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The molecular basis of neural crest axial identity

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Abstract

The neural crest is a migratory cell population that contributes to multiple tissues and organs during vertebrate embryonic development. It is remarkable in its ability to differentiate into an array of different cell types, including melanocytes, cartilage, bone, smooth muscle, and peripheral nerves. Although neural crest cells are formed along the entire anterior-posterior axis of the developing embryo, they can be divided into distinct subpopulations based on their axial level of origin. These groups of cells, which include the cranial, vagal, trunk, and sacral neural crest, display varied migratory patterns and contribute to multiple derivatives. While these subpopulations have been shown to be mostly plastic and to differentiate according to environmental cues, differences in their intrinsic potentials have also been identified. For instance, the cranial neural crest is unique in its ability to give rise to cartilage and bone. Here, we examine the molecular features that underlie such developmental restrictions and discuss the hypothesis that distinct gene regulatory networks operate in these subpopulations. We also consider how reconstructing the phylogeny of the trunk and cranial neural crest cells impacts our understanding of vertebrate evolution.

Introduction

The neural crest is a transient, multipotent progenitor population that plays a central role in vertebrate embryonic development. Formation of neural crest cells begins in the early gastrula with the establishment of the neural plate border territory amid the neural plate and the non-neural ectoderm. Subsequently, during neurulation, a subset of neural plate border is specified as the neural crest. This group of cells, which are initially organized in bilateral fields on both sides the neural plate, come to reside on the dorsal aspect of the neural tube as the roof plate closes (Gammill and Bronner-Fraser, 2003; Sauka-Spengler and Bronner-Fraser, 2008; Simoes-Costa and Bronner, 2015). Neural crest cells then undergo an epithelial-tomesenchymal transition and migrate through well-defined pathways to colonize specific locations within the developing embryo. Once they reach their destinations, these cells differentiate into diverse cell types (Martik and Bronner, 2017).

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Classical embryology studies, including ablations, cell transplantation, and dye-labeling experiments, have identified the vast array of neural crest derivatives Hall and Hörstadius, 1988; Le Douarin, 1982). These include the neurons and glia of the peripheral and enteric nervous system, craniofacial connective tissue, cartilage and bone, and pigment cells. Other derivatives include part of the smooth muscle of the great vessels, pericytes and secretory cells like the chromaffin cells of the adrenal gland (Etchevers et al., 2001; Le Douarin and Kalcheim, 1999). Indeed, neural crest progenitor cells have the remarkable ability to give rise to both ectodermal and mesodermal-like derivatives and are often referred to as the *fourth germ layer* (Hall, 2000). Furthermore, since most distinguishing features of vertebrates are neural crest-derived, these cells have important implications in deuterostome evolution and hence are considered a vertebrate synapomorphy (Green et al., 2015).

Neural crest cells are formed along most of the anteroposterior axis of the embryo, from the posterior diencephalon (Etchevers et al., 1999) to the lumbosacral region of the embryo (Osorio et al., 2009) (Fig. 1). They can be grouped into four distinct subpopulations according to the position where they are specified: *cranial*, *vagal*, *trunk* or *sacral* (Fig. 1). The cranial neural crest includes cells that are formed in the cephalic region, from the forebrain to the 6th rhombomere (R6) of the hindbrain. The vagal crest comprises progenitor cells that are specified next to the first seven somites (Le Douarin and Teillet, 1974) in fish, avian and murine embryos. The trunk crest includes the neural crest streams that migrate from the 8th somite until the 27th somite in avian embryos or the 24th somite in the mouse. The sacral neural crest, which has only been identified in amniotes, lies posterior to this boundary (Le Douarin and Kalcheim, 1999). This classification is not merely anatomical, as neural crest cells arising from different axial locations are not equivalent (See Box 1). These subpopulations differ in their migratory patterns, differentiation potentials, and gene expression profiles. For instance, cranial neural crest cells travel as a group, in a wave-like pattern. Alternatively, trunk cells form well-defined streams, and adopt either the ventral or the dorsolateral migratory pathways (Fig. 1b). Such differences in the migratory behaviors between axial levels have been recently reviewed (Kuo and Erickson, 2010; Theveneau and Mayor, 2012). Here, we will focus on the differences in developmental potential between neural crest cells of different axial levels, examining the molecular features that make each population unique. We will also discuss how the phylogeny of the neural crest subpopulations may have affected vertebrate evolution.

The developmental potential of neural crest subpopulations

The identification of the full array of neural crest derivatives was a considerable undertaking that spanned more than a century of experiments performed in multiple model organisms. Tissue ablation, cell labeling with vital dyes, cell transplantation, and genetic fate-mapping are among the techniques that were used to map the fate of neural crest cells (Chibon, 1964, 1967; Hörstadius, 1969; Johnston, 1966; Le Douarin, 1982; Le Lievre and Le Douarin, 1975; Noden, 1978; Weston, 1970). Such experiments revealed the extensive contribution of the neural crest to the vertebrate body plan. Furthermore, mapping the derivatives of neural crest cells from different axial levels revealed the specific contribution of each subpopulation (See Box 1). In this context, the chicken-quail chimera experiments pioneered by Nicole Le Douarin were particularly informative. While quail and chick embryos are very similar in

size and timing of development, cells from the quail have a unique nuclear morphology that is not observed in other birds. By transplanting quail tissue to chick embryo hosts, Le Douarin and colleagues were able to examine neural crest fate along the anteroposterior axis (Le Douarin and Kalcheim, 1999; Le Douarin et al., 2004). These studies revealed that the contributions of the cranial, vagal and trunk neural crest cells are remarkably diverse (Box 1) (Fig. 1c). However, it was not immediately clear whether those differences were due to cell-intrinsic differences in potential, or a consequence of the distinct environmental signals that are present at each axial level.

To address possible differences between neural crest cell fate and their differentiation potential, researchers employed heterotopic transplantations. In these studies, neural tube explants, containing the premigratory neural crest, were isolated from quail embryos and grafted into a distinct axial level of chick host embryo (Fig. 2) (Chibon, 1967; Le Douarin and Teillet, 1974). Embryos were cultured until late developmental stages and the contributions from the grafted tissue were assessed with histological analysis. The results from these experiments revealed a large degree of developmental plasticity, with neural crest cells often adopting the migratory routes and generating the derivatives of their new axial level (Le Douarin et al., 2004). For instance, when quail cranial or vagal neural crest cells were transplanted to the trunk of a host chick embryo they gave rise to trunk derivatives, differentiating into neurons, glia and pigment cells (Le Douarin and Teillet, 1974). Similarly, when trunk neural crest cells were transplanted to the vagal region, they colonized the gut and differentiated into the enteric nervous system. Thus, early neural crest cells exhibit a large degree of developmental plasticity, and their fate choice is controlled by the environmental signals they encounter during and after migration. While the potential of neural crest cells decrease as these cells migrate (see discussion below), these results are consistent with the idea that early-migrating neural crest cells of distinct axial levels are multipotent and can give rise to an array of cell types (Bronner-Fraser and Fraser, 1988, 1989; Fraser and Bronner-Fraser, 1991).

Restrictions of neural crest cell potential along the anteroposterior axis

While all neural crest cells display a high degree of developmental plasticity, cell transplantation experiments also uncovered important differences in potential between axial levels. These developmental restrictions are particularly evident for the mesenchymal derivatives that arise from the cranial and vagal neural crest. Experiments in multiple model organisms indicate that the cranial and vagal neural crest are unique in their potential to contribute to the craniofacial skeleton and the cardiovascular system, respectively (Kirby, 1989; Le Douarin et al., 2004). Several fate mapping studies have suggested that the trunk neural crest of basal vertebrates forms mesenchymal cells that reside in the fin (Collazo et al., 1993; Smith et al., 1994b), but this potential seems to have been lost in amniotes, with the possible exception of chelonians (see discussion below) (Cebra-Thomas et al., 2007).

