conserved transcriptional network operating within CNS and PNS neurons that has deep origins in early bilaterian animals. Genome wide-regulatory analysis comparing neural crest and placode programs with those governing mesodermal and neuroectodermal development across vertebrates and invertebrates will help address this possibility.

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Taxon	*Snail* in neural crest/neural plate border (1=present; 0=absent) †	*Snail* in placodes (1=present; 0=absent) †	*Snail* in CNS/PNS or nerve net (1=present; 0=absent) **	*Snail* in mesoderm (1=present; 0=absent)	References
Gnathostomata	1	1	0	1	[1-20]
Petromyzontiformes	1	1	1	1	[21-23]
Tunicata	1	1	1	1	[24-31]
Cephalochordata	0	0	1	1	[32]
Hemichordata	0	0	1	1	[33]
Echinodermata	0	0	0	1	[34, 35]
Annelida	0	0	1	1	[36-40]
Mollusca	0	0	1	1	[41-43]
Insecta	0	0	1	1	[44-52]
Arachnida	0	0	1	1	[53]
Cnidaria	0	0	0	1*	[54-56]

Table S1. Data matrix and supporting references for ancestral state reconstruction analysis of *Snail* expression domains across eumetazoans for Fig. 5 and Fig. S3.

Notes:

* Diploblasts such as cnidarians express *Snail* in endoderm.

† Although several protostome invertebrates have lateral neural borders [57, 58] that may be homologous to neural plate borders in vertebrates this column refers only to taxa having, *sensu stricto*, neural plate borders that generate neural crest cells and placodes (vertebrates) or "proto-neural crest" and "proto-placodes" (tunicates).

** PNS in this column refers to Snail expression in neurons that are of non-placode and non-neural crest origin.

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Off-target gene identity	Off-target sequence match	Off-target accession #	*Snail* gRNA1 matching sequence (PAM)
***Petromyzon marinus* blood plasma apolipoproitein LAL2 mRNA**	CCCGCACCTTG	M15892.1	(CCN)CGCACCTTGTGCA
***Petromyzon marinus* CD45 gene**	CCCCGCACCT	DQ008073.2	(CCN)CGCACCTTGTGCA
***Petromyzon marinus* clone 3_LRRV_Contig4573_25302878 variable lymphocyte receptor B cassette gene, partial cds**	CCCCGCACCT	EF529212.1	(CCN)CGCACCTTGTGCA

Table S2. Summary of potential *Snail* gRNA1 CRISPR off-target sites obtained by BLAST analysis of the sea lamprey genome.

FIGURE LEGENDS

Fig. 1. *Snail* patterns the neural plate border and establishes the pre-placodal ectoderm but is not required for formation of the neural plate. (A–F) Control T17 embryos with expression patterns delineating the neural plate border (npb, *Pax3/7*, *nMyc, ZicA, Tfap2a*), pre-placodal ectoderm (ppe, *DlxB*), and neural plate (np, *SoxB1a*). (G–L) T17 *Snail* CRISPR mutants ($Snail^{CR}$) show lateral expansion of *Pax3/7* expression (compare expression between np and npb in A and G), and loss of *nMyc* and *ZicA* expression in the np and npb (asterisks, H, I). (J) *Tfap2a* expression in the nbp appears normal in $Snail^{CR}$ embryos. (K) Loss of *DlxB* transcripts in ppe (asterisk) is observed in $Snail^{CR}$ embryos, whereas no obvious change in *SoxB1a* expression is observed in the np (L). (M) Quantitative comparisons of spatial gene expression indices in controls versus $Snail^{CR}$ embryos (see Materials and Methods for details). Asterisk in panel "M" indicates statistically significant difference ($\alpha = 0.05$), whereas "ns" indicates not significant.

Fig. 2. *Snail* is expressed in the cranial and trunk CNS throughout neurogenesis. (A,B) During early CNS neurogenesis (T24), *Snail* is enriched in the cranial CNS (ccns) ventricular zone (vz), and especially the marginal zone (mz; neural tube outlined, B), where Hu expression in ccns mz neurons appears (C; neural tube outlined in D). (E) *Snail* and Hu expression at T24 overlaps in neural tube mz (arrowheads, neural tube outlined). In older embryos (T26), *Snail* mRNA in the trunk CNS (tcns in F) includes the vz and mz (G, neural tube outlined). At T26, differentiated tcns neurons and neural crest-derived dorsal root ganglia (drg) express Hu (H), with tcns Hu expression mostly in the mz (I, neural tube outlined). *Snail* and Hu expression at T26 overlaps in

the mz (arrowheads in J, neural tube outlined), and weakly in drg (drg in J). Other abbreviations: n, notochord.

Fig. 3. *Snail* is not required for early CNS neurogenesis but is essential for formation of cranial PNS neurons. (A) T24 control embryo showing *SoxB1a* neurogenic expression in the cranial and trunk CNS (ccns, tcns). (B) *Snail* CRISPR mutant ($Snail^{CR}$) shows no obvious loss of *SoxB1a* expression in ccns or tcns. (C) *SoxB1a* expression at T25 showing continued neurogenic expression of *SoxB1a* in ccns and tcns with (D) no appreciable change in expression in $Snail^{CR}$ embryos. (E) *Six1/2* expression in neurons of the cranial PNS (pllg, epg), upper lip (ul) and pharyngeal arches (arrow) of control embryo. (F) $Snail^{CR}$ embryos show nearly complete loss of cranial neural expression in the PNS (asterisks, loss of epg; arrowhead, loss of pllg), and expression in pharyngeal arches (arrow). (G) Control embryo with *Pax3/7* expression in the CNS (arrowhead) and placode-derived portion of opV neurons in the PNS. (H) Mutant embryo showing abrogated *Pax3/7* neuronal expression in the opV (asterisk) but retention of CNS expression (arrowhead). (I) Control embryo with expression of *Phox2* in the CNS (arrowhead) and in PNS neurons in epg. (J) $Snail^{CR}$ embryos show complete loss of *Phox2* expression in epg (asterisks), but maintain CNS expression (arrowhead). Abbreviations: e, eye; epg, epibranchial ganglion; opV, ophthalmic division of the trigeminal ganglion; ov, otic vesicle; pllg, posterior lateral line ganglion.

Fig. 4. *Snail* is required for CNS neural differentiation by enhancing cell survival. (A) T26 control embryo expressing the neural differentiation marker Hu in cranial CNS (ccns) neurons,

cranial PNS neurons (opV, g, p, pllg, epg), and neurons of trunk dorsal root ganglia (drg). *Snail* CRISPR ($Snail^{CR}$) mutants lose Hu in the ccns (asterisks, B) and cranial PNS neurons (arrowheads, B). (C) Hu expression in the trunk CNS (tcns) at T26 is prominent in the marginal zone (D, mz, neural tube outlined). Mutant embryos show loss of Hu-positive neurons in the tcns (arrowhead, E and arrowhead, F, with neural tube outlined). (G) Control *NCAM* expression at T26 in ccns, tcns and cranial PNS neurons. $Snail^{CR}$ embryos lose *NCAM* expression in the ccns and tcns (asterisks in H), and in cranial PNS neurons (arrowheads, H). (I) Control T26 neural tube cross section showing no cell death in the tcns (neural tube outlined, arrowhead shows dying cell outside neural tube). (J) Control T26 neural tube cross section in the tcns showing normal *PCNA* expression in the ventricular zone (vz). (K, L) $Snail^{CR}$ embryos show increased apoptosis in the periphery of the tcns (arrowheads, K, neural tube outlined), but no appreciable change in *PCNA* expression (arrowhead, L, neural tube outlined). Abbreviations: epg, epibranchial ganglion; g, geniculate ganglion; mmV, maxillomandibular part of the trigeminal ganglion; n, notochord; opV, ophthalmic part of the trigeminal ganglion; p, petrosal ganglion; pllg, posterior lateral line ganglion.

Fig. 5. Model for the evolution of *Snail*-mediated regulation of neural crest and placodes from an ancient role in bilaterian neurogenesis. Terminal branches, from left to right, show representative bilaterian groups including bony fish (jawed vertebrate), lamprey (jawless vertebrate), tunicate (invertebrate chordate), amphioxus (invertebrate chordate), acorn worm (hemichordate), sea urchin (echinoderm), spider (arachnid), fly (insect), polychaete worm (annelid), snail (mollusc). Common ancestors, indicated by lower case letters at node points, include “a”, vertebrates; “b”,

olfactores; “c”, chordates; “d”, deuterostomes; “e”, ambulacraria; “f”, ecdysozoa; “g”, protostomia; “h”, lophotrochozoa; “i”, bilateria. Circles with color schemes at terminal branches and ancestral nodes denote *Snail* expression domains in placodes (purple), neural crest (green) and/or CNS/PNS neurons. Ancestral state reconstruction places *Snail* expression in the CNS/PNS/neuroectoderm as ancestral for bilaterians (black circle at “i”, 2 character changes required), with secondary loss (empty circle) of this domain in invertebrate deuterostomes such as echinoderms. In the olfactorean ancestor, *Snail* acquired expression in “proto-neural crest” and “proto-placodes” and patterned the neural plate and CNS. At the origin of vertebrates, evolutionarily conserved *Snail* CNS expression was retained and acquired downstream transcriptional targets such as *Pax3/7*, *SoxE*, *nMyc* and type II cadherins during the evolution of bona fide neural crest (see Results and Discussion). This ancestral CNS-neural crest-placode function for *Snail* is retained in lamprey.

