© 2020. Published by The Company of Biologists Ltd | Development (2020) 147, dev193888. doi:10.1242/dev.193888

REVIEW

From head to tail: regionalization of the neural crest

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ABSTRACT

The neural crest is regionalized along the anteroposterior axis, as demonstrated by foundational lineage-tracing experiments that showed the restricted developmental potential of neural crest cells originating in the head. Here, we explore how recent studies of experimental embryology, genetic circuits and stem cell differentiation have shaped our understanding of the mechanisms that establish axial-specific populations of neural crest cells. Additionally, we evaluate how comparative, anatomical and genomic approaches have informed our current understanding of the evolution of the neural crest and its contribution to the vertebrate body.

KEY WORDS: Neural crest, Ectomesenchyme, Stem cells, Gene regulatory networks, Patterning

Introduction

The neural crest (NC) is a transient, multipotent cell population that exhibits remarkable migratory capacity and gives rise to a vast array of cell types, including neurons, glia, pigment cells, chondrocytes and odontoblasts (Le Douarin and Kalcheim, 1999). The NC has long fascinated developmental biologists, who have used multi-disciplinary approaches ranging from classic embryological techniques to single cell transcriptomics to investigate its development. These studies have furthered our understanding of the mechanisms that underlie NC cell development (reviewed by Cheung et al., 2019; Le Douarin and Dupin, 2018; Mayor and Theveneau, 2013; Prasad et al., 2019; Simões-Costa and Bronner, 2015). However, the broad question of how the NC is regionalized into distinct cell populations along the anteroposterior (AP) axis remains a crucial topic for discussion.

The development of the quail-chick chimera system in the early 1970s by Nicole Le Douarin allowed for comprehensive analyses of NC migration and contributions. This system took advantage of the fact that the embryos of these closely related avian species are of a similar size during the early stages of development, yet their cells exhibit unique nuclear morphologies. Because the nuclei of quail cells show a large mass of condensed heterochromatin upon Feulgen-Rossenbeck staining, researchers could use them as natural, indelible lineage tracers (Le Douarin, 1973; see also Tang and Bronner, 2020). By generating quail-chick chimeras, Le Douarin and colleagues (summarized by Le Douarin and Kalcheim, 1999) and Noden (1975, 1978, 1983) elucidated NC cell migration pathways and derivatives along the AP axis (Fig. 1). Based on these and other studies, we now understand that the NC is regionalized along the AP axis into discrete subpopulations with distinct differentiation potential: cranial, vagal, trunk and sacral. Understanding the evolutionary and developmental origin of NC regionalization is essential, given that defects in NC formation that affect specific regional populations may lead to devastating diseases, such as Treacher Collins syndrome (Trainor, 2010) or Hirschsprung's disease (Bergeron et al., 2013; Butler Tjaden and Trainor, 2013).

In this article, we highlight how lineage-tracing experiments, primarily in avians, have revealed axial-specific differences in NC potential, and discuss how these differences may be explained by modifications to the gene regulatory network that underlies NC development. Next, we focus on recent studies of human pluripotent stem cell (hPSC) differentiation, which suggest that neuromesodermal progenitors (NMPs) – cells that form much of the trunk and tail – may be an important source of trunk NC cells. Finally, we present models for the evolution of NC regionalization and suggest experimental approaches to enhance our understanding of NC evolution.

Axial differences in neural crest differentiation potential

The pioneering lineage-tracing experiments using the quail-chick chimera system showed that NC cells from all levels of the body axis give rise to pigment cells, Schwann cells and neurons. NC cells that originate in the head migrate in broad streams and differentiate into neurons of the sensory and parasympathetic ganglia, as well as a wide array of ectomesenchymal cell types (Le Douarin and Kalcheim, 1999). By contrast, trunk NC cells delaminate from the neural tube to migrate along two distinct pathways: ventrally between the neural tube and the adjacent somite, or along a dorsolateral route between the somite and the overlying ectoderm. NC cells that migrate along the ventral route differentiate into sensory neurons of the dorsal root ganglia and sympathetic neurons of the sympathetic chain ganglia, whereas those that migrate along the dorsolateral pathway form pigment cells (Le Douarin and Kalcheim, 1999). Subsequently, complexity was added to this model by the finding that a large number of pigment cells also originate from Schwann cell precursors, which originate from NC cells that travel along the ventral pathway (Adameyko et al., 2009). In chick embryos, NC cells from the level of somites 1-7 also give rise to the parasympathetic neurons that innervate the gut, which are collectively termed the enteric nervous system (Le Douarin and Teillet, 1973). In addition, the sacral NC cells that originate posterior to somite 28 form enteric neurons, although these are limited to the posterior-most region of the gut.

One of the striking differences between NC cell populations along the AP axis is that – at least in amniotes – only cranial NC cells give rise to ectomesenchymal derivatives, including cartilage, connective tissues, dermis, dermal bone and teeth (for a discussion on the ectomesenchymal potential of NC cells in other species, please see the section below on 'The evolution of neural crest regionalization'). This ectomesenchymal potential of NC cells was first proposed in the late 19th century by Julia Platt based on her

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Development (2020) 147, dev193888. doi:10.1242/dev.193888

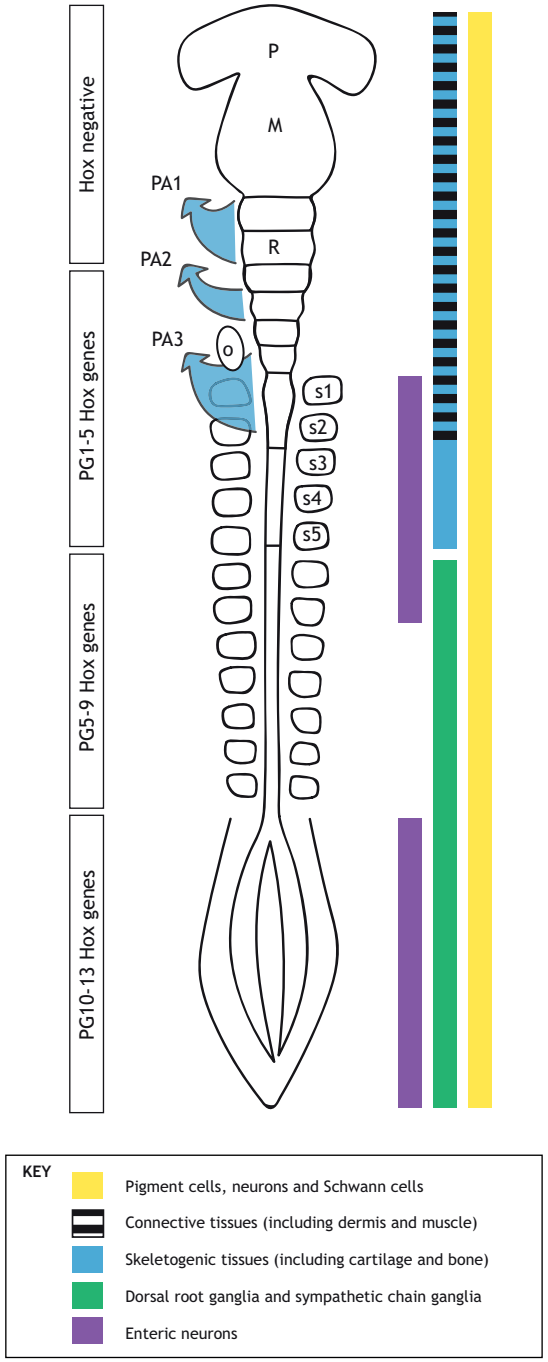

Fig. 1. Axial regionalization of the neural crest. Hox gene expression domains and neural crest derivatives are aligned to the body axis of a schematized amniote embryo. PG, paralog group. Fates of neural crest cells from all axial levels (yellow), cranial region only (blue, striped), trunk region only (green) and vagal/sacral regions (purple) are shown. The sacral neural crest occurs posterior to somite 28 in older embryos and is therefore shown alongside the unsegmented region of the pre-somitic mesoderm. The prosencephalon (P), metencephalon (M) and rhombencephalon (R) are labeled within the central nervous system. The otic vesicle (o), somites (s1-s5) and cranial neural crest streams migrating to the pharyngeal arches (PA) 1-3 are also indicated.

experiments in salamander (Platt, 1893). Her results were highly controversial at the time, as they countered the prevailing dogma of germ layer theory, which posited these tissues to be derived from the mesoderm. However, her findings were eventually confirmed by several researchers, including Hörstadius and Sellman, who used experimental embryology to elucidate the migration and contributions of NC cells (Hörstadius, 1950). Later, radiographic labeling with tritiated thymidine allowed researchers to characterize the extensive contributions of the NC to the vertebrate body and begin to demonstrate differences in potential between NC cells from various axial levels (reviewed by Le Douarin and Kalcheim, 1999). These studies also demonstrated that NC cells form embryonic facial processes and cartilage, and contribute to cranial ganglia (Johnston, 1966; Noden, 1975). However, as the radiolabel becomes diluted by cell proliferation, this method was of limited use in the analysis of late-developing tissues.