The neural crest origin of the craniofacial skeleton was identified more than a century ago (Platt, 1893), in experiments conducted in amphibians. This finding was quite controversial at the time (Noden, 1978) due to the popularity of the germ layer theory, which stated that bone and cartilage were of mesodermal origin (Oppenheimer, 1940). Nevertheless, the

contribution of cranial neural crest cells to the craniofacial skeleton was confirmed by subsequent experiments (Landacre, 1910; Stone, 1929). Studies in avian embryos allowed for the fate-mapping of the anteroposterior arrangement of the progenitors that form the distinct parts of the skull (Couly et al., 1993; Le Lievre and Le Douarin, 1975; Noden, 1978). Further experiments in birds and amphibians revealed that this potential is unique to the cephalic population. Neural crest cells from tissue grafts dissected from the trunk were unable to give rise to cartilage and bone when grafted to the cranial region (Chibon, 1967; Lwigale et al., 2004; Nakamura and Ayer-le Lievre, 1982) (Fig. 2). Even cardiac neural crest, which can otherwise differentiate into smooth muscle and cardiac tissue, fails to contribute to craniofacial structures when transplanted in the midbrain (Lwigale et al., 2004). Remarkably, quail neural crest cells from cranial neural tube grafts transplanted into the trunk of a chicken embryo can form ectopic cartilage nodules (Le Douarin and Teillet, 1974) (Fig. 2). This suggests that these cells are able to differentiate into cartilage even outside of the cephalic microenvironment.

A similar restriction in potential is observed at the vagal levels. The anterior-most vagal crest (adjacent to somites 1–3) contributes to the heart and the great vessels, hence being termed the *cardiac* neural crest (Kirby et al., 1983). Intra-vagal transplant experiments show a high degree of plasticity. Posterior vagal crest can occupy the cardiac outflow tract when transplanted to an anterior axial level, while the cardiac crest can populate the gut when grafted posteriorly. In contrast, cells from other axial levels are unable to give rise to cardiac tissue even when grafted in the vagal region. Cranial neural crest cells, when transplanted in the region of the cardiac crest, cannot give rise to a functional heart septum, and instead form ectopic cartilage similar to that found in the face (Kirby, 1989). Trunk derivatives also fail to contribute to the ectomesenchyme for septation of the outflow tract. These results suggest that the cardiac neural crest has diverged from the other subpopulations, acquiring the unique ability to contribute to the cardiovascular system.

Taken together, these studies suggest that the neural crest subpopulations are (i) plastic in respect to most derivatives, being able to differentiate into neural cell types and melanocytes regardless of their axial level of origin, and (ii) restricted in regards to mesenchymal potential. It is important to emphasize that the mesenchymal potentials observed in the cranial and cardiac subpopulations are not interchangeable, as these cells cannot substitute one another. Overall, there seems to be the trend where the neural crest becomes progressively restricted along the anterior-posterior axis (Lwigale et al., 2004), with cranial cells being able to give rise to most cell types and trunk/sacral populations having more restricted developmental potential.

Differences in timing of cell fate restriction

Neural crest subpopulations have essential differences in their patterns of migration, which, in turn influence their timing of cell fate restriction. In the cranial regions, the neural crest engages in collective cell migration, forming a migratory wave that occupies the space between the head mesoderm and the future epidermis (Kuo and Erickson, 2010; Le Douarin, 1980). The early migrating neural crest travels longer distances to populate the distal branchial arches and forms most of the facial structures, including cartilage and connective

tissue. The late-migrating crest remains close to the neural tube and contributes mainly to the cranial ganglia (Baker et al., 1997). Compared to its vagal and trunk counterparts, the cranial neural crest population maintains its plasticity the longest during migration. Grafting of early migrating branches of the trigeminal crest into the late-migrating branch and vice versa have shown that late neural crest cells can give rise to all derivatives formed by the early cells (Baker et al., 1997). This suggests that pre-migratory cranial crest is considerably plastic, and that its fate determination depends on the signals that the cell encounters along its migratory routes.

Unlike the cranial neural crest, ventrally migrating trunk crest cells travel within well-defined streams, which are delimited by signals and physical barriers provided by the cells of the somites and the neural tube. This constrains the possible routes that can be adopted by the neural crest cells (Debby-Brafman et al., 1999; Kalcheim and Teillet, 1989; Lallier and Bronner-Fraser, 1988). Furthermore, the timing of migration initiation and the pathways adopted synchronized with the development of the adjacent somites, which imposes strong developmental regulation on the temporal pattern of trunk neural crest migration (Sela-Donenfeld and Kalcheim, 2000). In contrast to cranial cells, the late-migrating trunk neural crest cannot form all the derivatives when transplanted in younger embryos (Artinger and Bronner-Fraser, 1992). These findings indicate that trunk cells become fate-restricted closer to the neural tube than the cranial neural crest. Given the evidence that trunk neural crest cells are still multipotent during migration (Baggiolini et al., 2015; Bronner-Fraser and Fraser, 1989), it is likely that the cells with a more limited repertoire of derivatives (trunk/sacral neural crest cells) commit to their final fates earlier than those with broader potential (cranial neural crest).

Molecular differences underlying neural crest axial identity

Studies from multiple laboratories have identified regulatory factors that control the induction, specification, and differentiation of the neural crest (Nelms and Labosky, 2010; Saint-Jeannet, 2006). Subsequent functional and biochemical analyses have shown that these molecules are connected by regulatory interactions, and thus are part of a gene regulatory network (GRN) that controls neural crest development (Betancur et al., 2010a; Meulemans and Bronner-Fraser, 2004; Simoes-Costa and Bronner, 2015). The neural crest GRN consists of multiple sub-circuits that are sequentially activated to control the major steps in neural crest formation. The earliest module of the network controls neural crest induction and describes the process by which early signalling pathways (Wnts, FGFs and BMPs) establish neural plate border identity by activating specifier genes like *Msx1*, *Pax7* and *Zic1* (Hong and Saint-Jeannet, 2007; Prasad et al., 2012) . In the next module of the GRN, the neural plate border genes cooperate to drive expression of a group of genes termed the neural crest specifiers, which include *bona fide* markers such as *FoxD3*, *Sox10* and *Ets1* (Cheung et al., 2005; Honore et al., 2003; Simoes-Costa and Bronner, 2015) Subsequently, these genes will activate transcriptional cascades that drive EMT and migration, cooperating with signaling systems to jumpstart the regulatory circuits that induce cell differentiation (Martik and Bronner, 2017; Simoes-Costa and Bronner, 2015).

The neural crest GRN is comprised of many of molecular players, including signalling proteins, transcription factors, and epigenetic modifiers. It has been compiled from regulatory studies performed in multiple model organisms, and comparative analysis indicates that is highly conserved amongst vertebrates (Sauka-Spengler et al., 2007). Although it is likely one the most comprehensive regulatory network described for any vertebrate cell type, it is far from complete. Transcriptomic analysis has uncovered dozens of uncharacterized neural crest genes (Lumb et al., 2017; Simoes-Costa and Bronner, 2016; Simoes-Costa et al., 2014), and new layers of regulation by epigenetic and post-transcriptional regulators have just begun to be incorporated in the network (Rada-Iglesias et al., 2012). Furthermore, differences in axial level-specific regulation have not been accounted for in the existing versions of the network (Martik and Bronner, 2017; Simoes-Costa and Bronner, 2015). Instead, the neural crest GRN has been typically presented as a composite of the regulatory interactions of different subpopulations, or has favoured the regulatory interactions that take place in the cranial region. This is a consequence of the sheer complexity of this regulatory program and a lack of thorough, comparative molecular studies contrasting neural crest subpopulations. Indeed, while we have just started to explore these molecular differences at a genomic level, there is mounting evidence that supports the hypothesis that the neural crest molecular toolkit differs between axial levels.