Fig. S1. Molecular phylogeny of the *Snail1*/*Snail2*/*Scratch* superfamily of zinc finger transcription factors. The sea lamprey, *P. marinus*, has a single *Snail* gene (Pm Snail) that is distinct from that in the Scratch clade (Pm Scratch). This tree was generated using MUSCLE alignment and maximum likelihood analysis, with results of 1000 bootstrap replicates at node points. The following abbreviations denote species (italics), with NCBI accession numbers in parentheses. Bf, *Branchiostoma floridae* (Snail: AAC35351.1); Dm, *Drosophila melanogaster* (C2H2 Zinc finger homeobox: CG15269, Snail: NP_476732); Dr, *Danio rerio* (Snail1a: NP_001300628.1, Snail1b: NP_571064.2, Snail2: NP_001008581.1, Scratch1a: NP_001107073.1, Scratch1b: NP_001014369.1, Scratch2: NP_998802.1); Gg, *Gallus gallus*

(Snail1: NP_990473.1, Snail2: CAA54679.1, Scratch2: ACO70860.1); Lj, *Lethenteron japonicum* (Scratch: retrieved from http://jlampreygenome.imcb.a-star.edu.sg/); Mm, *Mus musculus* (Snail1: NP_035557.1, Snail2: NP_035545.1, Scratch1: NP_570963.1, Scratch2: NP_001153882.1); Pm, *Petromyzon marinus* (Snail: ACL98051.1); Xl, *Xenopus laevis* (Snail1: NP_001079925.1, Snail2: NP_001079751.1, Scratch1-like: XP_018079536.1, Scratch2-like: XP_018090521.1).

Fig. S2. Lamprey *Snail* expression in the neuroectoderm prefigures neural crest, CNS, and placode territories. (A) At T17, *Snail* transcripts localize throughout the neural plate (np), neural plate border (npb), and pre-placodal ectoderm (ppe). Cross-sections from (A) at low (B) and high power (C) magnification show contiguous expression of *Snail* in the npb and np. (D) *Snail* expression remains in the cranial neural tube at T20. (E) Low power magnification of cross-section from (D) shows *Snail* mRNA in the neural tube (nt) and cranial mesoderm (arrowheads). (F) High power magnification of (E) details *Snail* expression in cells coalescing into dorsal-ventral columns within the neural tube (arrowheads in F, nt outlined). (G) By T21, *Snail* expression occurs in neural crest cells (ncc) in the anterior head and somites (s). (H) Low power magnification of cross-section from (G) shows *Snail* expression in the nt and in somitic mesoderm (arrowhead) with high power magnification (I) detailing expression throughout the nt in premigratory ncc and cells extending into the ventral neural tube (arrowheads, I; nt outlined). Other abbreviations: ar = archenteron; n = notochord; yp, yolk platelets.

Fig. S3. Ancestral state reconstruction of *Snail* expression domains in mesodermal/endodermal tissues mapped onto a eumetazoan phylogeny. Terminal branches, from left to right, show representative bilaterian groups including bony fish (jawed vertebrate), lamprey (jawless vertebrate), tunicate (invertebrate chordate), amphioxus (invertebrate chordate), acorn worm (hemichordate), sea urchin (echinoderm), spider (arachnid), fly (insect), polychaete worm (annelid), snail (mollusc), hydrozoan (cnidarian). Common ancestors, indicated by lower case letters at node points, include “a”, vertebrates; “b”, olfactores; “c”, chordates; “d”, deuterostomes; “e”, ambulacraria; “f”, ecdysozoa; “g”, protostomia; “h”, lophotrochozoa; “i”, bilateria; “j”, eumetazoa. Ancestral state analysis (see Materials and Methods; data matrix provided in Table S1) suggests that the last common ancestor of eumetazoans had *Snail* expression in endoderm-derived tissues (pink circle), which is retained in extant cnidarians. *Snail* retained a role in the development of mesoderm (black circle) with the advent of triploblasty and bilateral symmetry.

Fig. S4. Sequences of select individual *Snail* mutant lamprey embryos in Fig. 4. For each embryo, the lamprey *Snail* wildtype sequence (Pm Snail) is at the top with the gRNA target site in red and PAM site underlined. Four clones from each individual were sequenced to confirm mutagenesis, with the number of base pairs deleted (-) or unchanged (WT) listed to the right of each sequence. Deletions are indicated by dashed lines; transition/transversion substitutions are colored green. Overall mutagenesis efficiency was 95% (19/20 mutant alleles).

Fig. S5. Sequencing results from five randomly pooled *Snail* gRNA1-injected T26 lamprey embryos to estimate mutagenesis efficiency. Lamprey *Snail* wildtype sequence (Pm Snail) is at the top with the gRNA1 target site in red and PAM site underlined. Forty clones sequenced from the genomic DNA of the pooled embryos are listed below the wildtype sequence, with the number of base pairs deleted (-), inserted (+) or unchanged (WT) listed to the right of each sequence. Deletions are indicated by dashed lines; insertions are colored blue; transition/transversion substitutions are colored green. Mutagenesis efficiency is 90% (36/40 mutant alleles).

Fig. S6. Sequencing of potential *Snail* gRNA1 off-target sites in the lamprey genome. (A) Comparison of the lamprey *Snail* gRNA1 target locus and Apo LAL2 potential off-target reveals sequence similarity (red base pairs) in the 13 bp proximal to the PAM site (underlined). However, sequencing of 10 Apo LAL2 clones from *Snail* mutant embryos reveals no mutations. Similarly, *Snail* gRNA1 shares sequence similarity with CD45 (B) and VLRB (C) potential off-target loci (red base pairs) near the PAM site (underlined), but sequencing of 10 clones from each locus in *Snail* mutant embryos failed to uncover mutagenesis.

Figure 1

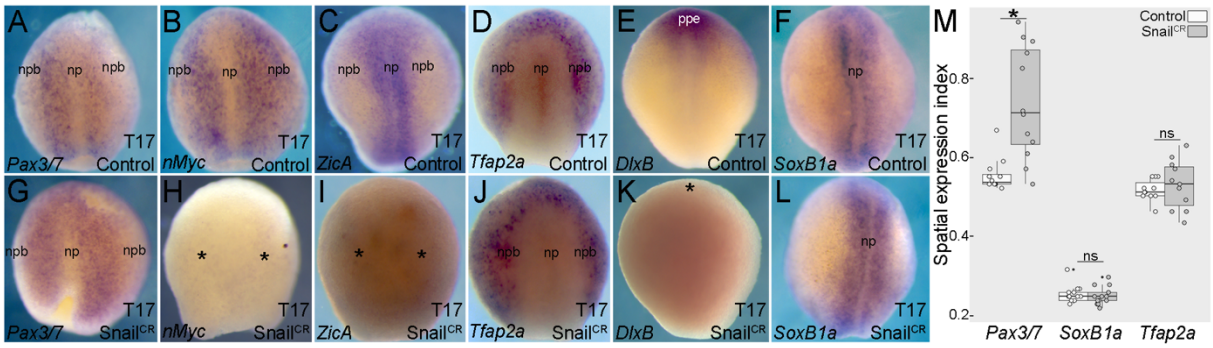
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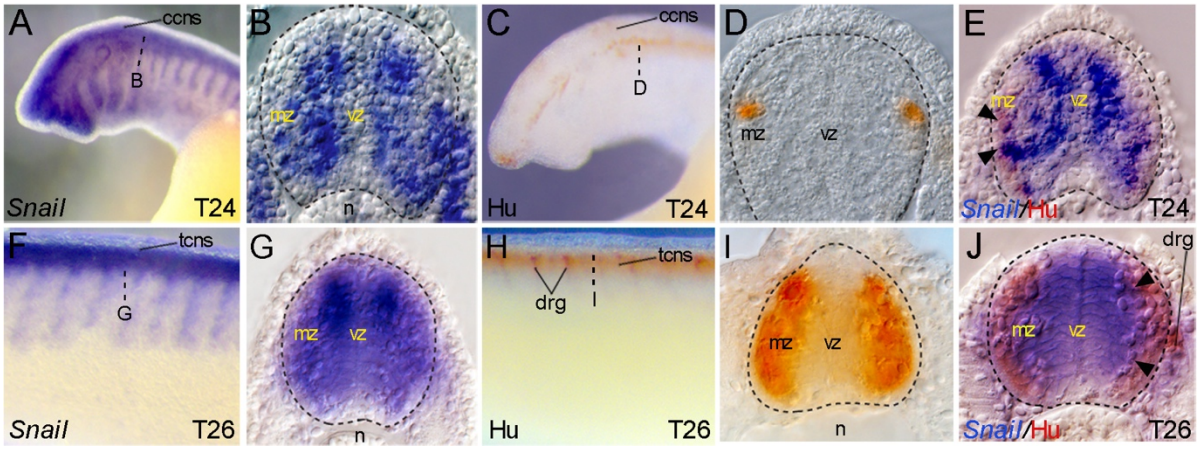
Figure 2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vz
Snail/Hu
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F
tcns
G
Snail
T26
G
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vz
n
H
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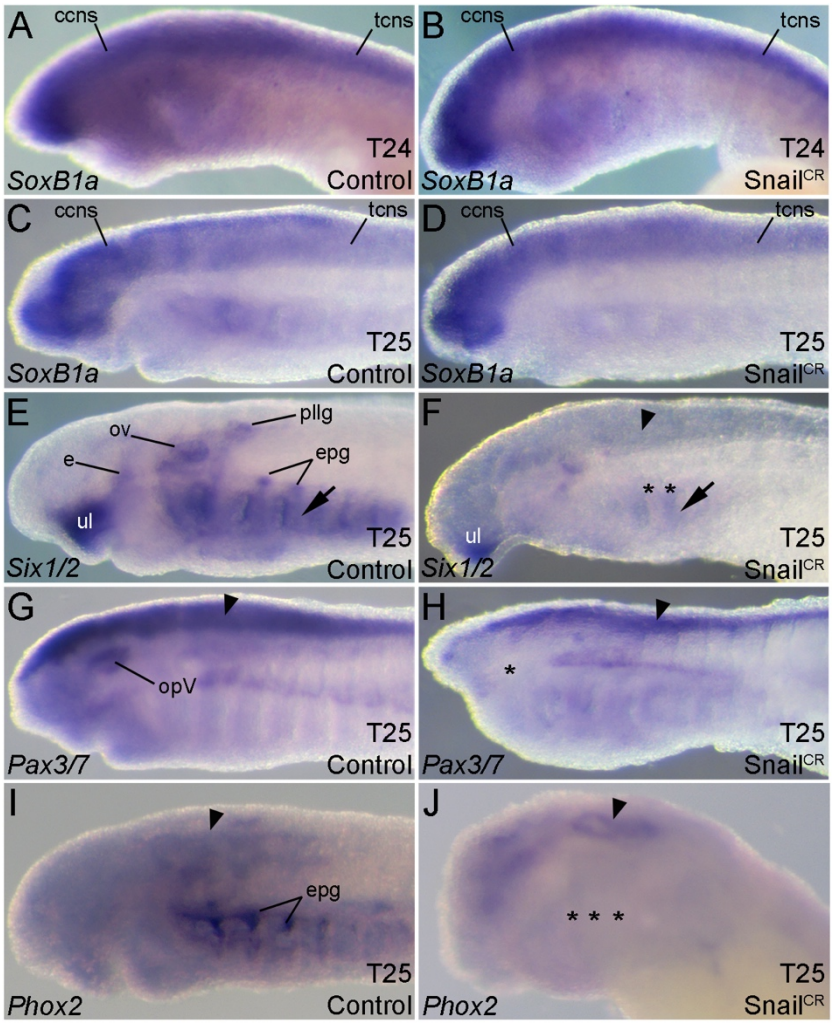
T26