The development of the quail-chick chimera system allowed Le Douarin and colleagues, as well as Noden, to demonstrate that cranial NC cells contribute to the facial and visceral skeleton, and its adjacent connective tissue. Briefly, NC cells from the prosencephalon and the mesenecephalon form the nasal and periorbital skeleton and contribute to the cranial vault (see Box 1). Mesencephalic NC cells additionally give rise to the skeleton of the upper and lower jaws, the palate, and the tongue. They also contribute to the pre-otic region, alongside rhombencephalic NC cells. Finally, cartilage of the hyoid and posterior pharyngeal arches is derived from rhombencephalic NC cells (Le Douarin and Kalcheim, 1999; Noden, 1978). Cranial NC cells also produce loose connective tissue of the lower jaw, tongue and ventrolateral part of the neck, as well as dermis and striated muscles of the branchial arches (Le Douarin and Kalcheim, 1999; Noden, 1978).

More-recent lineage-tracing experiments revealed that the cranial NC migration pathways (Lumsden et al., 1991) and craniofacial derivatives (Köntges and Lumsden, 1996) maintain the spatial organization of the rhombencephalon and mesencephalon from which they derive. Moreover, the contributions of cranial NC cells are influenced in part by the action of intrinsic factors, including the Hox genes. Hox genes play a crucial role in patterning the skeletal derivatives of NC cells arising from the posterior rhombencephalon [rhombomeres (r)4-r8] (see Fig. 1). By contrast, NC cells that arise from the prosencephalon, mesencephalon and anterior rhombencephalon (r1 and r2) – which form the bones of the cranial and facial skull – do not express Hox genes (Couly et al., 1998, 2002; Creuzet et al., 2002). Accordingly, transplanting Hox-expressing neural folds from r4-5 into the anterior Hox-negative domains (Couly et al., 1998), or ectopically expressing Hox genes in the diencephalic neural folds (Creuzet et al., 2002), causes defects in the lower jaw and facial skeleton. Thus, Hox expression is incompatible with proper development of the jaw or facial derivatives of NC cells.

In addition to the intrinsic functions of Hox genes, extrinsic signals from the surrounding tissues are instructive in the development of cranial NC skeletal derivatives. This was elegantly demonstrated by experimental manipulations of the chick foregut endoderm. When researchers ablated strips of foregut endoderm, specific cranial NC-derived skeletal structures failed to develop, while grafts of ectopic foregut endoderm altered the identity of the skeletal structures (Couly et al., 2002). Importantly, only anterior, Hox-negative NC cells can respond to these endoderm-derived cues, whereas posterior, Hox-expressing NC cells do not form bone and cartilage in response to anterior foregut endoderm grafts.

Strikingly, these studies indicated that the posterior limit of skeletogenic NC cells corresponds to the level of the 5th somite (Le Lièvre and Le Douarin, 1975), near the transition between the rhombencephalon and the spinal cord (Fig. 1). To determine whether this represents an intrinsic feature of the cranial NC or

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Box 1. The contribution of NC cells to the cranial vault

The role of NC cells in the development of the cranial vault – including the frontal and parietal bones – has been controversial. Both Noden (1978) and Le Lievre (1978) reported that the avian frontal bone is of combined mesodermal and NC origin, while Le Douarin (1982) concluded that both bones are derived entirely from the mesoderm (Le Douarin, 1982; Le Lievre, 1978; Noden, 1978). Subsequent fate mapping from an earlier stage of development – the late neurula – suggested that the frontal and parietal bones are derived exclusively from NC (Couly et al., 1993). However, the most recent chick data indicate a mixed origin of the frontal bone – with the supraorbital region derived from the NC and the calvarial region from the mesoderm – and a mesodermal origin of the parietal bone (Evans and Noden, 2006). Advances in lineage-tracing approaches in mice have allowed continued investigation of this issue. Wnt1-Cre/R26R transgenic-based NC lineage tracing, together with DiI labeling of the cephalic mesoderm, revealed that the mouse frontal bone is derived from NC cells, whereas the parietal bone originates from the mesoderm (Jiang et al., 2002). Recent experiments in amphibians have revealed yet more complexity (Maddin et al., 2016; Piekarski et al., 2014). In axolotls, the frontal, but not the parietal, bone originates from NC cells of the mandibular stream (Maddin et al., 2016; Piekarski et al., 2014), a pattern that largely reflects that of amniotes. However, *Xenopus* embryos exhibit a unique pattern characterized by extensive contribution of NC cells from the mandibular, hyoid and branchial streams to the osteocranium, including the frontoparietal bone (Piekarski et al., 2014). This pattern may have evolved after anurans diverged from other living amphibians (Piekarski et al., 2014). Lineage-tracing approaches will no doubt continue to be an important tool for investigating the role of the NC in the evolution of the cranium (reviewed by Teng et al., 2019).

results from different signaling environments, Le Douarin and colleagues performed a series of transplantation experiments (summarized in Fig. 2). When quail mesencephalic and anterior rhombencephalic primordia were grafted into the chick neural axis at the level of somites 18-24, donor quail cells differentiated into dermis, cartilage and connective tissues (Le Douarin and Teillet, 1974), suggesting that cranial NC are still capable of generating ectomesenchymal derivatives in an ectopic environment. Conversely, bilateral grafting of the trunk NC primordium into the anterior rhombencephalon resulted in the absence of facial and branchial skeletal elements (Le Douarin et al., 1977; Nakamura and Ayer-le Lievre, 1982). Similarly, when trunk dorsal neural tube was grafted to the midbrain, donor NC cells failed to form normal corneal derivatives, contributed fewer neurons to the trigeminal ganglion and did not form cartilage, even when grafted directly into the first branchial arch (Lwigale et al., 2004). These results demonstrate that chondrogenic potential is an intrinsic and distinguishing feature of cranial, but not trunk, NC.

Nevertheless, the signaling environment is also important in directing ectomesenchymal differentiation. Cranial and trunk NC cells differ in their survival and differentiation in response to various extracellular signals *in vitro* (Abzhanov et al., 2003). Yet when quail trunk NC fragments are unilaterally grafted to the anterior rhombencephalon of a chick host, donor NC cells migrate alongside host NC cells. In these chimerea, quail ectomesenchyme derivatives are detected in connective tissues, dermis and muscle, but not cartilage or bone (Fig. 2) (Nakamura and Ayer-le Lievre, 1982). These results suggest that the host cranial NC might provide extrinsic signals that allow trunk NC cells to give rise to a subset of ectomesenchymal derivatives. Moreover, when avian trunk NC cells are cultured in media commonly used for growing bone and cartilage cells, they generate ectomesenchymal derivatives *in vitro* (Coelho-Aguiar et al., 2013; McGonnell and Graham, 2002) and contribute to cranial skeletal components when transplanted into the head (McGonnell and Graham, 2002). Thus, although chondrogenic potential is an intrinsic feature of the cranial NC, the signaling environment contributes to promoting this fate.

Although much emphasis has been placed on the development of the cranial NC, it should be noted that trunk NC cells also give rise to unique cell types and exhibit distinct cellular behaviors. At the level of somites 18-24 in the chick, some NC cells form chromaffin cells: the neuroendocrine cells of the adrenal medulla (Le Douarin and Kalcheim, 1999). It was a commonly held view that sympathetic neurons and chromaffin cells are derived from a common lineage of catecholaminergic NC-derived progenitors, termed sympathoadrenal progenitors, that migrate to the dorsal aorta (reviewed by Huber et al., 2009). However, recent lineage-tracing and genetic ablation experiments have revealed that chromaffin cells are, in fact, largely generated by Schwann cell precursors: a NC-derived population of peripheral glial progenitors that migrate along motor nerve fibers (Furlan et al., 2017). Nevertheless, as graft-derived NC cells are detected in the adrenal medulla following transplantation of cranial neural primordium to the adrenomedullary region (Le Douarin and Teillet, 1974), the ability to generate chromaffin cells is not limited to trunk NC.