Axial-specific factors and enhancers

Studies in distinct model organisms have revealed a small number of neural crest factors that are specifically expressed or enriched at different axial levels. Some examples include transcription factors *ID2* and *ETS1*, and the proto-oncogene RET. *Id2*, which is present in the cranial neural crest and contributes to neural crest induction, was first characterized in avian embryos and is the first transcription factor reported to be axial-specific (Martinsen and Bronner-Fraser, 1998). Likewise, *ETS1* is a cranial-specific transcription factor, which contributes to neural crest delamination and serves as a regulator of several cranial-specific enhancers (Simoes-Costa and Bronner, 2015; Tahtakran and Selleck, 2003; Theveneau et al., 2007). The *receptor* tyrosine kinase RET, which had been previously linked to the formation of the enteric nervous system (Taraviras et al., 1999), was also shown to be enriched in the vagal neural crest (Simkin et al., 2013). In recent years, transcriptomic analysis has dramatically expanded the number of factors that are exclusively expressed, or enriched, at different axial levels (Lumb et al., 2017; Simoes-Costa and Bronner, 2016; Simoes-Costa et al., 2014). These studies suggest a higher degree of regulatory compartmentalization between the subpopulations than previously thought (see discussion below).

This idea is consistent with cis-regulatory studies, which have uncovered an unexpected degree of modularity in the enhancers controlling the expression of pan-neural crest genes *FoxD3* and *Sox10*. While both transcription factors are seamlessly expressed in neural crest cells at all axial levels, they are differentially controlled by enhancers that are active in either the cranial or the trunk region. This was first shown in *Sox10*, which is controlled by enhancers *Sox10E1* and *Sox10E2*. *Sox10E1* is a vagal/trunk enhancer present in late migratory neural crest cells, while *Sox10E2* is active in migratory cranial neural crest cells. These enhancers seem to be regulated by distinct inputs (Betancur et al., 2010b; Murko and Bronner, 2017). Similarly, the bona fide neural crest marker *FoxD3* is regulated by axial

level-specific enhancers, *NC1* and *NC2*. These elements are active in the cranial and vagal/trunk neural crest, respectively. Upon analysis of the regulation of each enhancer element, it was reported that *Pax7* and *Msx1* directly regulate NC1, in concert with cranial-specific transcription factor *Ets1*. In the trunk, NC2 is also regulated by *Pax7* and *Ms1*, but requires the additional binding of the trunk-enriched transcription factor, *Zic1* (Simoes-Costa et al., 2012). These results demonstrate that *Ets1* and *Zic1* have axial-specific roles in neural crest specification, contributing to the cranial and trunk regulatory programs, respectively.

Taken together, these findings highlight the nuanced regulation of pan-neural crest genes at different locations, and suggest that neural crest specification relies on different inputs according to each axial level. The biological significance of this modular regulation has not yet been fully explored. While it is possible that axial-specific enhancers generate subtle differences in the timing or the levels of expression of pan neural crest genes, these are not evident from transcriptomic analysis (Simoes-Costa and Bronner, 2016) or even *in situ* hybridization in whole mount embryos. Hence, the existence of cranial vs. trunk enhancers may reflect a requirement for distinct specification programs that are compatible with each axial level. Progenitor cells in the head and the trunk are exposed to distinct signalling inputs (retinoic acid, Wnts, FGFs), and have intrinsic regulatory differences (such as the Hox code, discussed below). Distinct enhancers might have been necessary to reconcile the high level of regulatory heterogeneity between axial levels, and to generate an equivalent transcriptional output in each subpopulation. Furthermore, the modular regulation of neural crest genes may also reflect the evolutionary history of these subpopulations (see discussion in evolution of neural crest subpopulations below). Additional studies that examine the functional consequences of which enhancer is deployed, as well as a survey of axial-specific enhancers in different species, are required to elucidate this intriguing property of the neural crest gene regulatory network.

Role of the Hox Code

The *homeobox-containing protein* (*Hox*) cluster plays a central role in patterning the vertebrate embryo along the anteroposterior axis, making it a likely player in the definition of neural crest subpopulations. In particular, Hox genes have been shown to play a central part in the segmentation of the cranial crest. The cranial neural crest can be subdivided into an anterior Hox− population and a posterior Hox+ population (Trainor and Krumlauf, 2000). Importantly, Hox gene expression also impacts the potential of neural crest cells. The absence of nested Hox gene expression in the anterior cranial neural crest (midbrain/r1-r2 of the hindbrain) has been shown to be necessary for the formation of ectomesenchymal derivatives. Ectopic expression of Hoxa2 in the mesencephalic and r1-r2 streams of chick embryos results in complete loss of jaw and frontonasal structures, while overexpression of Hoxa3 and Hoxb4 displays drastic reduction in the formation of the facial skeleton (Creuzet et al., 2002). Conversely, Hox expression in the posterior cranial neural crest (r4-r6), as well as the vagal and trunk neural crest appears to be required for differentiation. Studies in mouse, Xenopus, and zebrafish embryos have shown that inactivation of Hoxa2 in the r4 neural crest streams results in the transformation of pharyngeal arch (PA) 2 elements into structures of PA1, such as Merkel's cartilage and malleus and incus bones of middle ear(Minoux et al., 2009).

The apparent significance of Hox patterning in the morphogenesis of neural crest-derived tissues has raised the question of how this gene family is regulated and how its expression relates to the neural crest gene regulatory network(Parker et al., 2018). A recent study comparing the transcriptomes of Hox+ and Hox− cranial crest in mice reported an enrichment of genes responsive to the retinoic acid signaling pathway in the Hox− population. Further, several other members of the Hox family proteins including *Hoxb1*, *Hoxc5* and *Hoxc11* were also found to be upregulated in the r4 stream (Lumb et al., 2017). It has been previously reported that Hox1 family members are responsive to retinoic acid signaling, and thus it likely that in the Hox+ cranial neural crest, Hoxa2 functions downstream of the retinoic acid pathway to activate transcription of other Hox family proteins. This generates a unique “Hox code”, which fine-tunes the migration and differentiation of r4 and r6 streams of cranial crest, patterning the developing vertebrate head(Parker et al., 2018).

It is possible that Hox genes may act directly on neural crest genes to modulate the neural crest specification program. In mice, microarray analysis of Hoxa1 null embryos revealed a downregulation in neural crest genes, Zic1, Hnf1b and Foxd3(Makki and Capecchi, 2011). Furthermore, knockdown of Hoxa2 results in the ectopic expansion of the expression domain of the ectomesenchymal factor, Sox9. Sox9 transcriptionally activates the cartilage-specific gene, *Col2a1*, and epistatic experiments have shown that Sox9 is responsible for ectopic cartilage formation in Hoxa2 mutants(Bell et al., 1997).