Figure 3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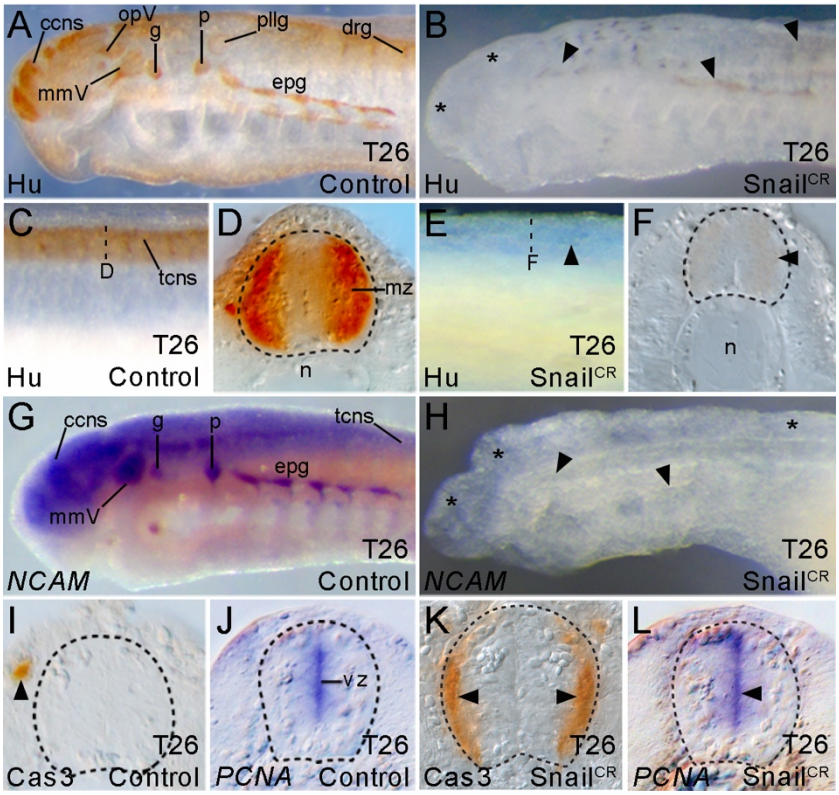
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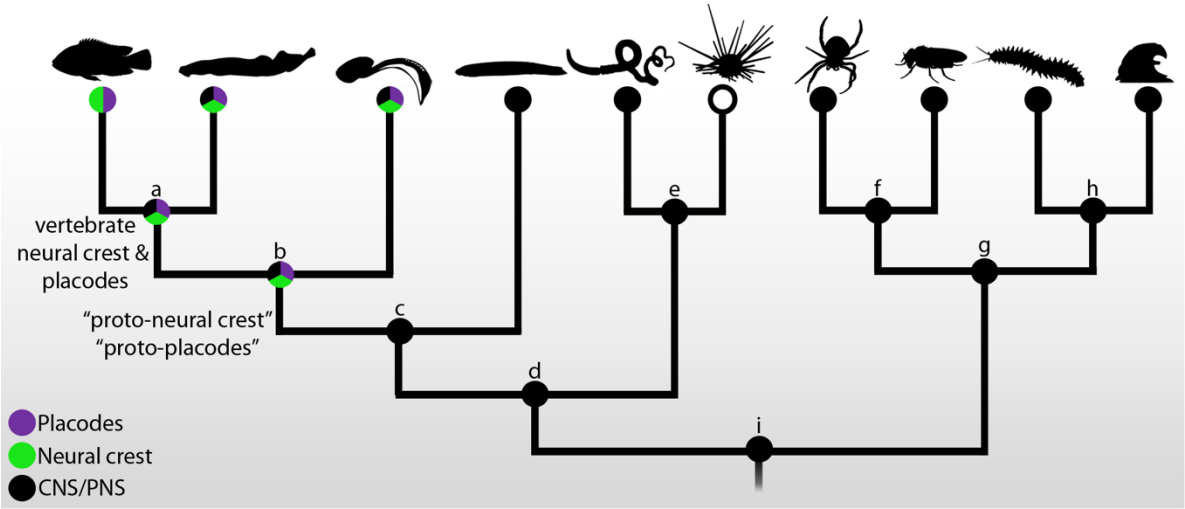
Figure 5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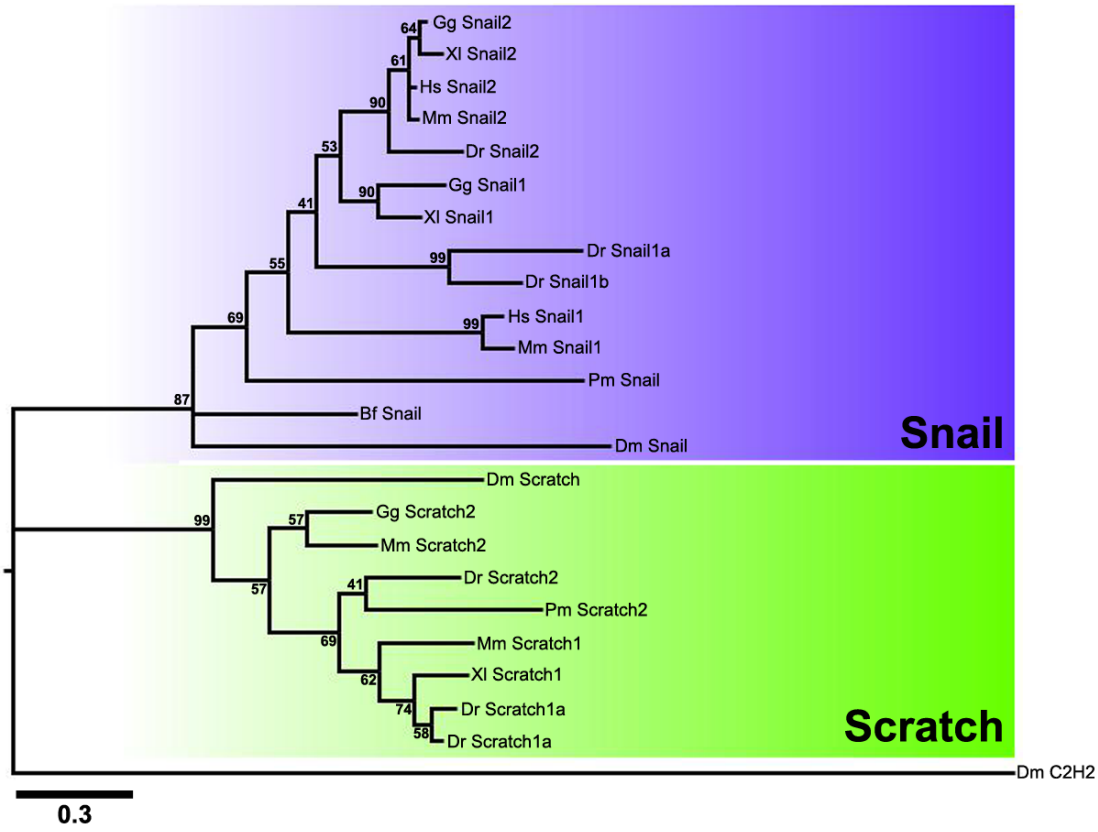
Placodes
Neural crest
CNS/PNS