Axial-specific gene regulatory networks

A gene regulatory network (GRN) is a powerful tool used to describe the genetic basis of cell fate specification. Indeed, the distinct properties of NC cells at various axial levels may be explained by axial differences in their GRNs (Simões-Costa and Bronner, 2015). Several transcription factors, including Id2 (Martinsen and Bronner-Fraser, 1998) and Ets-1 (Tahtakran and Selleck, 2003; Théveneau et al., 2007), are expressed in cranial, but not trunk, NC cells in chick embryos. However, it should be noted that chick Id2 is also expressed in cardiac NC cells (Martinsen et al., 2004) and Ets1 is expressed in zebrafish and hPSC-derived trunk NC cells (Frith et al., 2018; Gomez et al., 2019a; Martik et al., 2019). Thus, a greater understanding of the regulatory functions of these factors, as well as the species-specific variation in the mechanisms that establish axial identity, is still needed. Nevertheless, Ets-1 is both necessary and sufficient to confer cranial-specific delamination properties on NC cells in chick embryos (Théveneau et al., 2007). In addition, the regulatory regions of two key NC specifier genes in chick – *Foxd3* (Simões-Costa et al., 2012) and *Sox10* (Betancur et al., 2010) – have axial-specific enhancers that drive their expression in either the cranial or the trunk NC (Betancur et al., 2010; Simões-Costa et al., 2012). Notably, both cranial enhancers are directly activated by Ets-1.

In recent years, next-generation sequencing approaches, primarily in amniote model systems, have enabled researchers to evaluate the hypothesis that axial-specific GRNs pattern the NC. Specifically, transcriptional profiling has further elucidated the gene regulatory differences between cranial and trunk NC cell populations. For example, Simões-Costa and Bronner (2016) uncovered a cranial-specific transcriptional circuit in chick embryos. This GRN includes *Brn3c*, *Lhx5* and *Dmbx1*, which are expressed in the anterior region of gastrula-stage embryos and persist throughout NC specification. Subsequently, *Tfap2b*, *Sox8* and *Ets-1* are detected in NC progenitors in the cranial neural folds and in migrating NC cells. Introducing the latter three components of this network into the trunk is sufficient to reprogram trunk NC cells to a cranial identity and leads to the acquisition of

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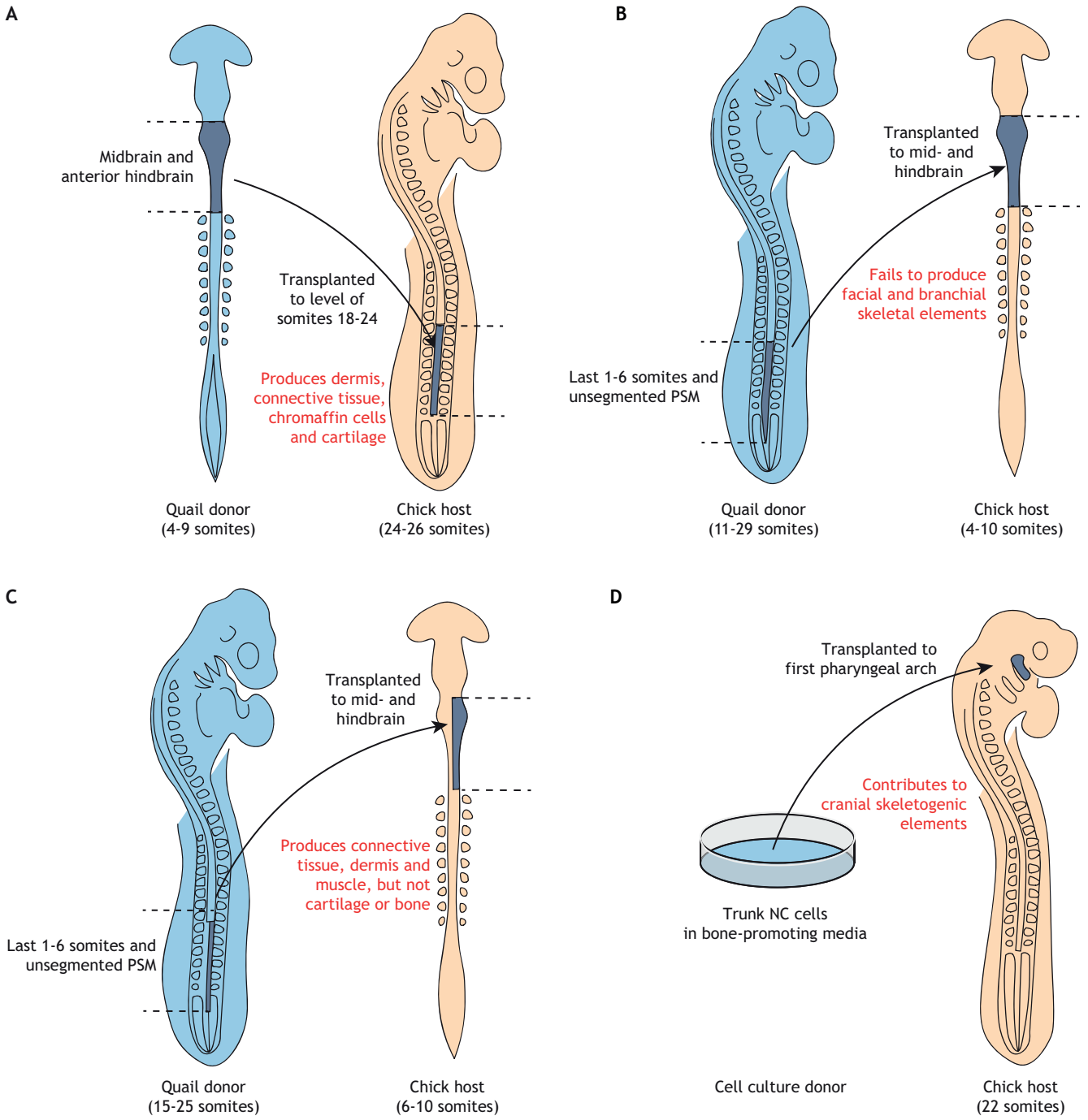

Fig. 2. Transplantation approaches reveal that intrinsic factors and extrinsic signals underlie differences between cranial and trunk NC cells. Transplantation experiments reveal differences in the contributions of cranial and trunk neural crest. Derivatives of transplanted tissue are in red; quail embryos are shown in blue, chick embryos in orange. (A) Bilateral and heterotopic transplant of cranial (midbrain and anterior hindbrain) neural primordium from a quail donor (4-9 somite stage; ss) to the trunk of a chick host (24-26 ss) leads to the formation of skeletogenic derivatives, as well as chromaffin cells, at ectopic posterior positions. (B) The reciprocal transplant of trunk neural tube from a quail donor (11-29 ss) to the cranial (mid- and hindbrain) region of a chick host (4-10 ss) shows that trunk NC does not form skeletogenic derivatives. (C) A unilateral version of the transplant experiment shown in B demonstrates that host tissue can influence the migration and potential of transplanted cells. Donor cells form connective tissues alongside the host NC but cannot form skeletogenic derivatives. (D) When trunk NC cells cultured in bone-promoting media are transplanted into the mandibular and maxillary primordium of a chick host, the transplanted cells are able to form skeletogenic derivatives, demonstrating the importance of the NC signaling environment for cell fate decisions.

chondrogenic potential (Simões-Costa and Bronner, 2016). A similar approach identified a transcriptional subcircuit comprising *Tgif1*, *Ets1* and *Sox8* that imparts cardiac NC identity and is necessary for proper heart development. Ectopic expression of this subcircuit is sufficient to reprogram trunk NC cells to a cardiac fate and enables them to rescue defects in heart formation caused by cardiac NC ablation (Gandhi et al., 2020).

By coupling transcriptional and epigenomic profiling in cranial NC cells at population and single-cell levels, Williams et al. (2019) reverse engineered the global NC GRN with remarkable resolution.