The vagal and trunk neural crest also comprise the Hox+ neural crest population. However, these neural crest components differ from the cranial Hox+ population. Indeed, while Hoxa2 is the predominant Hox family protein expressed in the cephalic neural crest, Hoxb5 has been shown to be enriched in vagal and trunk neural crest (Kam and Lui, 2015). Hoxb5 promotes the transcription of *RET*, and inhibition of Hoxb5 specifically in the vagal neural crest results is reduced crest migration and delayed gut development (Lui et al., 2008). In the trunk neural crest, Hoxb5 is hypothesized to induce the expression of classical neural crest factors Sox9 and FoxD3 to promote neurogenic fate in the early migrating trunk neural crest cells(Kam and Lui, 2015). Moreover, posterior Hox genes such as Hox6, Hox7, Hox8, and Hox9 are also exclusively expressed in the vagal and trunk neural crest. These proteins are essential for posterior neural tube closure and migration of neural crest cells originating therein. Interestingly, ubiquitous expression of Hoxa7 results in severe craniofacial defects (Balling et al., 1989), which highlights how the topological restriction of specific Hox genes recapitulates anatomical differences inherent in neural crest cells. Additionally, unlike members of Hox1-Hox5 family, the above proteins respond to FGF signaling and not retinoic acid signaling(Nelms and Labosky, 2010), which is supportive of the hypothesis that environmental cues contribute to differences in development of neural crest subpopulations.

The absence of Hox expression in the anterior cranial neural crest has been implicated in the inherent plasticity of this cell population(Helms et al., 2005). For example, transplants of single neural crest cells from r2 to r6 in the developing zebrafish embryo revealed a striking switch in Hox gene expression of the transplanted cell, such that it adopted the profile of the surrounding tissue(Schilling et al., 2001). These cells also acquired the ability to form the neuronal and skeletal derivatives reminiscent of the donor site, indicating that Hox status

may play a role in dictating the differentiation potential among neural crest subpopulations along the anteroposterior axis. The plasticity of the anterior Hox− cranial crest has established this progenitor pool as a potential candidate for regenerative medicine. In the case of bone regeneration, skeletal progenitor cells derived from the Hox− neural crest are able to adopt the Hox status of the surrounding tissue when grafted to an injury site(Leucht et al., 2008). Upon the acquisition of this identity, these cells maintain this gene expression profile throughout their lifetime

Hox gene expression along the anteroposterior axis serves as an exciting avenue for exploring axial level differences among neural crest subpopulations. Additionally, the existence of Hox− and Hox+ cranial neural crest populations showcase the nuanced regulation of axial level-specific gene expression even within the same neural crest subpopulation. Though many parallels have been drawn between the Hox Code and differentiation of neural crest progenitors, particularly in the case of cranial neural crest derivatives, many of the regulatory interactions between these two gene sets remain to be discovered(Parker et al., 2018).

Axial-specific gene regulatory networks

To further examine the regulatory programs that control distinct neural crest subpopulations, we recently employed axial-specific enhancers to perform a comprehensive transcriptome analysis of cranial vs. trunk neural crest cells. Comparative transcriptional profiling of these two neural crest cell populations, which were isolated using the FoxD3 *NC1* and *NC2* enhancers, revealed hundreds of cranial and trunk-enriched genes (Simoes-Costa and Bronner, 2016). Functional and biochemical analysis of a group of transcription factors found to be cranially enriched allowed for the delineation of a cranial-specific regulatory sub-circuit (Fig. 3a). Remarkably, ectopic expression of three components of this circuit (*Ets1*, *Sox8*, and *Tfap2b)* in the trunk neural crest cells were able to form ectopic cartilage nodules when transplanted to the head, while engraftment of untreated trunk neural crest could only give rise to neurons and melanocytes (Simoes-Costa and Bronner, 2016). This study emphasizes the importance of axial-specific genetic programs in cell fate determination within the neural crest.

Analysis of the cranial and trunk neural crest transcriptomes may reveal the axial level-specific identity of existing players within the neural crest GRN (Simoes-Costa and Bronner, 2015). For example, in addition to the cranial-specific factors identified in Simoes-Costa et al, 2016 (*Brn3c*, LHX5, *Dmbx1*, *ETS1*, *Sox8*, and *TFAP2b)* other factors such as *Zic1*, *GATA2* and *Sox6* were found to be enriched in the trunk neural crest (Fig. 3b). Lastly, neural crest genes found not to be differentially expressed in either the cranial or trunk neural crest (such as Sox10 and FoxD3) presumably coordinate both axial levels (Fig. 3c). By combining information from the existing neural crest GRN and the cranial and trunk transcriptomes, it is possible to predict gene regulatory subcircuits for axial-specific programs (Fig. 3). While the direct interactions of these cranial-specific factors with the promoters of their downstream targets have been shown in cranial neural crest (Simoes-Costa and Bronner, 2015), much less is known in the context of the trunk. Intriguingly, a comparison of the trunk and cranial neural crest transcriptomes showed that there is larger number of

transcripts enriched in the posterior population (327 trunk genes *vs.* 216 cranial genes). This surplus of trunk-enriched genes may indicate the existence of a number of regulatory mechanisms unique to the posterior neural crest. First, the nested expression of Hox genes, which are highly transcribed in the posterior region of the embryo, may modulate the developmental potential of these cells. Second, the complex migratory patterns exhibited by the trunk crest may require a larger molecular toolkit, including membrane-bound receptors that trigger responses to attractant and repellent cues. Finally, it is also possible that a trunk-specific regulatory program acts to suppress skeletogenic potential. Additional genomic and functional analyses (Cheung et al., 2005) and biochemical studies will be necessary to delineate the structure and importance of this putative trunk regulatory program.

The architecture of cranial and trunk regulatory sub-circuits may inform upon the specific migratory patterns, fate decisions, and clarify the phylogeny of neural crest axial levels. It is likely that a number of factors present at several axial levels contribute to multiple programs, but may have different inputs/outputs in each context. The evidence detailed above supports the existence of subpopulation-specific genetic programs governing neural crest specification. However, axial level-specific regulation has not been studied in the context of terminal differentiation. It will be interesting to explore the network architecture governing differentiation of common neural crest derivatives, such as neurons, glia, and melanocytes, which are derived from every subpopulation. Given the differences observed in specification, it is possible that the formation of same cell type requires divergent regulatory programs at distinct axial levels. Thus, network analysis remains the suggested strategy for decoding the regulatory basis of neural crest axial identity.

The evolutionary history of neural crest subpopulations

Neural crest cells give rise to multiple elements that characterize the body plan of vertebrates, and thus are thought to have played a central role in their emergence and diversification. This idea was initially proposed in ‘New Head’ hypothesis, which suggests that the origin and diversification of vertebrates is linked to the transition from a passive to an active mode of predation (Gans and Northcutt, 1983). According to this idea, this shift is characterized by a number of co-opted and newly established structures that are of neural crest and placodal origin, including the formation of the jaw and the acquisition of an elaborate set of sense organs, replacing filter-feeding with selective predation. These new feeding mechanisms came with drastic implications, including increased body size and metabolic output. The New Head hypothesis distinguishes the neural crest as one of the defining factors giving rise to these new structures, ultimately driving the diversification of the vertebrate clade.

Given the indispensable role of the neural crest in vertebrate evolution, researchers have long sought to discover the origins of this intriguing cell population and how it attained its unique properties. While neural crest cells are a vertebrate innovation, work conducted in urochordates and cephalochordates, the closest invertebrate relatives to vertebrates, has suggested that the neural crest has even more ancient roots. Indeed, homologs to a number of *bona fide* neural crest genes, including BMP2/4, Pax3/7, Msx, and Snail are expressed at the neural plate border in the cephalochordate, amphioxus (Holland and Holland, 2001).