Figure S1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Snail
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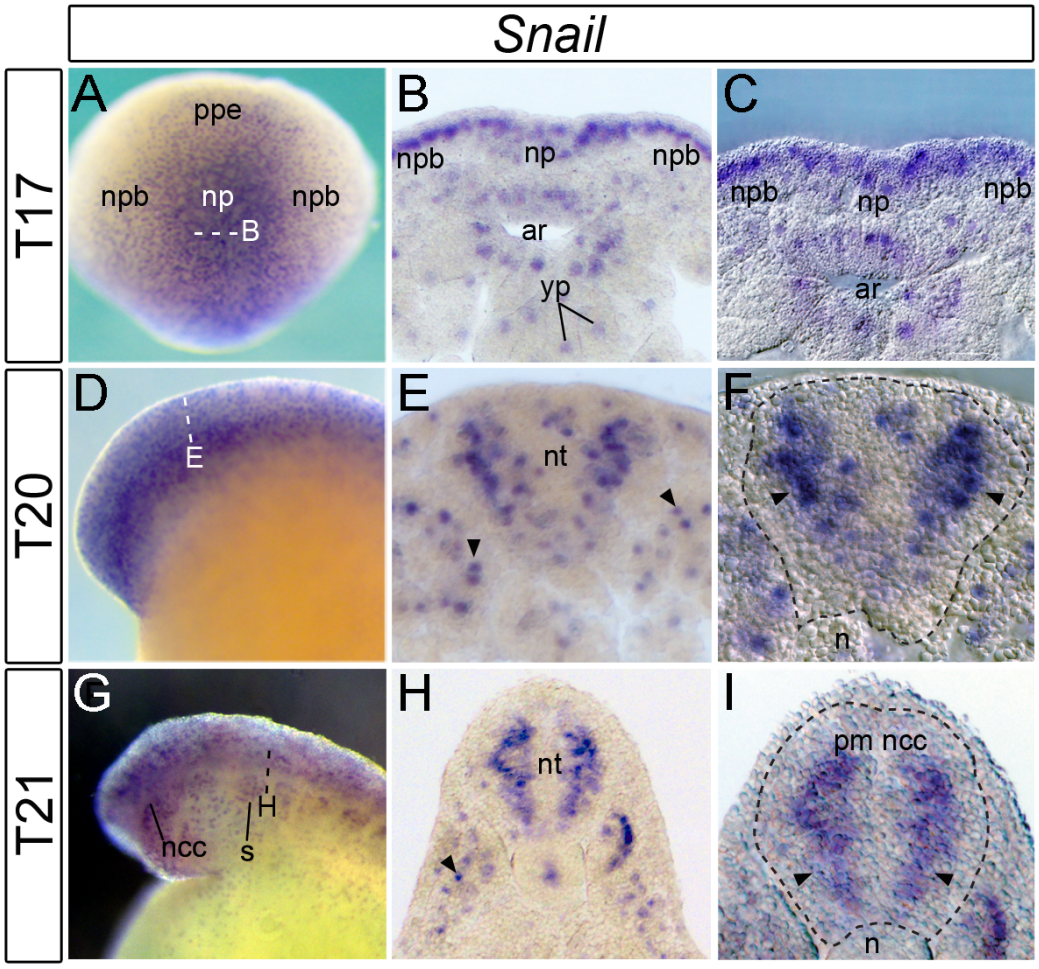
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Figure S3

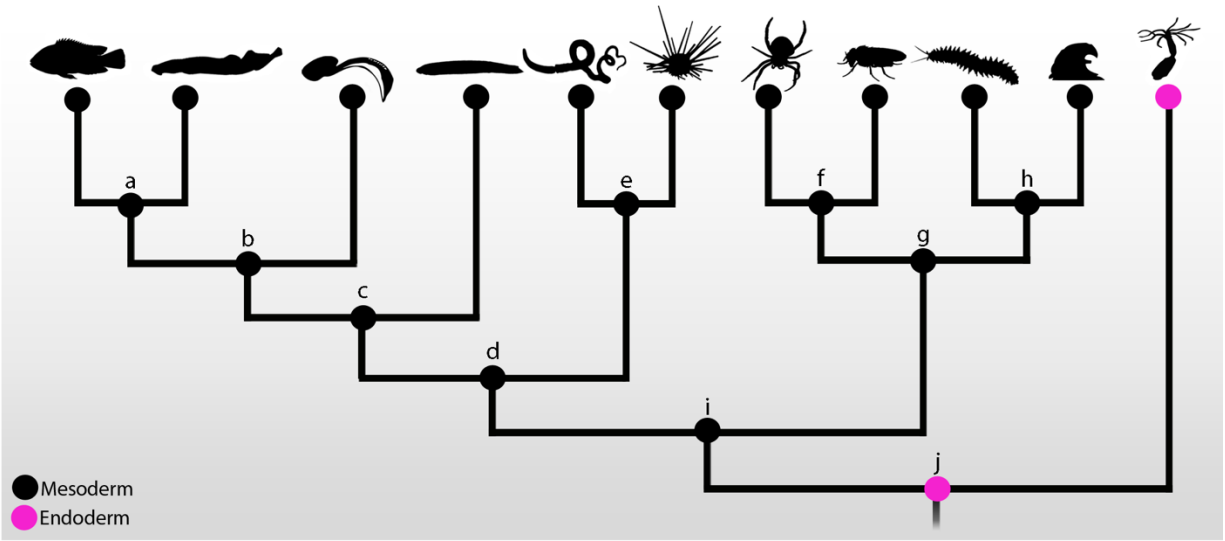

Figure S4

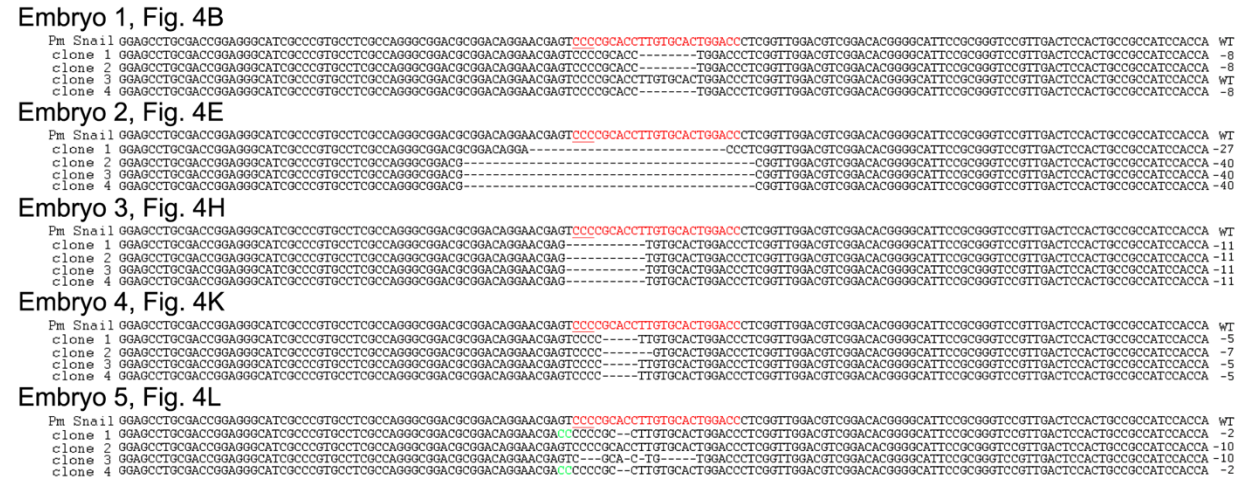
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Figure S5

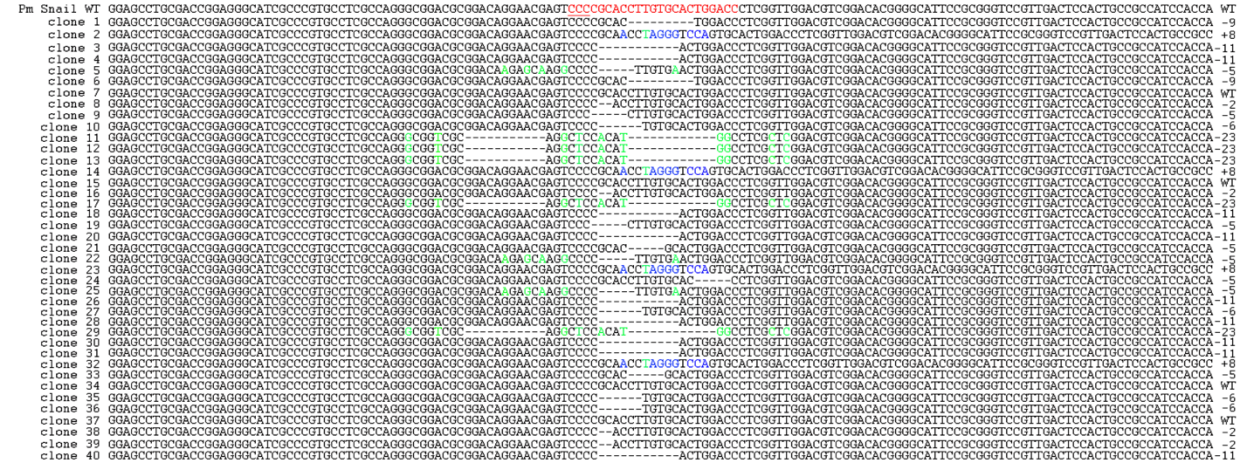
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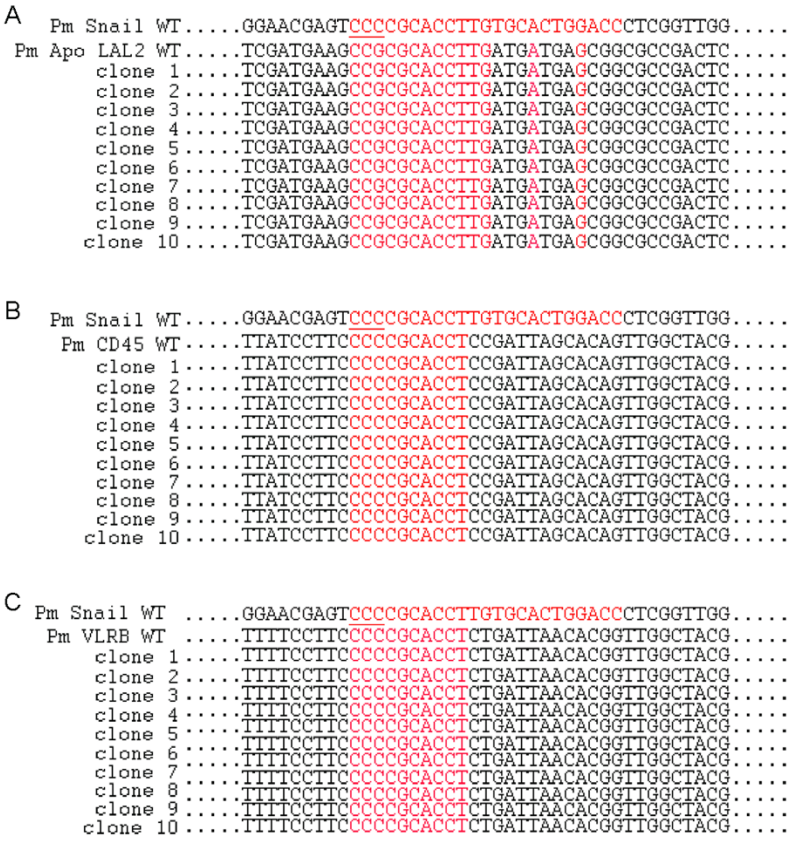
Figure S6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clone 1.....TCGATGAAGCCGCGCACCTTGATGATGAGCGGCGCCGACTC.....
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C
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Pm VLRB WT .....TTTTCCTTCCCCCGCACCTCTGATTAACACGGTTGGCTACG.....
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CHAPTER 6: EVOLUTION OF THE VERTEBRATE NEURAL CREST FROM AN ANCESTRAL PAN-NEURAL CELL POPULATION