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Their analysis of chromatin dynamics revealed three distinct classes of regulatory elements: one that is accessible in premigratory and/or migratory NC, one that is accessible in both NC and neuroepithelial cells, and one that is accessible in naive epiblast and premigratory NC cells but inaccessible at later stages (Williams et al., 2019). This study also uncovered an early cis-regulatory split between mesenchymal and neural progenitors, which was confirmed by single cell transcriptomics (Williams et al., 2019). Relevant to these findings, Weston and colleagues have posited that the cranial cell types typically attributed to the NC are in fact derived from two spatially, temporally and molecularly distinct pools of progenitors. The first is the 'metablast', which encompasses non-neural epithelium that lies lateral to the developing neural folds and gives rise to the cranial ectomesenchymal lineage. The second is the more medial, neuroepithelial-derived 'authentic' NC that migrates at a slightly later stage to produce neurogenic and melanogenic cell types (Breau et al., 2008; Lee et al., 2013a; Weston and Thiery, 2015; Weston et al., 2004). This model has been disputed by Dupin et al. (2018), who contend that single NC cells can give rise to both ectomesenchymal and neural-melanocytic derivatives *in vitro*. These competing models do have important implications regarding the patterning of the NC. The model put forth by Dupin and colleagues reflects a traditional view in which the potential of NC cells is axially regionalized. By contrast, the model of Weston and colleagues would argue for a more uniform 'authentic' NC being present at all axial levels alongside a 'metablast' that is restricted to the cranial region. The results obtained by Williams and colleagues (2019) highlight how systems-biology approaches may serve as an important tool for informing this discussion.

Using a similar approach, Ling and Sauka-Spengler (2019) dissected the GRN that governs the development of the vagal NC. Their study showed that this heterogeneous cell population can be separated into a $Sox10^{high}$/FoxD3+ sub-population capable of forming neural, mesenchymal and neuronal derivatives, and a $Sox10^{low}$/FoxD3^{-} sub-population that is restricted to neuronal and mesenchymal fates. By incorporating chromatin accessibility and genetic interactions, this study identified the Tfap2, Sox, Hbox and bHLH families of transcription factors as core regulators of the vagal crest GRN and validated their function by genetic knockout (Ling and Sauka-Spengler, 2019).

Single-cell analyses of mouse embryos have revealed that NC cells at distinct axial positions exhibit largely similar transcriptional profiles over time, yet they also have important axial-specific biases (Soldatov et al., 2019). For example, cranial NC cells are biased towards a mesenchymal fate, whereas trunk NC cells are biased towards sensory and autonomic neuronal fates. These biases emerge during delamination, with mesenchymal fates resulting from sustained high levels of expression of *Twist1* in the cranial region (Soldatov et al., 2019). Interestingly, cranial and trunk NC cells become transcriptionally distinct at different times in mouse and chick: while the mouse cranial program is established during delamination, the chick cranial GRN initiates during the early stages of NC specification (Simões-Costa and Bronner, 2016). It will be important to establish whether this apparent offset in timing is a technical artefact – e.g. due to inconsistent labeling techniques or inconsistencies in staging – or reflects species-specific biological differences.

Cranial NC cells in zebrafish also express Twist1, which promotes ectomesenchymal fate at the expense of other genetic programs (Das and Crump, 2012). In mice, *Twist1* mutants show impaired skeletogenic differentiation and fail to form bones of the snout, upper face and skull vault (Bildsoe et al., 2009; Soo et al., 2002). In both species, Twist1 deficiency leads to persistent expression of Sox10 and a loss of ectomesenchymal differentiation markers (Bildsoe et al., 2009; Das and Crump, 2012; Soo et al., 2002). Soldatov and colleagues also showed that loss of Twist1 in mouse cranial NC results in a reduction of mesenchymal derivatives and an increase in glial and neuronal fates. Conversely, ectopic expression of Twist1 in the mouse trunk NC, starting from pre-EMT stages, results in the expression of a mesenchymal marker (*Prrx1*) at the expense of neuronal sensory, autonomic and glial fates (Soldatov et al., 2019). Together, these results indicate that Twist1 is sufficient to drive the acquisition of some ectomensenchymal fates.

Recent advances in the dissection of genetic circuits and interrogation of transcriptional profiles have been invaluable in uncovering the molecular basis of NC axial identity. These approaches have revealed that intrinsic differences in gene expression mediate at least some axial-specific properties of NC cells, including ectomesenchymal potential, and have begun to establish the regulatory logic that underlies the cranial genetic circuit.

Lessons from stem cells

The ability to differentiate human pluripotent stem cells (hPSCs) into NC cells *in vitro* has provided novel insights into the mechanisms by which the NC is patterned along the AP axis. Importantly, it has also proven an important tool for studying human NC biology and NC-associated developmental disorders. Early methods for deriving NC cells from hPSCs relied on stromal co-culture (Jiang et al., 2009; Lee et al., 2007; Pomp et al., 2005) or induction of neural rosettes (Chambers et al., 2009; Lee et al., 2010). However, these protocols yielded limited numbers of NC cells and often required FACS isolation using the cell surface markers HNK-1 and p75. More recently, several protocols have described feeder-free conditions for generating NC cells with high efficiency using small molecules and growth factors (Hackland et al., 2017; Lee et al., 2010; Leung et al., 2016; Menendez et al., 2011, 2013; Mica et al., 2013).

Remarkably, these protocols yield hPSC-derived NC cells that possess cranial identity by default, indicated by their ability to give rise to chondrocytes and their lack of Hox expression (Fig. 3) (Fukuta et al., 2014; Hackland et al., 2017; Lee et al., 2007, 2010; Leung et al., 2016; Menendez et al., 2011; Mica et al., 2013). Treatment of hPSCs with retinoic acid (RA) during differentiation yields a subpopulation of NC cells with characteristics of cardiac and/or vagal NC, including expression of paralog group (PG) 1-5 Hox genes (Figs 1 and 3) (Frith et al., 2018; Fukuta et al., 2014; Mica et al., 2013). In particular, these conditions yield cultures with the potential to form enteric neurons, a cell type that defines the vagal NC (Barber et al., 2019; Fattahi et al., 2016; Workman et al., 2017). Huang et al. (2016) reported that, when combined with both TGFβ inhibition and Wnt signaling activation, treatment with RA generates NC cells that express PG6-9 Hox genes in addition to PG2-5 Hox genes (Figs 1 and 3). These NC cells activate the *Sox10E1* enhancer, which is expressed in both vagal and trunk NC, and they are capable of differentiating into TH^{+} sympathoadrenal cells (Huang et al., 2016). However, the expression of PG6-9 in these cells is relatively low and they are unlikely to efficiently generate trunk NC cells. Finally, when NC cells are derived from stem cells in the presence of RA, they give rise to enteric neurons when grown together with human intestinal organoids, or colonize the foregut when transplanted into chick embryos (Workman et al., 2017). These findings are consistent with the known role of endogenous RA, which is necessary for proper development and gut colonization of the enteric NC (Niederreither et al., 2003; Uribe

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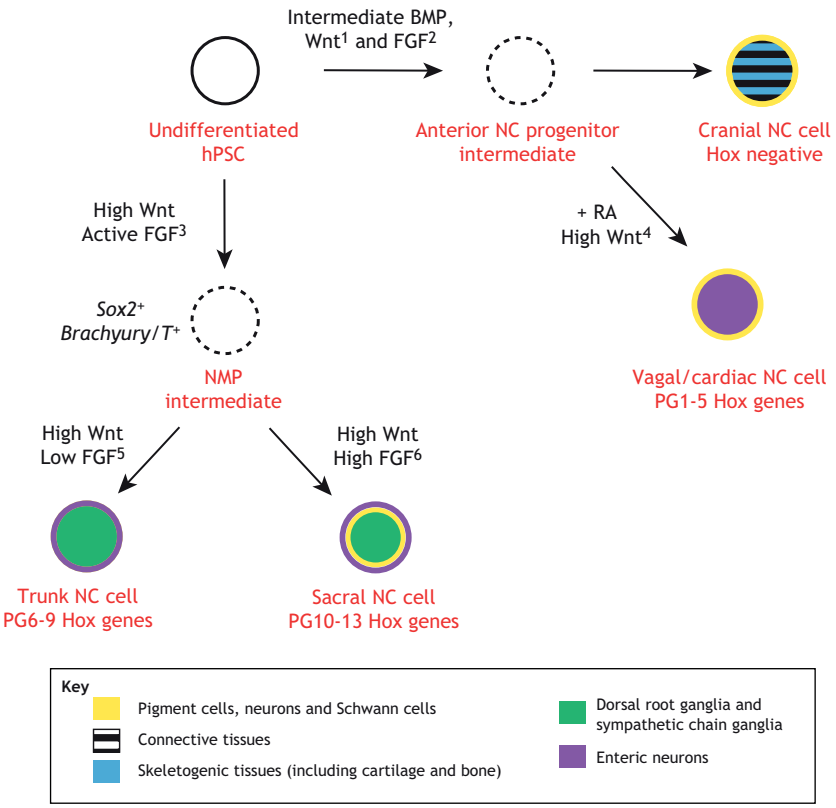

Fig. 3. Differentiation of hSPCs into distinct axial subpopulations of NC. An undifferentiated human pluripotent stem cell (hPSC) passes through different intermediate states en route to a cranial or trunk neural crest cell fate. Each cell type and its characteristic gene expression is in red. The signals needed to promote each cell type are indicated next to the arrows. Derivatives formed by each cell type are color coded. [1]Hackland et al., 2017; [2]Lee et al., 2007; [3]Frith et al., 2018; [4]Fattahi et al., 2016; [5]Gomez et al., 2019b; [6]Hackland et al., 2019.

et al., 2018). Together, these studies indicate that treatment with RA during differentiation yields vagal NC cells.