Likewise, the urochordate, *Ciona intestinalis*, expresses Snail at the dorsal neural tube (Nieto, 2002). This perhaps indicates that though these invertebrate chordate species do not have neural crest, at least some of the gene regulatory machinery was present prior to its induction (Manzanares and Nieto, 2003; Yu et al., 2008). The first evidence of migratory neural crest-like cells was identified in the ascidian urochordate, *Ecteinascidia turbinate*. These cells were found to migrate from the neural tube and populate the body wall, giving rise to pigment cells. They also expressed neural crest markers, HNK-1 and *Zic*, suggesting that they may share a common ancestor with the neural crest (Jeffery et al., 2004). Since these cells were only able to contribute to pigmentation, it is possible that the other lineages of the neural crest were only acquired within vertebrate chordates. More recently, studies in *Ciona* have presented evidence of neural crest like-cells, giving rise to both melanocytes and peripheral neurons (Abitua et al., 2012; Stolfi et al., 2015).

Rebuilding the phylogeny of neural crest subpopulations

The evolutionary history of neural crest subpopulations is still an open question, although recent findings have shed light on the order in which these populations appeared. Studies on the agnathan vertebrate, *Petromyzon marinus* (sea lamprey), indicate that it lacks vagal neural crest cells despite having both cranial and trunk subpopulations (Green et al., 2017). Furthermore, fate mapping experiments with DiI-labelling revealed that in these animals the enteric nervous system is partly derived from Schwann cell precursors that are formed from the trunk neural crest (Green et al., 2017). These results indicate that the split between the cranial and trunk neural crest took place during early neural crest evolution. In contrast, the vagal neural crest, which is the most recent neural crest subpopulation, is a shared gnasthome trait.

The acquisition of skeletogenic potential by the neural crest is crucial for clarifying the phylogeny of neural crest subpopulations. Elucidating the developmental potential of the ‘ancient neural crest’ will provide insight into which subpopulation arose first during vertebrate evolution. Here, we present two scenarios for the evolution of the neural crest subpopulations (Fig. 4). The first scenario proposes that the ancient neural crest possessed sketogenic potential and thus would be a more plastic cell population akin to the cranial neural crest. Subsequently, the posterior neural crest subpopulations diverged to develop their unique properties and lost most of this ectomesenchymal potential. The second scenario postulates that the ancient neural crest displayed restricted developmental potential reminiscent of the trunk neural crest. This suggests that skeletogenic potential is a derived feature, arising later during evolution. The following sections summarize the evidence in support of each scenario.

Scenario 1: A skeletogenic ancient crest

Early theories of an ancient neural crest that displayed chondrocytic potential came from observations of the fossil record. Ostracoderms (armored jawless fish) were amongst the earliest vertebrates and possessed a mineralized exoskeleton composed of dermal bone and dentine. Though this primitive armour has subsequently been lost in extant species, it has been suggested that teleost and cartilaginous fish retain components of this skeleton in the form of scales, fin rays, or dermal denticles. These skeletal elements could have played an

important role in vertebrate evolution, functioning as protection or for sensory organ enhancement (Donoghue and Sansom, 2002). Were the exoskeletons of the earliest vertebrates neural crest-derived? This would suggest that the ancient neural crest possessed skeletogenic potential, not only on the cephalic regions, but also in the trunk. Observations of a *bona fide* cranial neural crest origin of dentine in higher vertebrates suggests that the ostracoderm exoskeleton was derived from neural crest (Smith and Hall, 1990). While controversial (see discussion of alternative scenario below) this idea is supported by fate mapping studies showing the contribution of the trunk neural crest to the caudal fin mesenchyme of zebrafish (Kague et al., 2012) and *Xenopus* (Collazo et al., 1993). More recently, the trunk neural crest has also been shown to contribute to the dermal denticles of the little skate, *Leucoraja erinacea* (Gillis et al., 2017; Smith et al., 1994a).

Studies that argue for the existence of ectomesenchymal capacity in the trunk neural crest also exist in amniotes. Chelonians, or turtles, are among the few vertebrate species that possess an extensive trunk exoskeleton. For this reason, the embryonic origins of the turtle shell have been studied extensively to determine whether it may be neural crest-derived. Multiple studies have reported the contribution of trunk neural crest cells to the turtle plastron bones, located on the ventral surface of the shell (Cebra-Thomas et al., 2007; Cebra-Thomas et al., 2013; Clark et al., 2001; Gilbert et al., 2007). These studies suggest that a late-migrating population of trunk neural crest cells, expressing similar markers to those of the cranial neural crest, populate the developing plastron (Cebra-Thomas et al., 2013).

It is possible that if the ancient neural crest had originally possessed ectomesenchymal potential, remnants may still exist in all neural crest subpopulations. Following this premise, much work has been done *in vitro* in attempts to induce skeletogenesis in trunk neural crest cells. Several studies have revealed that under certain culture conditions, avian trunk neural crest cells may be induced to produce bone and cartilage, often by modifying growth factor signaling (Calloni et al., 2007; Coelho-Aguiar et al., 2013; McGonnell and Graham, 2002). These studies have suggested that trunk neural crest cells possess latent chondrocytic potential that is suppressed *in vivo* in higher vertebrates (Coelho-Aguiar et al., 2013). These studies raise the possibility that the basal neural crest evolved with the potential to form ectomesenchyme, but that regulatory mechanisms arose during evolution to suppress skeletogenesis. The molecular mechanisms governing this suppression and pathways by which it may resurface remain unresolved. Nevertheless, assignment of skeletogenic potential to the ancient neural crest provides a compelling evolutionary scenario that bridges information obtained the fossil record with results from developmental studies.

Scenario 2: The ectomesenchyme as an evolutionary novelty

Early heterotopic grafting experiments conducted in amphibians and amniotes have established that, *in vivo*, skeletogenic potential is restricted to the cranial neural crest. Accordingly, upon their presentation of the ‘new head’ hypothesis, Gans Northcutt proposed that many changes in the vertebrates body plan occurred anterior to the existing nerve tube (Gans and Northcutt, 1983). They postulated that the vertebrate head, derived from cranial neural crest, represents an addition rather than a modification of the trunk. This supports the

idea that a skeletogenic cranial subpopulation emerged later during evolution, and that the ancient neural crest possessed a more restricted differentiation potential, reminiscent of the trunk. According to this hypothesis, the cranial neural crest subsequently co-opted mesenchymal regulatory circuits from other cell types, which allowed for the emergence of an elaborate craniofacial skeleton.

Consistent with this, a number of studies have refuted the idea that the trunk neural crest displays ectomesenchymal potential. For example, experiments exploring the contribution of the trunk neural crest to the fin/scale mesenchyme in teleost fish have reported an exclusively mesodermal origin of these tissues (Lee et al., 2013a; Lee et al., 2013b; Mongera and Nusslein-Volhard, 2013; Shimada et al., 2013). While much of the data supporting a trunk neural crest origin of skeletal tissues in extant species has relied heavily on fate mapping via administration of lipophilic fluorescent dye, genetic fate-mapping has continuously contested these arguments. In amniotes, some studies suggest a mesodermal origin for the turtle carapace, being derived from endoskeletal ribs rather than from a skeletogenic trunk neural crest (Hirasawa et al., 2013). Furthermore, while some studies have used the HNK-1 antibody to identify neural crest derivatives in chelonians, it is important to consider the limitations of this reagent as a diagnostic marker for the neural crest lineage. Since the HNK-1 epitope is present in multiple cell types (Bronner-Fraser, 1986; Tucker et al., 1984), its use for the mapping of neural crest derivatives has been strongly questioned. Thus, the observation that trunk neural crest cells are able to give rise to ectomesenchyme *in vivo* is still contentious.