(formatted for submission to *Nature*)

SUMMARY PARAGRAPH

The origin of neural crest cells was a catalyst for the evolution and elaboration of the vertebrate body plan[1]. In jawed vertebrates, neural crest cells arise exclusively in the dorsal neural tube, and then migrate throughout the embryo where they form much of the peripheral nervous system, pigment, and craniofacial cartilage and bone[2]. The current paradigm for the origin of neural crest cells is that they evolved within the embryonic dorsal neural folds of ancestral vertebrates[2,3]. Here, using a basal vertebrate, the sea lamprey, we show that a core set of transcription factors controlling neural crest specification and migration are expressed throughout the entire dorsoventral axis of the embryonic lamprey neural tube. We show that a marker of migratory crest in vertebrates, *n-Myc*[4], is expressed in cells emigrating from the lamprey ventral neural tube in a neural crest-like stream, and CRISPR/Cas9-mediated knockout of n-Myc results in reduction of premigratory neural crest cells and loss of the neural crest-derived head skeleton. Vital dye labeling *in vivo* reveals that cells in both the dorsal and ventral neural tube migrate in a neural crest-like manner into the embryonic pharynx, and *in vitro* slice culture assays confirm that individual cells can migrate from the ventral neural tube. We propose a new paradigm in which neural crest cells in an ancestral vertebrate arose throughout the entire embryonic neural tube—rather than from the dorsal neural tube alone—and were able to migrate from any position along the neural dorsal-ventral axis, a feature that may been inherited from invertebrate chordates. This new model challenges 150 years of thinking in neural crest and vertebrate evolution[5] and predicts that in jawed vertebrates these ancestral migratory domains were lost, with neural crest becoming secondarily localized to the dorsal neural tube.

MAIN TEXT

Classical hypotheses for the evolutionary origin of the neural crest suggest that this cell population evolved within the embryonic dorsal neural folds of ancestral vertebrates, culminating in their migration from the dorsal neural tube as a stem-cell-like population (Fig. 1a) [2,3,6-9]. Evolutionary-developmental studies on the closest extant relatives of vertebrates, the invertebrate chordates (tunicates and amphioxus), have identified homologues of several genes that specify neural crest fate in vertebrates, the expression patterns of which are highly variable and can be found in disparate tissues [2,10]. Because the expression patterns of these genes localize exclusively to the dorsal neural folds in vertebrates during stages of neural crest formation, the current model for the evolution of the neural crest suggests that these expression domains were co-opted into the neural folds in stem vertebrates[11,12]. Although this model has been useful for outlining a broad evolutionary history of neural crest cells (Fig. 1a), we currently have no insight into the ancestral cellular and genetic basis for the origin of bona fide migratory neural crest in the first vertebrates. In an attempt to address this knowledge gap, we re-examined the transcriptional control of neural crest development in a basal jawless vertebrate, the sea lamprey—an ideal model for deciphering the developmental evolution of early vertebrates[13].

Previous findings have suggested that lamprey neural crest development is similar to that of jawed vertebrates, and that the gene regulatory network orchestrating neural crest development is conserved to the base of vertebrates[14-16]. In jawed vertebrates, the transcription factors Snail, Sox10, FoxD3, Id and Sip1 specify neural crest cell fate and mediate neural crest migration by activation of type II cadherins in the dorsal neural tube[4,17]. Although lamprey deploys homologues of these same regulatory genes to commence neural crest development[14,17],

our results show that their spatial expression in the lamprey neural tube is radically different from that of jawed vertebrates[17]. We found that these transcription factors in lamprey (Snail, SoxE2, FoxD-A, Id-B, Sip1, CadIIA) are instead expressed throughout the entire dorsoventral axis of the neural tube (Fig. 1b–k, n, o) in Tahara stage 22[18] (T22) embryos. Nevertheless, lamprey also has dorsally-restricted expression of the neural crest transcription factors SoxE1 and Tfap2a, similar to their homologs in jawed vertebrates (Fig. 1l, m, p, q)[4]. The collective expression of neural crest specifiers and effectors of migration (Fig. 1r) suggests that the core regulatory program for neural crest specification and migration in lamprey may not be restricted to the dorsal-most region of the neural tube, as in jawed vertebrates.

The transcription factor n-Myc, is a marker of bona fide migratory neural crest in jawed vertebrates and is required for early neural crest development[4]. In contrast to jawed vertebrates, we observed *n-Myc*-positive cells throughout the neural tube, with the exception of the floor plate (Fig. 2b) and in a discrete stream exiting from the ventral neural tube in a neural crest-like pattern (Fig. 2a, b). We then asked if these cells emigrating from the ventral neural tube in lamprey embryos are functionally required for neural crest development by using CRISPR-Cas9[17,19-21] mediated deletions to knock out (KO) n-Myc function. Functional perturbation of n-Myc caused a reduction of *Snail* expression in premigratory and migratory neural crest (Fig. 2c, d, n=15/20), and led to complete loss of neural crest-derived *SoxE1*-positive prechondrocytes in the pharyngeal arches among 60% of embryos analyzed (Fig. 2e, f, n=12/20), compared to negative control CRISPR injections. To determine that putative n-Myc CRISPR phenotypes were directly related to deletion of DNA sequence from the n-Myc target locus, we isolated and sequenced genomic DNA surrounding the CRISPR target site. (Fig. S1). Taken together, these

results suggest that migratory cells emigrating from the ventral neural tube are required for both neural crest specification and formation of neural crest-derived structures.

Because our gene expression and functional analyses suggested that cells throughout the lamprey neural tube express canonical markers of neural crest cells (Figs. 1, 2), and are required for neural crest development (Fig. 2), we sought to verify that cells throughout the neural tube were capable of migrating *in vivo*, a hallmark of vertebrate neural crest[22]. To test this notion, we performed vital dye labeling using lipophilic DiI. Focal DiI injections in the dorsal neural tube of T22 embryos (n=5 dorsal labeled embryos) confirmed that "canonical" neural crest from the dorsal domain migrated as a stream of mesenchymal cells ventrally toward the pharyngeal arch mesenchyme (Fig. 3a, b). We then focally injected DiI into the ventral neural tube at T22 (n=3 ventral labeled embryos) and observed streams of cells exiting from along the entire lateral axis of the neural tube (Fig. 3d, left side) including the ventral most region of the neural tube, and moving ventrally into the pharyngeal arch mesenchyme (Fig. 3c, d), similar to that of labeled neural crest cells migrating from the dorsal neural tube (Fig. 3a, b).

To corroborate our *in vivo* DiI labeling experiments, we developed an *in vitro* slice culture assay[23] that allowed us to specifically label and monitor in real time the migration of mesenchymal cells from the lamprey ventral neural tube. Briefly, we sectioned live lamprey embryos in the cranial region at migratory neural crest stages (T22) and microinjected these sections with a small bolus of DiI focally in the ventral neural tube. Successfully labeled sections (n=2) were explanted in cell culture medium and monitored for cell migration by time-lapse microscopy. Within 6 h, individual cells in ventral neural tubes had begun to migrate laterally and ventrally away from the labeled site (Fig. 3e–k, Supplementary Movie S1). By 6.5 h, labeled

migratory cells had exited the neural tube and by 9 h were observed moving along a ventral pathway (Fig. 3i–k, Supplementary Movie S1). This pattern of migration from the ventral neural tube was similar to *n-Myc*-positive cells and DiI-labeled cells emigrating from the ventral domain of the lamprey neural tube (Fig. 2a, b; Fig. 3a–d). To rule out the possibility that labeled cells in the ventral neural tube only migrate as an artifact of culture conditions, we cultured DiI-labeled cross-sections from the hindbrain of older lamprey embryos (T25) when neural crest cells no longer migrate from this region[16]. We found that labeled cells in these experiments never migrate from the ventral neural tube (Supplementary Movie and Figure S2, n=2).

Our findings reveal unprecedented features regarding the formation and migration of neural crest cells in a basal jawless vertebrate, the sea lamprey, compared to their jawed vertebrate counterparts. By virtue of its basal phylogenetic position among vertebrates[13,24], comparative developmental studies in lamprey embryos can inform our understanding of the origin and evolution of vertebrate innovations, including neural crest cells and their unique migratory properties[13]. Collectively, our gene expression, functional analysis, and cell labeling experiments suggest that a population of cells with the genetic and phenotypic hallmarks of vertebrate neural crest cells can form throughout and migrate from any dorsoventral axial position within the neural tube in lamprey embryos.