In recent years, increasing evidence has suggested that the production of bona fide trunk NC cells from hPSCs requires cells to pass through an intermediate state that resembles neuromesodermal progenitors (NMPs). NMPs are bipotent stem cells found in the primitive streak and tailbud that produce much of the trunk and tail (see Fig. 4 and Box 2). Initial studies suggested that hPSC-derived NMP-like cells, marked by robust co-expression of Sox2 and Brachyury/T, can be differentiated into trunk NC cells capable of differentiating into chromaffin cells *in vitro*, as well as *in vivo* upon transplantation into chick embryos (Abu-Bonsrah et al., 2018; Denham et al., 2015). Subsequently, Frith and colleagues demonstrated that hPSC-derived NMPs (Gouti et al., 2014) can differentiate into trunk NC cells and their derivatives (Frith and Tsakiridis, 2019; Frith et al., 2018). Interestingly, several known markers of neural plate border and early NC identity are also detected in these NMPs (Frith et al., 2018). Under differentiation conditions, the hPSC-derived NMPs give rise to NC cells that express PG5-9 Hox genes – typical of the thoracic neurectoderm – and can also give rise to sympathoadrenal cells (Frith et al., 2018). Other protocols for generating trunk NC cells – defined by HoxC9 expression, limited mesenchymal potential, and the ability to produce sympathoadrenal cells – again report the presence of an NMP-like intermediate state (Gomez et al., 2019b; Hackland et al., 2019). Notably, these trunk NC cell cultures exhibit a wider developmental potential than do avian trunk NC cells, as they are capable of forming smooth muscle and osteoblasts (Gomez et al., 2019b; Hackland et al., 2019). Finally, NMP-derived pre-neural progenitors give rise to trunk NC cells with progressively more posterior identity over increasing passages (Cooper et al., 2020 preprint), perhaps reflecting the co-linear expression of Hox genes observed *in vivo*.

Additional experiments have revealed that Wnt and Fgf signaling, which are necessary for maintaining the NMP niche *in vivo* (reviewed by Wilson et al., 2009) are also critical for specifying the axial identity of hPSC-derived NC cells *in vitro*. Wnt signaling levels are crucial for determining cranial versus trunk fate of hPSC-derived NC cells (Gomez et al., 2019b; Hackland et al., 2019). hPSCs exhibit a bimodal response to Wnt signaling, whereby low Wnt signaling leads to anterior Hox-negative NC cells, and high Wnt signaling results in posterior Hox-expressing NC cells (Gomez et al., 2019b). Furthermore, the magnitude of Wnt stimulus dictates the degree of NC posterior identity based on Hox gene expression, suggesting a rheostat response. Within the trunk compartment, Fgf signaling determines axial identities: treatment of hPSC cultures with Fgf2 during the first 2 days of NC induction leads to expression of the sacral HoxA10-13 genes (Figs 1 and 3), whereas the Fgf inhibitor PD17 abrogates all Hox expression (Hackland et al., 2019).

The finding that NMPs produce trunk NC cells *in vitro* is consistent with the results of lineage-tracing studies *in vivo*. Based on their analyses of chick and mouse embryos, colleagues (Schoenwolf and Nichols, 1984; Schoenwolf et al., 1985) first proposed that cells in the tail bud might give rise to NC cells in the tail. This hypothesis was later substantiated by grafting quail tissue into the tailbud of 25-somite stage chick hosts, which revealed that the cells in the chordoneural hinge region of the tailbud contribute not only to the spinal cord and somitic mesoderm – as expected of NMPs – but also to the NC and its derivatives (Catala et al., 1995). More recently, fate mapping of the mouse primitive streak and tailbud, either by grafting GFP-labeled cells or by permanent genetic cell labeling, has also shown that NMPs give rise to trunk and tail NC cells (Javali et al., 2017; Rodrigo Albors et al., 2018; Tzouanacou et al., 2009; Wymeersch et al., 2016), as well as NC-derived sensory neurons of the dorsal root ganglia in the sacral

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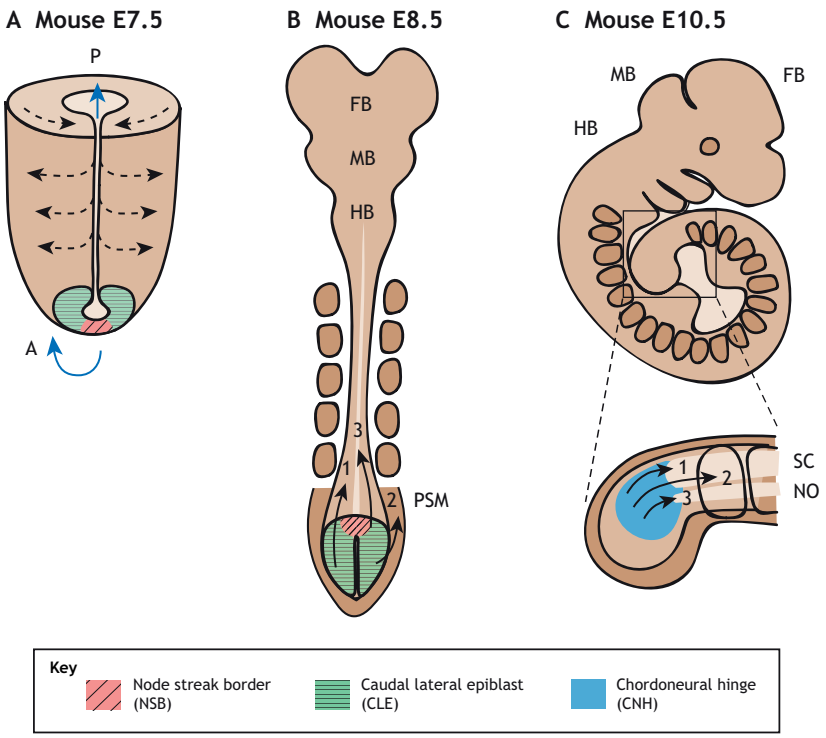

Fig. 4. Bipotent NMPs in the tailbud give rise to posterior tissues. (A) Gastrulation stage (E7.5) mouse embryo showing the primitive streak and the location of neuromesodermal progenitors (NMPs) within the node streak border (NSB) and caudal lateral epiblast (CLE). Gastrulation movements through and away from the primitive streak are shown using dashed arrows, while the anterior (A) to posterior (P) axis of the developing body is labeled with a blue arrow. (B) Early somite-stage mouse embryo (E8.5). Arrows show contributions of bipotent NMPs located in the NSB and CLE to both neuroectodermal tissues, such as the spinal cord (1), and mesodermal tissues, such as the somitic mesoderm (2) and notochord (3). (C) At later stages (E9.5-14.5), NMPs located in the chordoneural hinge (CNH) of the developing tailbud continue to contribute to both mesodermal and neuroectodermal tissues. A, anterior; FB, forebrain; HB, hindbrain; MB, midbrain; NC, notochord; P, posterior; PSM, pre-somitic mesoderm; SC, spinal cord.

region (Shaker et al., 2020 preprint). However, the extent to which NMPs contribute to the NC remains unclear, as these analyses are based on only a small number of clones. Cre-based lineage tracing driven by *Tbx6* and *Nkx2* regulatory sequences, which are expressed in NMPs and early mesodermal and neural progenitors, labeled NC cells in the anterior trunk (Javali et al., 2017; Rodrigo Albors et al., 2018), although Javali et al. (2017) reported a higher contribution to NC cells at the sacral level. Clonal analysis using the *R26nlaacZ* system – in which an inactive variant of *LacZ* is driven by the ubiquitous Rosa26 promoter and rare spontaneous deletions restore β-galactosidase activity in single cells – yielded clones that labeled the NC along the entire extent of the trunk and tail (Tzouanacou et al., 2009). Collectively, these results provide compelling evidence that NMPs contribute to at least a subset of trunk NC cells *in vivo*.