Despite the body of research conducted in efforts to elucidate the status of the ancient neural crest, its nature still remains controversial. It is possible the ancient neural crest was neither cranial nor trunk in nature, but that these subpopulations both diverged from a common ancestral cell type. Thus far, we have discussed the cranial vs trunk identity of the ancient neural crest only in terms of its skeletogenic potential. However, these two subpopulations differ in many aspects, including their migratory patterns, cis-regulatory inputs, and gene expression profiles (Kuo and Erickson, 2010; Simoes-Costa and Bronner, 2016). Though many questions still remain unanswered, it is clear that the analysis of an array of chordate species will continue be indispensable in elucidating the origins of neural crest subpopulations. To date, many studies have drawn conclusions from classical developmental biology techniques such as fate mapping via heterotropic grafting or DiI labeling. Conflicting results from these experiments highlight how technically complex fate-mapping is as a technique, and the need of additional strategies to scrutinize neural crest potential. In this context, understanding the molecular circuits controlling skeletogenic differentiation in the early neural crest (Simoes-Costa and Bronner, 2016) may be particularly informative. New technologies allowing for comprehensive genomic analysis of non-traditional model organisms may provide insight as to the molecular mechanisms governing the divergence of neural crest subpopulations over evolutionary time. Additionally, paleontological studies may continue to uncover morphological features in extinct species that will clarify the developmental potential of neural crest cells, shedding light in the evolutionary history of these cell populations.

Conclusions

The unique properties of the neural crest set vertebrates apart from other chordate species. The broad potential of this cell type and its importance in the establishment of the vertebrate body plan has fascinated scientists for decades. Though much work has been conducted to characterize this cell population in great detail, to date, we still have a limited understanding of the molecular asymmetries that result from axial-specific differences in potential and behaviour. However, with the advent of improved genomic techniques, it is becoming increasingly easier to investigate the molecular mechanisms governing the emergence of the neural crest subpopulations. Here, we have described in detail the demarcation of the neural crest into four distinct anatomical components, the cranial, vagal, trunk, and sacral neural crest. It is important to emphasize that this demarcation only partially recapitulates the heterogeneity characteristic of this stem cell population. Indeed, as we have highlighted above, even cells within the same neural crest population differ considerably in their temporal and spatial patterns of migration, lineage plasticity and differentiation abilities. These differences are a consequence of the unique transcriptional circuits that are active in individual neural crest components. Ultimately, much remains unknown regarding the molecular underpinnings of neural crest subpopulations. Further extrapolation of these mechanisms will have important consequences regarding our understanding of the emergence of the vertebrate clade.

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Overview of Neural Crest Subpopulations

Extensive fate map studies performed in the 1960s and 1970s allowed for the identification of the four distinct neural crest subpopulations, distributed along the anteroposterior axis of the vertebrate embryo. Subsequently, a fifth population, the cardiac neural crest, was identified; it is now considered a subset of the vagal neural crest. Here we present a summary distinctive features of each subpopulation, focusing on their migratory patterns and their respective cellular derivatives.

Cranial Neural Crest

The cranial neural crest is the first subpopulation to undergo an epithelial to mesenchymal transition and delaminate from the neural tube. It gives rise to the majority of the skeletal elements of the face, melanocytes and the neurons and glia of the cranial ganglia (Graham et al., 2004). In amniotes, cranial neural crest cells migrate dorsolaterally and adopt different routes, depending on their axial location along the neural tube. The cells from the forebrain and midbrain regions travel as a continuous mesenchymal sheet of cells (Couly et al., 1995), moving anteriorly over the optic primordia to form fronto-nasal structures. In contrast, neural crest cells from the hindbrain travel laterally and ventrally in defined segmented streams to populate the pharyngeal arches (PA). The developing hindbrain can be divided into 7 segments or rhombomeres (r1–7), and the neural crest streams are formed adjacent to even-numbered rhombomeres, resulting in this segmented pattern. The first stream, also dubbed the trigeminal neural crest, emanates from the midbrain and r1–2. The second stream, or hyoid crest, originates from r4, while the third stream or post-otic crest migrates from r6–7(Kontges and Lumsden, 1996; Kuo and Erickson, 2010; Lumsden et al., 1991). These streams sequentially fill the corresponding pharyngeal arches; trigeminal crest fill PA1 to give rise to jawbones and malleus and incus bones of the middle ear, hyoid crest migrates to PA2 to form hyoid bone and stapes bone of the ear, and post-otic crest travels to PA3 and contributes to thyroid and parathyroid glands. Each of these streams also gives rise to neurons of cranial and jugular ganglion(Noden, 1983).

Vagal neural crest

This subpopulation arises from the axial level of somites 1–7 in the developing embryo and is positioned between the cranial and trunk neural crest. A subset of the anterior vagal crest (somites 1–3) comprises the cardiac neural crest, which is unique in its ability to contribute to the septation of the outflow tract of the heart. This population follows a stereotypic dorsolateral pathway to fill the heart, great vessels and pharyngeal arches 3–6, wherein it differentiates into smooth muscle cells(Kirby et al., 1983; Kirby and Stewart, 1983). In contrast, the late-migrating cells from the same axial level preferentially give rise to melanocytes(Yntema and Hammond, 1954). Two additional populations of vagal neural crest cells arising from somites 1–3 and somites 4–6 travel ventrally to form the enteric nervous system of the foregut and stomach, respectively.(Epstein et al., 1994; Le Douarin and Teillet, 1973) The cells formed next to somite seven only contribute to the dorsal root ganglia of the peripheral nervous system and melanocytes(Reedy et al., 1998).

Trunk Neural Crest

Trunk neural crest cells originate from the posterior part of the embryo and primarily differentiate into neural and glial cells of the peripheral nervous system, adrenomedullary cells, endoneurial fibroblasts and melanocytes (Kuo and Erickson, 2010; Vega-Lopez et al., 2017). The migration of the trunk neural crest initiates in a crowded environment around the posterior neural tube, in which the cells are in close contact with the prospective epidermis and paraxial mesoderm. This restricts the migration of trunk neural crest cells to specific predetermined routes and makes them especially susceptible to the physical and molecular cues afforded by the surrounding environment. In particular, the somites play an essential role in determining the path and timing of migration by functioning as a source of signaling molecules, as a physical obstacle or as a migration substrate for different streams of trunk neural crest cells(Loring and Erickson, 1987). The trunk neural crest can travel through three different pathways. In mammalian and avian embryos, these are the ventrolateral pathway between the neural tube and somites, the ventromedial pathway between neural tube and sclerotome, and the dorsolateral pathway between the ectoderm and anterior sclerotome. Among those that take the ventral routes, the early migrating cells take the ventromedial/ventrolateral pathway and give rise to dorsal root ganglia of the peripheral nervous system, while the late-migrating cells from the posterior sacral region travel ventrally to differentiate into non-neuronal derivatives (Artinger and Bronner-Fraser, 1992). The cells of the melanocytic lineage migrate through the dorsolateral pathway, being the only trunk crest cells to do so (Henion and Weston, 1997; Reedy et al., 1998).

Sacral Neural Crest

The sacral neural crest has a crucial contribution to the enteric nervous system, giving rise to the ganglia that innervate the hindgut. The sacral crest migrates ventrally and colonizes the gut after the vagal crest. Interestingly, although the sacral neural crest can populate the gut in the absence of vagal crest, it cannot compensate for the lack of vagal derivatives, suggesting molecular differences between the two populations(Burns et al., 2000). The ability to migrate to the hindgut is distinctive of the sacral neural crest, as cranial and trunk neural crest stop migrating at a location much dorsal to the gut. Transplantation experiments have shown that when located at the sacral level, even thoracic crest can invade the gut, while sacral crest grafted in the thorax fails to enter gut (Erickson and Goins, 2000). Thus, the migratory route adopted by neural crest cells located in the sacral region determines their final fate. Indeed, the dorsal location of the gut in the posterior end of the embryo allows the sacral crest to take a more direct route to the gut, populating the enteric mesenchyme at the right developmental window

Highlights

- Neural crest cells are divided in distinct subpopulations that differ in migratory behaviour and developmental potential;
- We postulate that axial-specific genetic circuits operate in each subpopulation and underlie their unique features;
- We discuss how the phylogeny of neural crest subpopulations impacts our understanding of vertebrate evolution.