In light of our findings, we propose a new evolutionary hypothesis for the origin of vertebrate neural crest cells (Fig. 4). We suggest that the neural crest evolved as a migratory and multipotent cell population in the neural tube of ancestral vertebrates after diverging from the invertebrate chordates, which lack migratory neural crest (Fig. 4a–c). However, we propose that neural crest cells did not initially appear in the dorsal neural tube alone, as is currently accepted

[8,22,25-27], but rather were capable of forming throughout the entire dorsoventral axis of the embryonic central nervous system in ancestral vertebrates, with this condition being largely retained in lamprey (Fig. 4d). This pan-neural domain of the neural crest is also present during the earliest stages of lamprey neural crest development, as observed by the expression of *Snail*, *Wnt6*, *Pax3/7*, *n-Myc*, *ZicA* and *BMP2/4b* throughout the neural plate and neural plate border in lamprey embryos[14]. The combinatorial expression of these early patterning genes prefigures the dorsoventral expression domains of neural crest specifiers that we observed throughout the lamprey neural tube (Figs. 1, 2), thereby rendering these cells competent to produce migratory neural crest (Fig. 4d).

We hypothesize that along stem lineages leading to crown-group jawed vertebrates, loss of regulation controlling specification and migration in the mid- and ventral neural tube localized the neural crest exclusively to the dorsal neural tube where they are specified and migrate in extant jawed vertebrates (Fig. 4e). Although cells in the ventral neural tube of jawed vertebrates never endogenously form migratory neural crest, there is evidence that they are still competent to do so. Forced expression of the neural crest transcription factors Sox10[28] or Sox5[29] throughout the neural tube in jawed vertebrate embryos produces a "lamprey-like" pattern of ectopic migratory cells throughout the neural tube that express *Sox10*, HNK-1 and *RhoB*, all of which are markers of migratory crest in vertebrates[4]. We suggest that these findings may be interpreted as experimental re-activation of an ancient vertebrate neural crest domain that is retained as a vestige in jawless vertebrates such as lamprey.

Although no jawed vertebrate embryo shows comparable pan-neural expression of neural crest transcriptional regulators, the invertebrate chordate amphioxus has expression patterns of

Snail[30] and SoxE[10] that are strikingly similar to that observed for neural crest specifiers and migration regulators throughout the neural tube in lamprey[7,30]. These genes are enriched throughout the neural tube in amphioxus, despite the lack of neural crest in this animal. Similarly, tunicates, the sister group to vertebrates show pan-neural plate expression of neural crest factors such as Snail, FoxD, Myc and others [7], reminiscent of the condition we observe in lamprey. The most parsimonious explanation for these plesiomorphic patterns between lamprey and invertebrate chordates is that migratory proto-neural crest cells evolved from an ancestral cell population having a protochordate-like expression pattern of neural crest homologues throughout the neural plate and neural tube in stem vertebrates, rather than being co-opted directly to the dorsal neural folds. In summary, our results identify a new developmental-evolutionary sequence for positioning the vertebrate neural crest into neural folds and the dorsal neural tube, and provide insight into how neural crest regulatory mechanisms controlling specification and migration have evolved and diversified across 500 million years of vertebrate evolution.

FIGURE LEGENDS

Figure 1. Evidence for a neural crest-like domain throughout the lamprey neural tube. (a), Illustration of canonical neural crest development in jawed vertebrates . Markers[4] of pre-migratory and migratory neural crest are expressed throughout much of the neural tube in T22 lamprey embryos (arrowheads, b–k, n, o) and in the dorsal neural tube (l, m, p, q), whereas cognates of these genes in jawed vertebrates are restricted dorsally[4]. (r) Cartoon of lamprey neural tube section summarizing spatial expression of neural crest regulators in this study (Figs.1, 2) and elsewhere (Pax3/7, Id-A, Zeb1)[14,31,32]. Blue ovals denote expression domains within the neural tube; blue tear-drops denote migratory cells from the neural tube. In all panels, dorsal is up and ventral is down. E, epidermis; N, notochord; NF, neural folds; NP, neural plate; MNCC, migratory neural crest; pMNCC, premigratory neural crest.

Figure 2. Cells expressing the transcription factor n-Myc emigrate from the ventral neural tube in a stream from the ventral neural tube and are required for neural crest development in lamprey. *n-Myc*-positive cells are in migratory neural crest (a), and these cells exit from the neural tube (NT, dotted outline) in a thin stream ventrally (arrowheads, b), just over the floorplate (FP) and notochord (N). n-Myc CRISPR knockout embryos (n-Myc^{CR}) reduced *Snail* expression in premigratory (arrowhead) and migratory (asterisk) neural crest (c), and lose SoxE1-positive pharyngeal prechondrocytes (arrowhead, e) compared to control embryos (Cont^{CR}) (d, f).

Figure 3. Lamprey neural crest-like cells migrate from any position within the neural tube. DiI was injected into the dorsal (a) or ventral (c) hindbrain of T22 embryos, which were fixed and sectioned for imaging. By ~6h post injection (hpi), DiI+ cells in both dorsal and ventral labeled embryos migrated into the pharyngeal arch mesenchyme (b, e, autofluorescent tissue is magenta). (e–f), Hindbrain vivo-sections (T22 embryos) were DiI-labeled in the ventral neural tube (dotted outline) and explanted for time-lapse microscopy (see Methods). DiI+ cells (blue arrowheads) emerge from the labeled region by 6 hpi (g) and migrate toward ventral boundary of the neural tube (red hatching) (a-d). By ~9 hpi post labeling, cells have exited the neural tube (h–k) from the region where neural crest markers are expressed (compare with Figs. 1, 2). N, notochord. Asterisks in (b) and (e) indicate the angle of injection in to the hindbrain.

Figure 4. A new scenario for the origin of migratory neural crest cells. (a) Last common chordate ancestor. According to our scenario, after splitting from invertebrate chordates (b), ancestral vertebrates (c) produced neural crest-like cells throughout the entire dorsoventral axis of the neural tube. This domain of is similar in scope to that of *Snail* and *SoxE* expression in the invertebrate chordate, amphioxus, which lacks migratory neural crest (b). The ancestral condition of producing neural crest-like cells throughout the embryonic neural tube is largely retained in the basal jawless vertebrate, lamprey (d), but was lost in early jawed vertebrate groups, in which the neural crest became localized exclusively to the dorsal neural tube (e).

Figure S1. Sequencing results from five randomly pooled n-Myc gRNA1-injected T26 lamprey embryos. Lamprey n-Myc wildtype sequence (WT) is at the top with the gRNA1 target site in

red and PAM site underlined. Fifteen clones sequenced from the genomic DNA of the pooled embryos are listed below the WT sequence, with the number of base pairs deleted (-) or unchanged (WT) listed to the right of each sequence. Deletions are indicated by dashed lines.

Figure S2. Still images of time-lapse series from Supplementary Movie S2. n, notochord. Arrowheads show the lack of any DiI-positive cells emigrating from the ventral neural tube.

Supplementary Move S1. Quicktime Movie of the embryonic slice culture assay shown in Figure 3e–k.

Supplementary Movie S2. Quicktime Movie of two embryonic slice culture assays for a T25 lamprey embryo in which the ventral hindbrain neural tube was focally labeled with DiI. Note that the labeled cells never migrate out of the neural tube.

METHODS

To collect embryos, gravid adult sea lampreys were obtained from the Hammond Bay Biological Station, Millersburg, MI, and shipped to the University of Oklahoma. Adults were housed at 14 °C in a recirculating water system. We manually stripped eggs from females into a beaker of water and then expressed sperm from a male directly onto the eggs. Embryos developed in small Pyrex dishes under a constant flow of water supplemented with 0.05X Marc's Modified Ringers solution (MMR) chilled to 19 °C. All clones used for gene expression analysis were identified from the 2010 version of the *Petromyzon marinus* genome assembly[24], amplified from a sea

lamprey cDNA library (kindly provided by Dr. James Langeland), ligated into a pGEM-T easy vector, sequenced and verified by BLAST. For *in situ* hybridization we used a previously described protocol[33]; sectioning (20-25 μm) was performed on a Vibratome with embryos embedded in 5% agarose.

For CRISPR-Cas9-mediated mutagenesis experiments targeting n-Myc coding sequence individual lamprey zygotes were microinjected with approximately 1 ng ul^{-1} Cas9 protein (PNA Bio), 500 pg guideRNA (gRNA) and 10% fluorescein dextran in nuclease-free water. We targeted the coding sequence using two different gRNA constructs (gRNA1: 5'-GCCCCGCCAAAAAGAGCGTGG-3'; gRNA3: 5'- CCGAGAACGGGGTGATGTCTC -3'; PAM sequence underlined) that were carefully selected to avoid off-target cleavage effects based on stringency criteria found elsewhere[19]. For negative control experiments, we injected embryos with two different randomly generated gRNA constructs (gRNA1: 5'-TGGAAACCCCAACTTATT-3'; gRNA2: 5'-CGGGTAACGTCGTTAGCC-3') to demonstrate specificity of n-Myc CRISPR phenotypes. After injection, embryos were reared to the desired stage, fixed in MEMFA, and then dehydrated and stored in 100% methanol. In addition to negative control experiments, we used a recently described protocol[17] to genotype individual putatively mutant embryos so that we could directly link a mutant genotype to a specific phenotype (e.g., loss or reduction of gene expression). Oligonucleotides (Sigma) flanking the n-Mycg1 (forward: 5′-GCTAACTGCATGCATGTTGC-3′; reverse: 5′-CTTGTCCTGGTCCTCCGAGT-3′) genomic CRISPR target site were used to PCR amplify and sequence a 564 bp fragment of the genomic locus to verify mutagenesis (Fig. S1). Taken together, our stringent criteria for gRNA selection [19], direct validation of mutant genotype-

phenotype relationships for individual embryos, and use of negative control experiments make it unlikely that observed n-Myc mutant phenotypes are attributable to off-target effects.