Despite the evidence for a role for NMPs in generating trunk NC cells, it remains unclear whether NMP intermediates are necessary for trunk NC identity *in vivo*. In fact, Gomez et al. (2019b) also showed that cells exposed to high Wnt can still adopt a trunk NC fate even when expression of NMP markers is compromised following inhibition of Fgf signaling. Additionally, it is unclear how the NMP state might imbue trunk-specific features of NC cell development, such as the restriction of ectomesenchymal potential. Thus, it will be important to better elucidate the regulatory link between the NMP state and trunk NC identity. To address this issue, it will be valuable to use systems biology approaches to determine how components of the NMP genetic circuit establish trunk NC cell fate. Moreover, while the contribution of NMPs to axial elongation and NC cell populations has been well documented in amniotes, whether equivalent cells play a role in the development of non-amniotes, such as zebrafish (Attardi et al., 2019; Kanki and Ho, 1997; Martin and Kimelman, 2012) and *Xenopus* (Gont et al., 1993), remains controversial. Nevertheless, some have proposed that the molecular mechanisms governing posterior extension of the embryo – including the gene regulatory state that defines NMPs – are conserved across vertebrates (Kimelman, 2016; Steventon and Martinez Arias, 2017; Wilson et al., 2009). As current experimental evidence for this assertion remains inconclusive, it will be important to investigate the cell lineages that give rise to trunk NC cells in zebrafish and *Xenopus*, and determine whether they pass through an NMP-like state.

The evolution of neural crest regionalization

Our understanding of NC regionalization along the AP axis is largely based on studies from a small set of model organisms. An evolutionary framework is therefore required to compare regionalization mechanisms between these organisms and reconstruct the ancestral state of NC development. In this context, Gans and Northcutt (1983) hypothesized that the vertebrate head is an evolutionary novelty, the emergence of which was facilitated by the acquisition of NC cells and neurogenic placodes in a vertebrate ancestor. New lines of research continue to test predictions of this hypothesis. Fortunately, the ectomesenchymal derivatives of NC cells are preserved in the fossil record as a diverse array of skeletal structures (Smith and Hall, 1990). This provides a dataset that complements findings from living embryos. In addition, different approaches – such as the comparative analysis of ectomesenchymal tissues and the examination of genetic circuits – have shed light on the evolutionary pattern of NC fates and potential regulatory processes underlying these patterns. Below, we highlight results derived from both these lines of questioning and discuss additional tests that could be used to validate emerging models and synthesize new hypotheses.

Paleontology and comparative anatomy suggest ectomesenchymal potential is ancestral

Histological examination of the vertebrate dermal skeleton in fossils and extant vertebrates led Smith and Hall (1990, 1993) to postulate that the ancestral NC possessed ectomesenchymal potential at all axial levels. Both fossil and extant vertebrates show a remarkable

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Box 2. Neuromesodermal progenitors contribute to the post-cranial body

In mouse and chick, tissues posterior to the head are in large part generated by multipotent stem cells, termed neuromesodermal progenitors (NMPs). This idea was first supported by lineage tracing of Hensen's node in the chick embryo, which showed that single cells can contribute to more than one tissue type (Selleck and Stern, 1991). Similarly, labeling of small groups of cells in the caudal lateral epiblast yielded clones that contribute to the neural tube and somitic mesoderm (Brown and Storey, 2000). Clonal analyses of mouse somites (Nicolas et al., 1996) and the spinal cord (Mathis and Nicolas, 2000), using the *LaacZ* system (see main text) uncovered long clones spanning many segments, suggesting that these progenitors must persist over extended periods. Later, Cambray and Wilson showed that cells located in several discrete regions of the mouse primitive streak and the adjacent epiblast – the caudal lateral epiblast and the node-streak border – give rise to both neural and mesodermal derivatives (Fig. 4), and can be serially transplanted into younger hosts. The descendants of cells from these regions are later found within the chordoneural hinge of the tailbud (Fig. 4) and exhibit similar properties (Cambray and Wilson, 2002, 2007). In chick embryos, tailbud progenitors are capable of resetting their Hox gene expression to match the surrounding tissue upon heterochronic transplantation into younger hosts, indicating that NMPs change their Hox gene expression profile over time and that this process is reversible (McGrew et al., 2008). NMPs are characterized by co-expression of the mesodermal marker *Brachyury*/*T* and the neural marker *Sox2* (Cambray and Wilson, 2007; Garriock et al., 2015; Wymeersch et al., 2016), and recent studies have begun to elucidate the genetic circuits that govern NMP formation, differentiation and maintenance (Amin et al., 2016; Gouti et al., 2017).

diversity of mineralized scales and dermal bones along the AP axis. The mineralized dermal tissues in vertebrates are derived from different combinations of odontogenic (dentine-forming) and mesodermally derived osteogenic (bone-forming) units (Sire et al., 2009; Smith and Hall, 1990). These two cell condensations are primarily distinguished based on their position within the dermis, the state of polarization of the extracellular matrix and their modes of development (Fig. 5).

The role of cranial NC cells in the formation of dentine in oral teeth has been well established in amphibians (Graveson et al., 1997; Smith and Hall, 1990) and mice (Chai et al., 2000; Lumsden, 1988). Experimental embryology approaches in amphibians revealed that the dentine-producing dental mesenchyme is derived from cranial NC cells, while the overlying enamel develops from oral ectodermal epithelium. In addition, tissue recombination studies in mice showed that teeth can form when cranial NC cells are co-cultured with ectodermal epithelium from the mandibular arch, but not with limb epithelium (Lumsden, 1988). More recently, genetic-based lineage tracing using the Wnt1-Cre system demonstrated that cranial NC cells contribute to the condensed dental mesenchyme, dental papilla, odontoblasts, dentine matrix, pulp, cementum and periodontal ligaments (Chai et al., 2000). Because dentine found in oral teeth is derived from NC cells, Smith and Hall (1990) concluded that the odontogenic tissues of dermal scales are similarly derived from post-cranial NC cells. This conclusion is further supported by reports that post-cranial NC cells can form dentine under appropriate signaling conditions (Graveson et al., 1997; Lumsden, 1988).

Differential losses and elaborations of osteogenic and odontogenic components have led to the variety of dermal tissues found in extinct and extant vertebrates (Fig. 5). Chondrichthyan species – cartilaginous fishes including sharks and skates – have a complete dermal armor of small dentinous scales and have lost the osteogenic layer (Gillis et al., 2017; Sire et al., 2009). Thus, they offer tractable models for elucidating the relationship between oral teeth and scales. Oral teeth and trunk scales in sharks display similar expression patterns of Dlx transcription factors during development (Debiais-Thibaud et al., 2011), shedding light on the gene-regulatory basis of the long-recognized histological and morphological similarities between skin denticles and oral teeth. Consistent with this, lineage-tracing experiments suggest that trunk NC cells give rise to odontoblasts of trunk dermal denticles in the little skate (Gillis et al., 2017). However, the extended developmental period of skate embryos – in this case requiring analysis 4-5 months after dye injections (Gillis et al., 2017) – presents a formidable challenge to precise and comprehensive labeling, and to securing robust controls that could rule out a mesodermal contribution.

In contrast to the findings in chondrichthyans, lineage-tracing studies in teleosts indicate a mesodermal origin for trunk scales. Contrary to early reports that zebrafish trunk NC cells contribute to fin ectomesenchyme (Kague et al., 2012; Smith et al., 1994) and scales (Sire and Akimenko, 2004; Smith and Hall, 1990), recent analyses have clarified that these tissues are derived exclusively from the mesoderm in both zebrafish (Lee et al., 2013b,c; Mongera and Nüsslein-Volhard, 2013) and medaka (Shimada et al., 2013). However, the superficial odontogenic layer has been highly reduced or eliminated in teleosts, implying that the analysis of teleost scale development may be of limited use for evaluating the broader evolutionary pattern of ectomesenchymal potential of trunk NC cells across vertebrates.