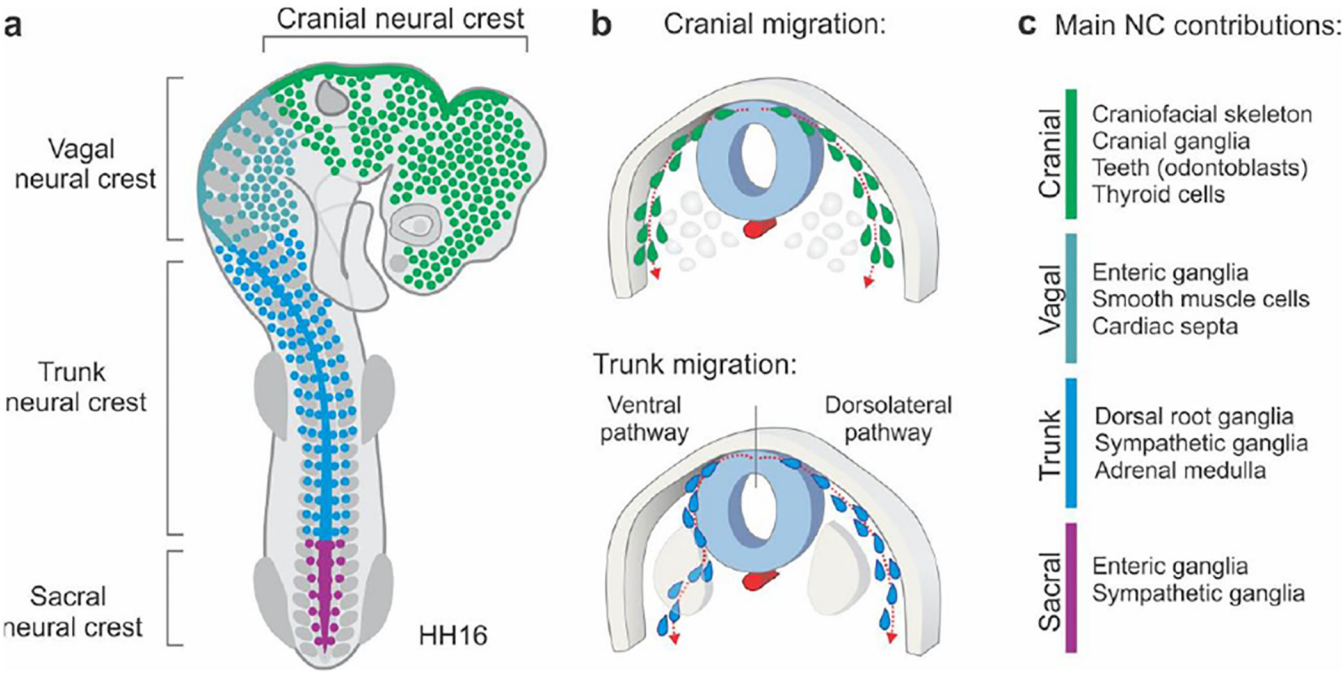

Figure 1. Overview of neural crest subpopulations along the anteroposterior axis.

(A) Diagram of neural crest subpopulations in an HH16 chick embryo. The cranial neural crest subpopulation includes neural crest cells that are formed between the forebrain and the 6th rhombomere (R6) of the hindbrain. The vagal crest is adjacent to the first seven somites. The trunk neural crest spans from the 8th to the 27th somite, while the sacral neural crest is positioned posterior to this boundary. (B) Differences in migratory patterns among neural crest subpopulations. While the cranial neural crest migrates dorsolaterally, the trunk neural crest, which is present adjacent to the developing somites, may take either the dorsolateral or ventral pathway of migration. (C) Major derivatives of neural crest subpopulations. The cranial neural crest gives rise to the craniofacial skeleton and is the only subpopulation possessing ectomesenchymal potential in amniotes. HH: Hamburger and Hamilton developmental stages.

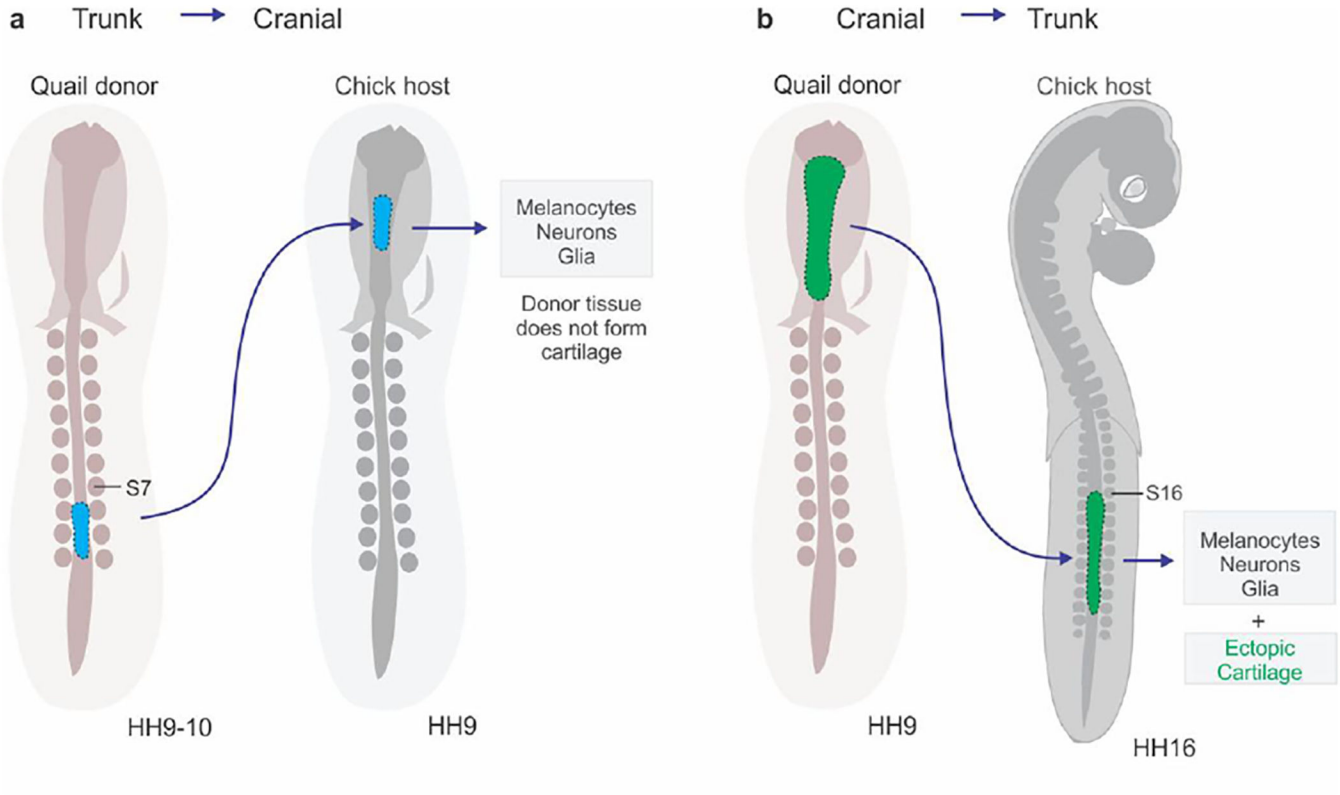

Figure 2: The use of quail-chick chimeras in establishing the differentiation potential of neural crest subpopulations.