Focal injections in vivo of the lipophilic dye DiI (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ('DiI'; $DiIC_{18}(3)$) were performed as described previously[16]on Tahara stage (T) T22 embryos. Embryos were fixed in MEMFA 6-7 hours post-injection, stored in PBST, and then sectioned on a Vibratome as described above. Embryonic tissue morphology of DiI-labeled sections was visualized by autoflouresence of lamprey tissue.

For time-lapse imaging of lamprey sections we modified a slice culture assay used for chick spinal cord sections [23]. Live lamprey embryos (E7-7.5) were manually dechorionated in 0.05X MMR and then washed briefly in Leibovitz's L-15 medium supplemented with antibiotics (LL15+). Embryos were then embedded in 7% low melting point agarose in LL15+, immediately mounted and then sectioned (200-300 μm) through cranial region on a Vibratome. Sections were washed carefully several times in LL15+ and then a small bolus of DiI was microinjected into the ventral neural tube. Injected sections were immediately evaluated both for tissue integrity and verification that only the ventral neural tube was labeled with fluorescent dye. DiI-labeled sections were then placed in a 35 mm petri dish with LL15+ that had been previously incubated with Fibronectin overnight at 4 degrees. Embryo sections were held beneath bridges, using glass coverslips placed on the sides and on above sections, and then allowed to attach to the fibronectin substrate for 2 h. Petri dishes containing labeled sections were visualized by time-lapse imaging at 10X magnification (DIC optics) with images taken every 10 m on a Zeiss Axioimager Z1, equipped with an Apotome module for optical sectioning.

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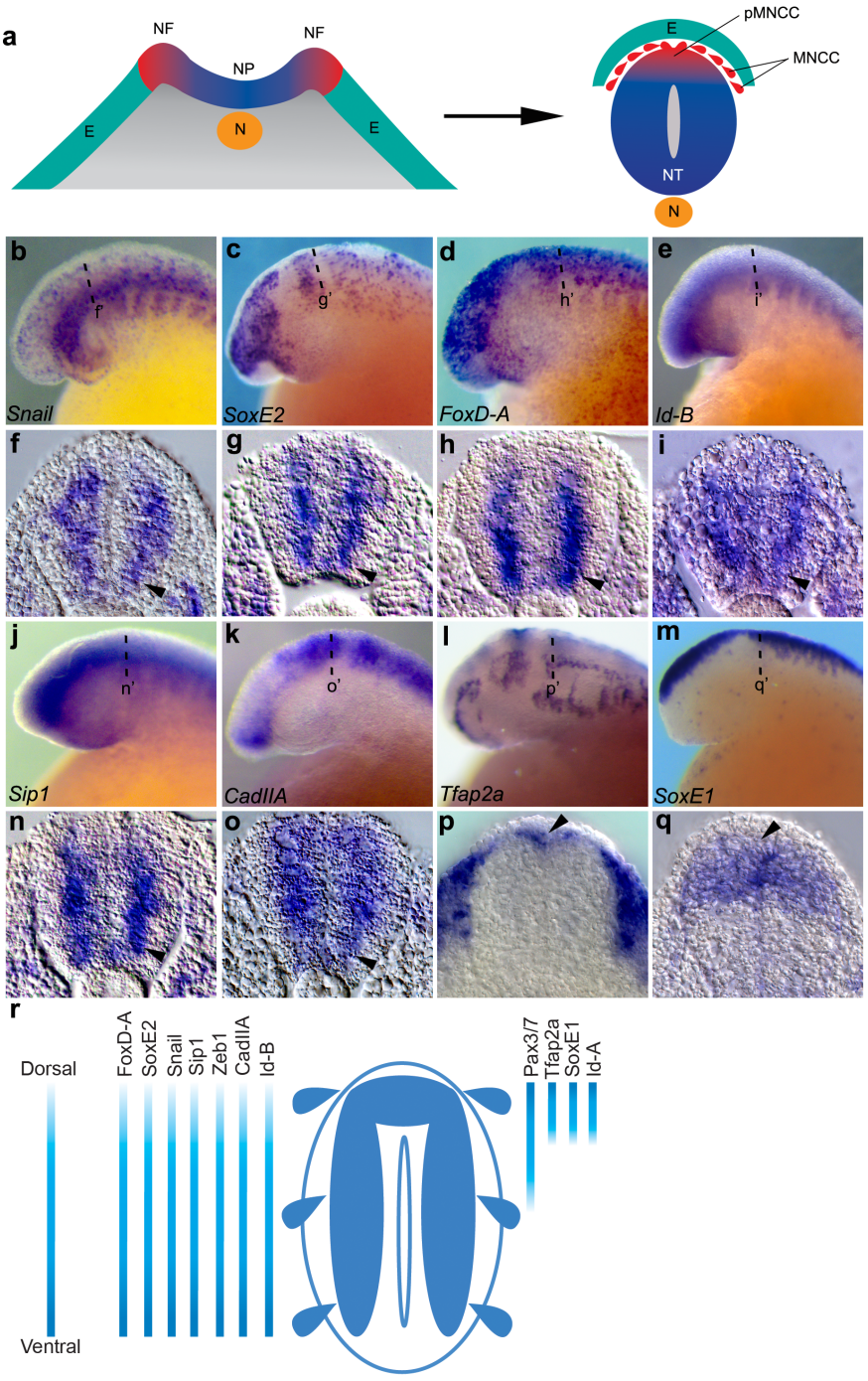
Figure 1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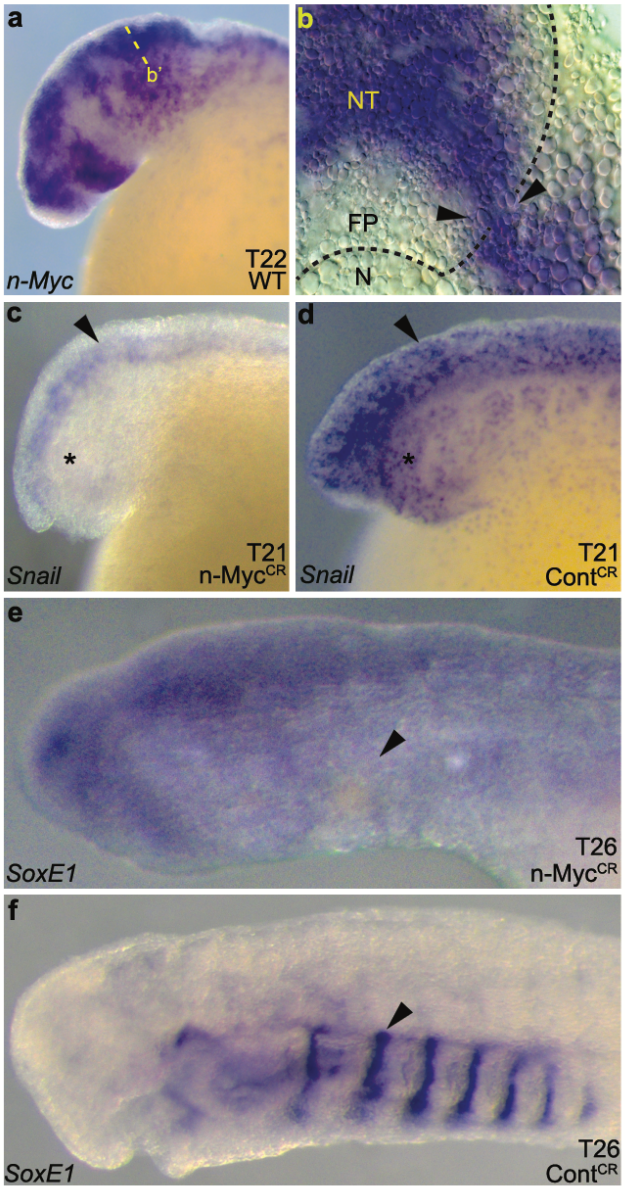
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Figure 3

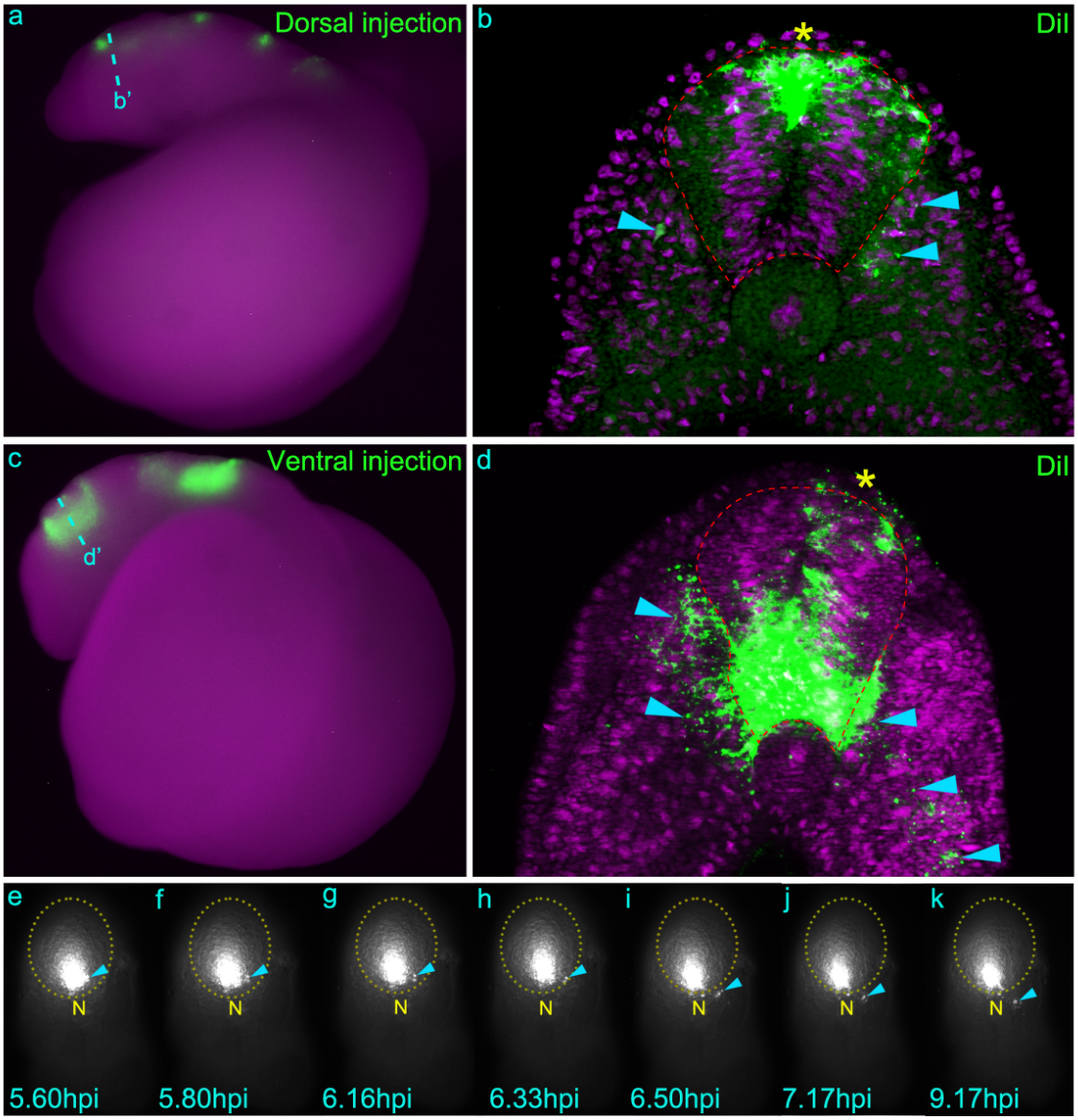
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Figure 4