In summary, the results from comparative anatomy approaches suggest that the ancestral NC possessed ectomesenchymal capacity throughout the body axis, but that this potential was restricted to the head as a result of evolutionary loss (Fig. 6A). In fact, the trend towards reduction and restriction of ectomesenchymal potential of NC cells to cranial and oral domains is observed across multiple lineages, including cyclostomes (discussed below) and ray-finned fishes, and within lobe-finned fishes (Fig. 5). Nevertheless, it is important to note that although the regulatory framework underlying odontogenesis by NC cells has been studied in mammals and amphibians (Chai et al., 2000; Graveson et al., 1997; Lumsden, 1988; Smith and Hall, 1990), it has not been well characterized in cartilaginous and non-teleost bony fishes, which have a clearly identifiable dentine layer in their scales (Sire and Huysseune, 2003). If dentine in scales is not derived from NC cells, then a fundamental assumption of the hypothesis that ancestral NC possessed ectomesenchymal potential along the entire body axis is violated.

Comparative genetics suggests that ectomesenchymal potential was restricted by modifying subcircuits

Recent studies of the NC GRN in a broad variety of extant vertebrates support an increased degree of axial regionalization and elaboration of the NC in jawed (versus jawless) vertebrates. Such molecular axial regionalization may provide an explanatory mechanism for the restriction of ectomesenchymal potential to cranial NC in some lineages. Key insights have come from a jawless (agnathan) species, the lamprey – a member of the early diverging vertebrate cyclostome lineage. Lampreys express core genes of the NC GRN (Hockman et al., 2019; Nikitina et al., 2008; Sauka-Spengler et al., 2007). In fact, it has been suggested that some components of the NC GRN may predate vertebrate origins (York and McCauley, 2020). Yet multiple components of the avian cranial

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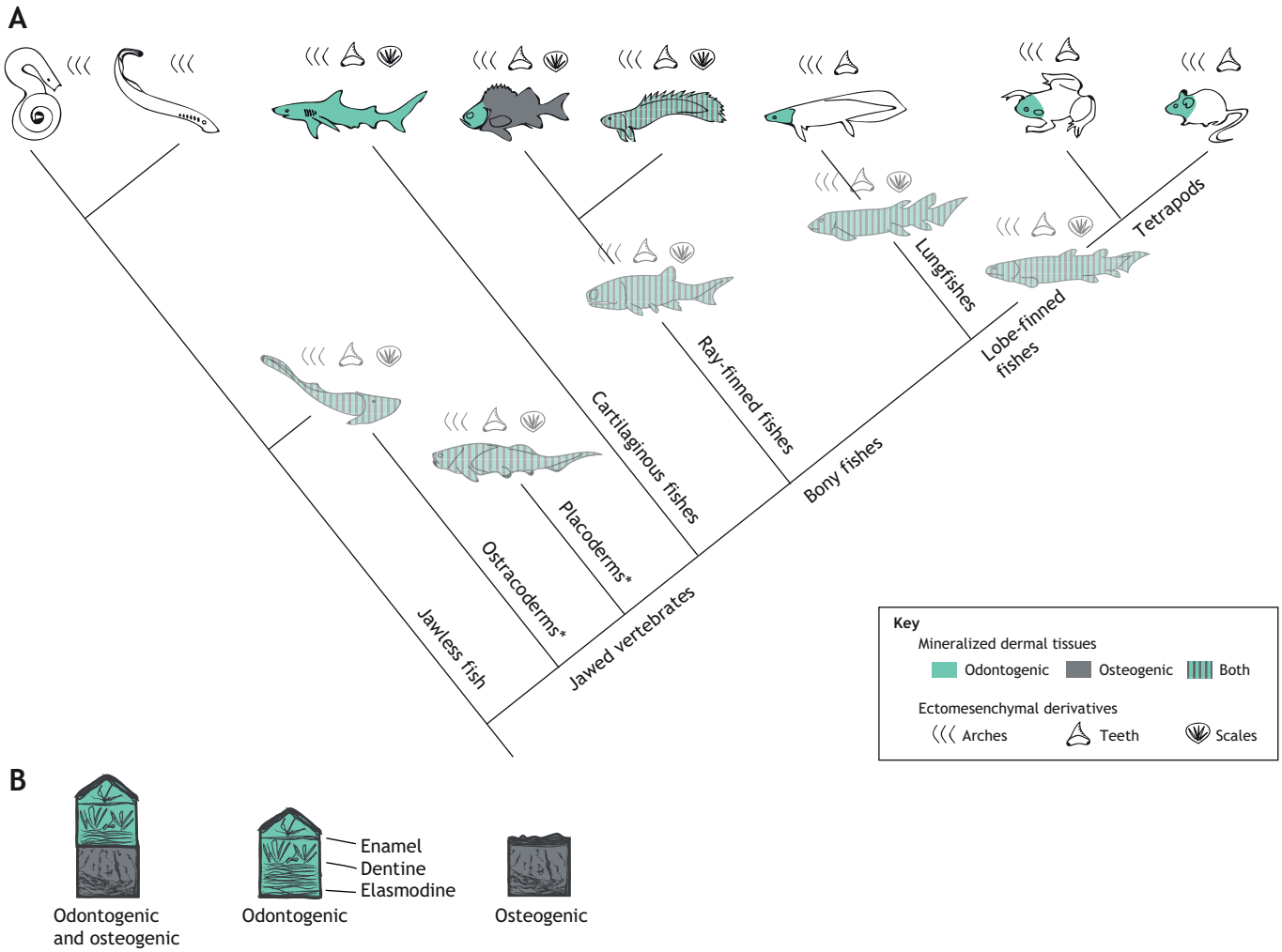

Fig. 5. Distribution of mineralized dermal tissues and neural crest derivatives across the vertebrate evolutionary tree. (A) Mineralized dermal tissues can be odontogenic (green) or osteogenic (gray), or both (striped). The extent of mineralized dermal tissues along the body axis of each species is indicated by the distribution of color. Contributions of the neural crest to cartilage in the pharyngeal arches and to odontogenic tissues of the teeth and scales are represented by symbols located above each species silhouette. Representative species are depicted for each branch of the tree. Extant groups (left to right): hagfish, lampreys, cartilaginous fishes, teleosts, non-teleost ray-finned fishes (e.g. *Polypterus*), lungfishes, anamniotes (e.g. *Xenopus*) and amniotes. Extinct groups (left to right): ostracoderms, placoderms, fossil ray-finned fishes (e.g. *Cheirolepis*), fossil lungfishes (e.g. *Dipterus*) and fossil tetrapods (e.g. *Osteolepis*). Asterisks indicate that ostracoderms and placoderms are both paraphyletic. (B) Representative cross-section of dermal skeletal elements with both odontogenic and osteogenic layers (left), odontogenic layer only (middle) and osteogenic layer only (right).

NC GRN (discussed above) are curiously absent in lamprey pre-migratory and migratory cranial NC, although they are present at later stages in the pharyngeal arches (Martik et al., 2019). Hierarchical clustering of transcriptional profiles revealed that early lamprey cranial NC shares more similarities with chick trunk NC than with chick cranial NC (Martik et al., 2019). Finally, lineage-tracing experiments have revealed that lampreys lack vagal NC, and the enteric nervous system is instead derived from late-migrating trunk NC cells (Green et al., 2017). Together, these findings suggest that axial regionalization of the NC may have been a gradual ongoing process during vertebrate evolution.

To better understand how the cranial NC GRN emerged during evolution, Martik and colleagues examined the expression of components of the avian cranial GRN in embryos of skate (a cartilaginous fish) and zebrafish (a bony fish). In both species, NC cells express two cranial GRN genes, *Tfap2b* and *Ets-1*; however, these genes are expressed in trunk NC as well as cranial NC in these taxa. Additionally, zebrafish express: (1) *lhx5* and *dmbx1* in the early cranial NC, but not in the pharyngeal arches; and (2) *sox8b* at all axial levels, rather than exclusively in the cranial NC (Martik et al., 2019). Moreover, axial-specific enhancers of *Sox10* are conserved in avians and mammals, but are absent in amphibians and teleosts (Betancur et al., 2010). In comparison, the cranial enhancer of *Foxd3* is highly conserved in chick, human, mouse and *Xenopus*, but not zebrafish (Simões-Costa et al., 2012). These results suggest that NC patterning may have evolved via the progressive addition of a 'cranial-specific' circuit onto an ancestral, generalized, trunk-like GRN that is retained in lampreys (Fig. 6B).