(A) Heterotopic grafting of HH9/10 quail trunk neural crest tissue into the midbrain of an HH9 chick host revealed the constrained potential of the trunk, which is not capable of forming cartilage. (B) In the converse experiment, HH9 quail cranial neural crest tissue transplanted into the trunk region of an HH16 chick host gave rise to ectopic cartilage nodules (adapted from Lwigale et. al, 2004 and Le Douarin and Teillet, 1974).

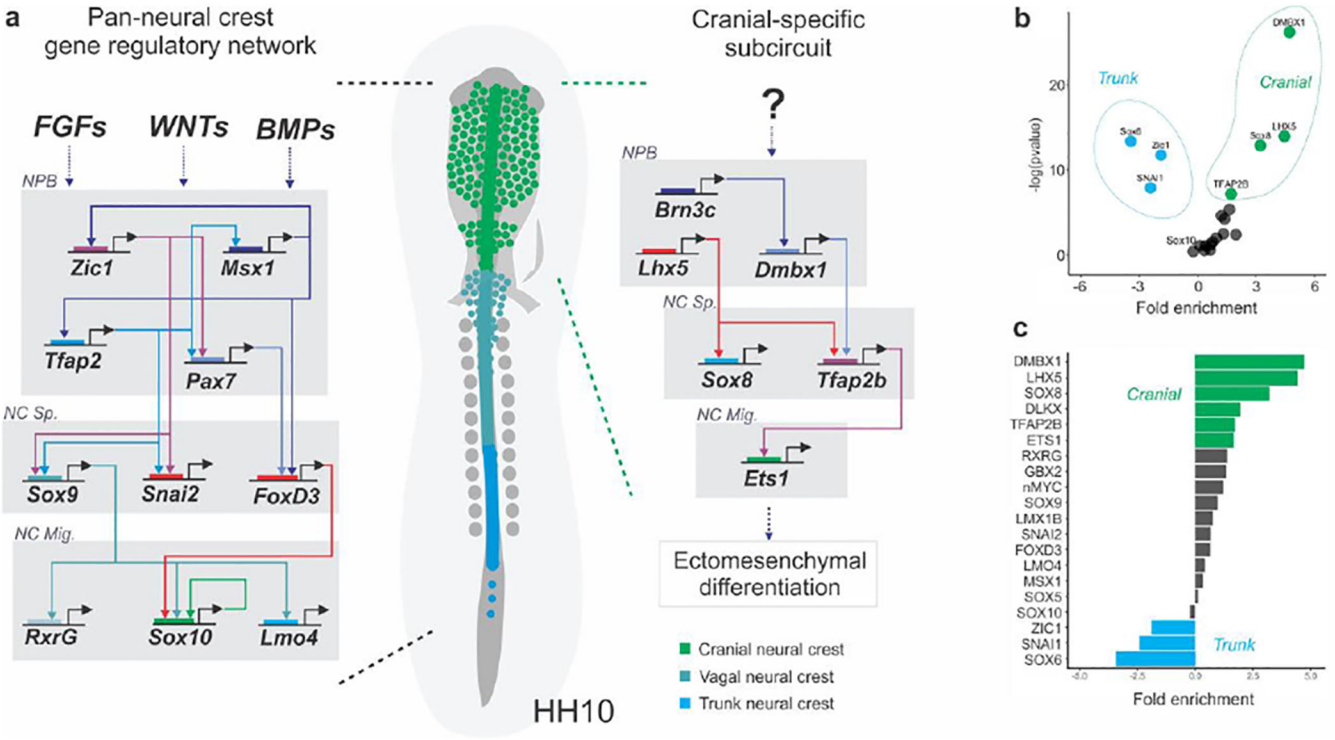

Figure 3. Axial-specific features of the neural crest gene regulatory network (GRN).

(A) Simplified diagram of the pan-neural crest gene regulatory network, which does not account for differences among subpopulations, and the cranial-specific subcircuit contributing to ectomesenchymal differentiation. Neural plate border genes (HH5–6) regulate the expression of neural crest specification and migration genes (HH8–10) (B-C) Enrichment of neural crest genes in the cranial vs. trunk populations suggests the presence of axial-specific circuits. NPB: Neural Plate Border; NC Sp: Neural Crest Specification; NC. Mig: Neural Crest Migration

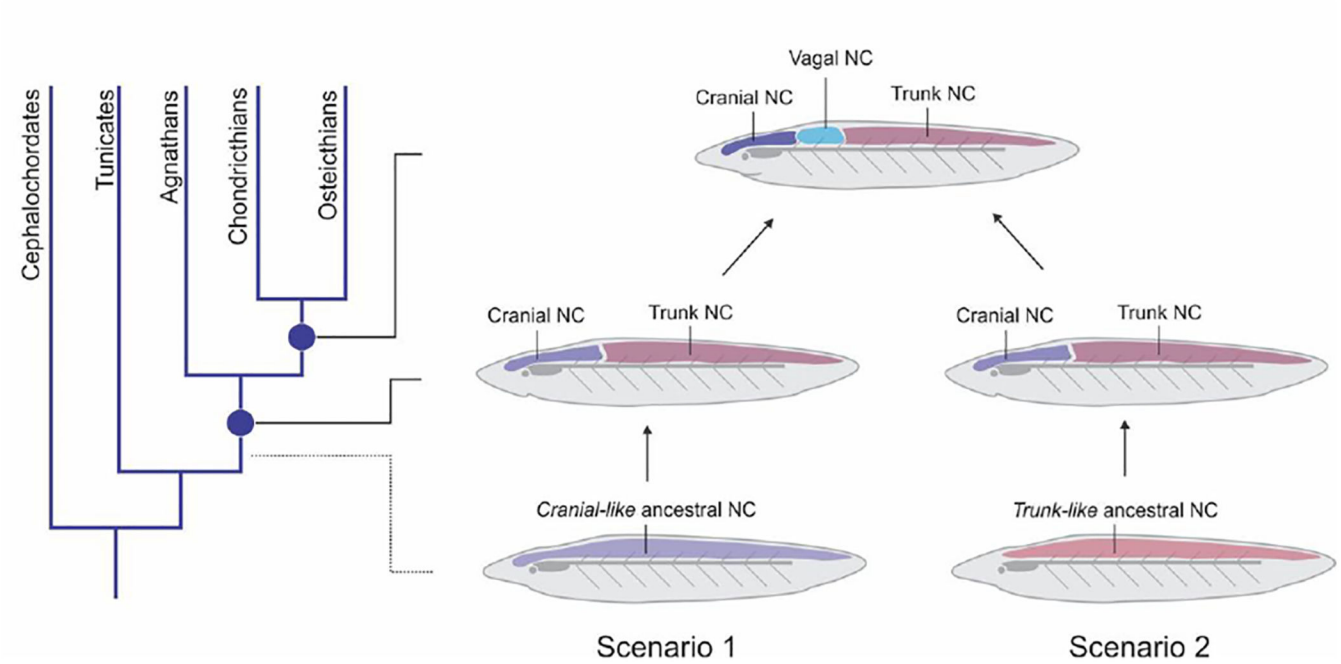

Figure 4. Scenarios for the evolution of neural crest subpopulations.

Skeletogenic potential impacts the phylogeny of the neural crest subpopulations. A skeletogenic ancient crest implies that the trunk subpopulation gradually lost the potential to give rise to ectomesenchyme *in vivo* (scenario 1), during vertebrate evolution. Alternatively, if the ancient crest was trunk-like, it is likely that a skeletogenic cranial neural crest appeared much later (scenario 2), possibly co-opting mesenchymal genetic programs that exist in other cell types.