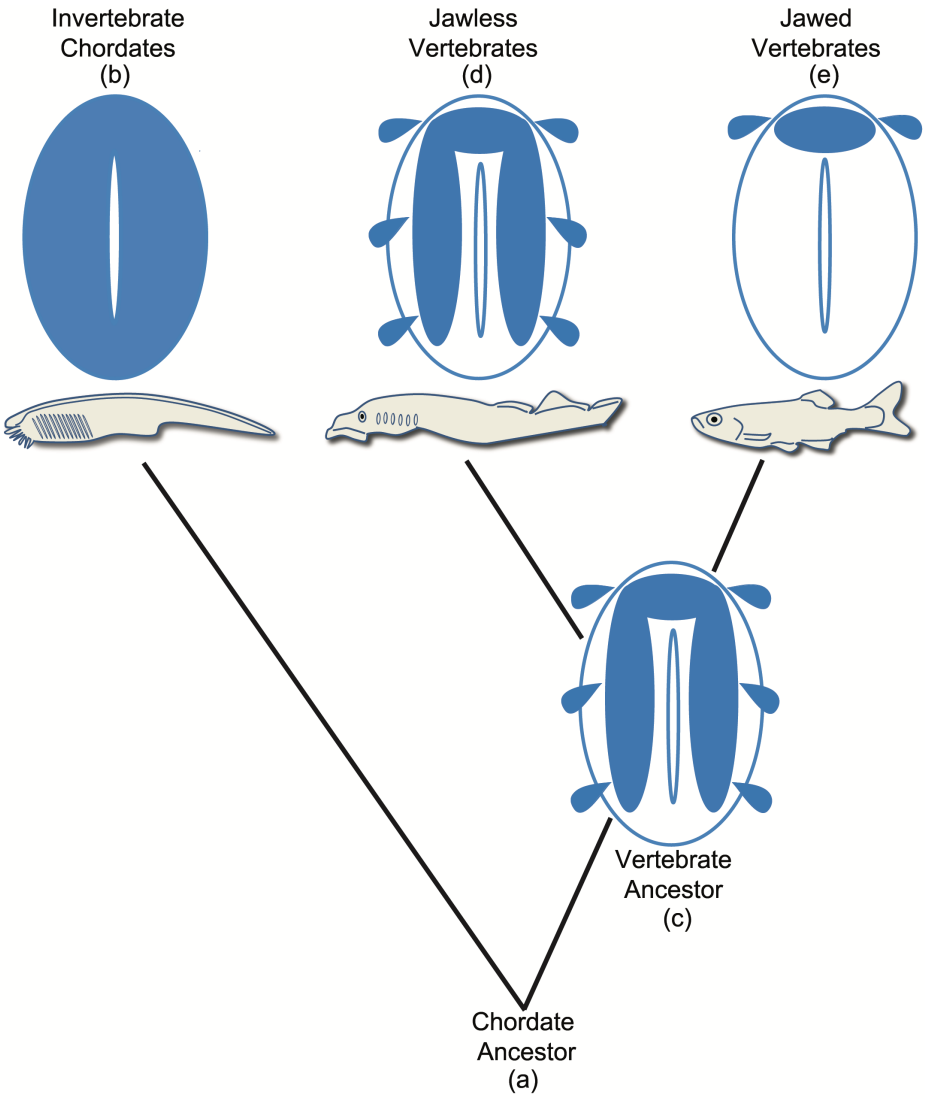
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Figure S1

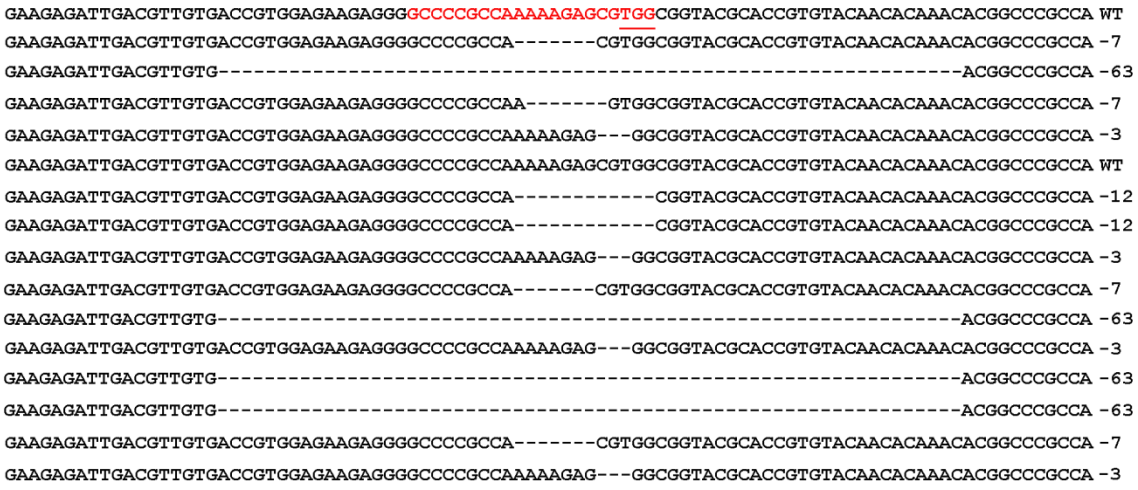
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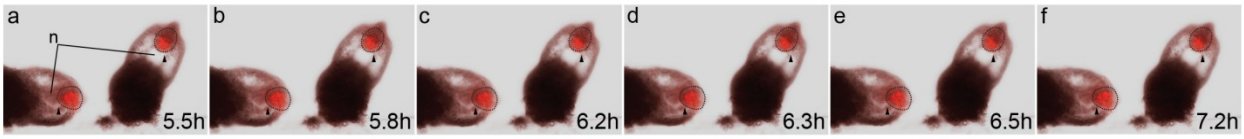
Figure S2



CONCLUSIONS

Originally discovered by Wilhelm His in 1868, the *Zwischenstrang*, or "cord in between" was the term he used to describe what we now refer to as the neural crest. In the over 150 years since His' discovery, the neural crest has been an important focus for embryologists interested in deciphering both the proximate (mechanistic) and ultimate (evolutionary) processes underlying the emergence of the vertebrate body plan. From an evolutionary perspective, the origin of the neural crest is almost synonymous with the origin of the vertebrates. Identifying the ancestral mechanisms for neural crest developmental genetics has the potential, therefore, to explain within a comparative embryological framework the origin and evolution of the vertebrates and vertebrate neural crest cells.

To this end, the findings presented here in my dissertation highlight important themes demonstrating shared mechanisms of neural crest development across vertebrates, a result which suggests a great deal of evolutionary conservation, ranging from the initiation of EMT and migration to the use of common signaling pathways to construct key morphological characters (Ch. 2–4). This is to be expected for a cell type that plays multiple important roles throughout vertebrate development. Yet, my work also reveals fundamental differences in neural crest regulatory mechanisms between jawed and jawless vertebrates, including an unexpected developmental link between CNS/neural cells and neural crest (Ch. 5), and the ability of neural crest cells to form within and migrate from almost any position along the dorsal-ventral axis of the embryonic CNS (Ch. 6). These differences have the potential to fundamentally change our understanding not only of how neural crest cells form in vertebrate embryos but how this cell type evolved in our earliest vertebrate ancestors. Although these findings paints a more

complicated picture for the evolutionary origins of both neural crest cells and the vertebrate clade, it also opens up new and promising areas of research, and a new conceptual framework within which to study how neural crest regulatory mechanisms were assembled during early vertebrate evolution and how modifications to the neural crest gene regulatory network facilitated the advent of key vertebrate innovations. Going forward, it will be crucial for the field of evolutionary-developmental biology broadly, and lamprey "evo-devo" more generally, to take advantage of rapidly emerging genomic and transcriptomic technologies, including single cell sequencing, spatial transcriptomics, genome-wide chromatin profiling, and many others to continue to make fundamental advances in the study of developmental evolution in early vertebrates.