Based on these and other findings, Martik and colleagues (2019) suggested that the primitive pan-axial distribution of ectomesenchymal tissues – seen in fossil vertebrates and to the best of our current understanding retained in living cartilaginous fishes (discussed above) – has become restricted to cranial axial levels during evolution by changes to the spatiotemporal expression of network components. This is best illustrated by the amniote-specific restriction of Ets-1 to the cranial NC. However, this model

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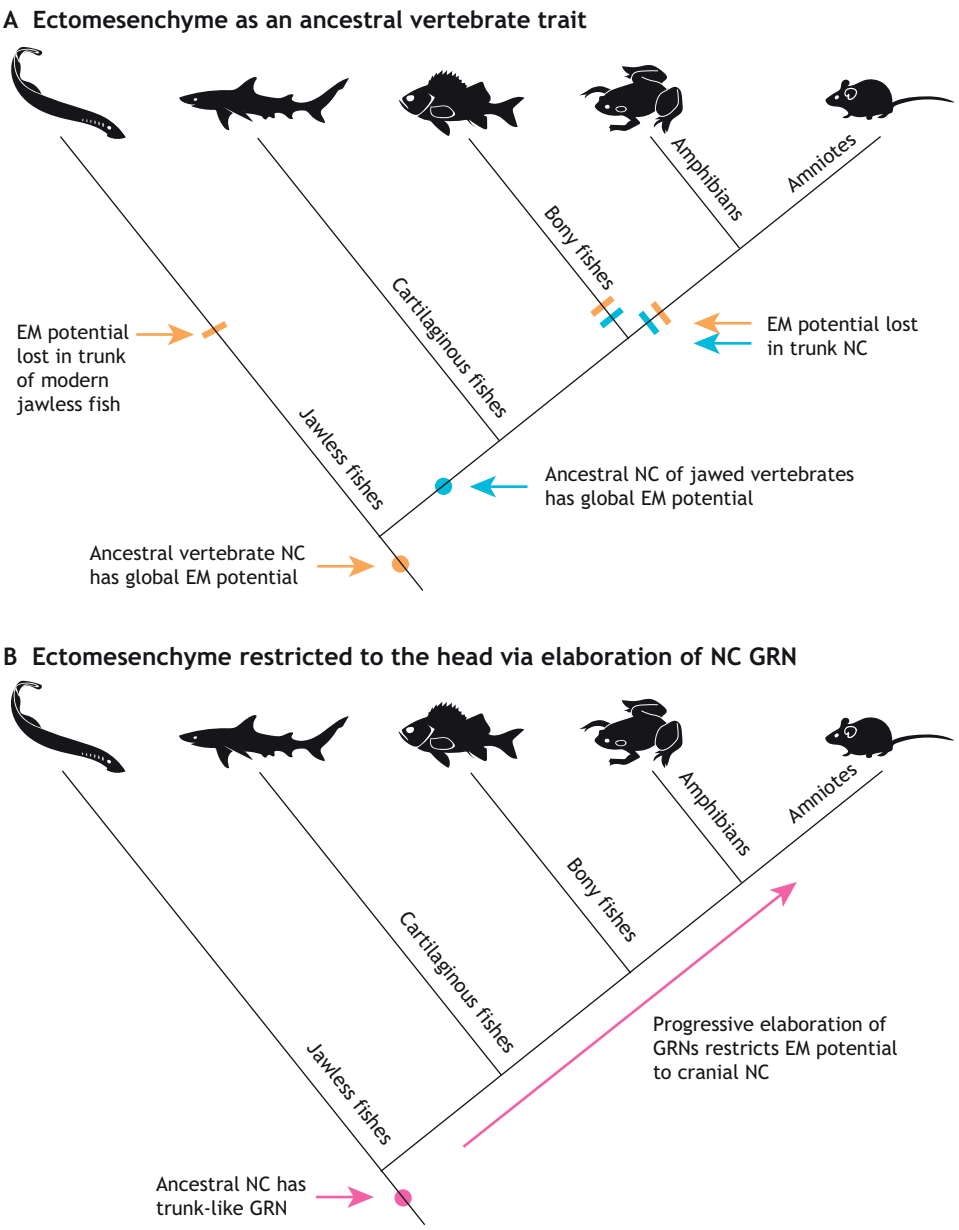

Fig. 6. Evolutionary models for axial regionalization of the neural crest. (A) Paleontological data on the location of mineralized dermal tissues along the body axis suggest that ectomesenchymal (EM) potential originated either: (1) at the base of the vertebrate tree (orange); or (2) in the last common ancestor of jawed vertebrates (blue). Circles indicate acquisition of a trait, while lines indicate a loss. In both cases, EM potential would have been lost in an ancestor of modern teleosts and modern tetrapods. (B) Genetic data from a variety of species, including lamprey, shark, zebrafish and chick, suggest that multiple heterochronic shifts in transcription factor expression may have led to the restriction of EM potential to the cranial neural crest. In this scenario, the ancestral vertebrate neural crest would have resembled that found in the modern amniote trunk (pink).

does have some caveats. First, it is based solely on expression data and requires functional validation. Second, despite the absence of a 'cranial' GRN in early cranial NC cells of lamprey, these cells nevertheless form pharyngeal cartilage (McCauley and Bronner-Fraser, 2003). Thus, even with an apparently simple GRN, lampreys exhibit distinctions between cranial and trunk NC. Third, the lamprey NC GRN may be secondarily simplified. Although extant lampreys lack scales altogether, extinct taxa from the cyclostome stem possessed dentinous scales (Fig. 5) (Keating and Donoghue, 2016; Miyashita et al., 2019). This fossil evidence implies that the ability to form dentine preceded the jawless-jawed vertebrate split, and that the absence of ectomesenchyme in the lamprey trunk is a secondary loss (Fig. 6A).

Conclusions and perspectives

Since the early realization that the NC is regionalized along the body axis, the issue of how distinct axial NC cell populations are established has fascinated researchers. Recent systems biology approaches have uncovered the gene regulatory basis for the unique ectomesenchymal potential exhibited by cranial NC cells, while advances in stem cell differentiation and lineage-tracing methods have revealed the importance of NMPs as a source of trunk NC cells. Moving forward, we propose that multidisciplinary approaches that integrate distinct subfields and incorporate evolutionary data could further our understanding of NC regionalization.

Although analyses of GRNs are invaluable, subsequent functional characterization is necessary to explain how GRNs confer disparate differentiation potential along the AP axis. As illustrated by Simões-Costa and Bronner (2016) and Soldatov et al. (2019), experimental manipulations that couple early transcriptional differences with readouts of NC fate are especially informative. Therefore, future analyses must be complemented by experimental approaches to evaluate the functions of axial-specific genetic circuits in a variety of species. Although zebrafish appear to have a simpler cranial GRN than do chicks, this does not preclude the

existence of a distinct, teleost-specific cranial NC GRN. Indeed, the zebrafish GRN is 'simplified' only with respect to chick, and both model species represent equally ancient lineages that diverged from their last common ancestor.

Understanding the evolution of ectomesenchymal potential also remains a central topic for future investigation. Important insights into this may come from exploring whether trunk NC cells exhibit ectomesenchymal potential in non-teleost fishes with dentinous scales. Specifically, the developmental origin of scales in non-teleost ray-finned fishes such as sturgeons, gars and bichirs warrants examination. The bichir *Polypterus* is especially interesting, both because its scales have an extensive layer of dentine (Sire and Huysseune, 2003), and because its embryos have recently become accessible (Stundl et al., 2019), potentially paving the way for lineage tracing and evaluating whether trunk NC cells give rise to dermal scales. Such experimental data would complement our understanding of tissue development from the fossil record (Giles et al., 2013; Sire et al., 2009) and help bridge the evolutionary gap of more than 425 million years since the last common ancestor of zebrafish and amniotes. Similarly, the teleost group itself warrants more-detailed comparative studies. For example, investigation of the development of dentinous dermal armor in some catfish (Sire and Huysseune, 1996), which may represent retention or redeployment of an ectomesenchymal program, would be of significant value.

Axial patterning of the NC provides a rich system for integrating the genetic, morphological and evolutionary underpinnings of vertebrate development and diversity. The results presented here demonstrate how integrating findings from disparate subfields may illuminate complex questions in developmental biology. We have highlighted the importance of integrating findings from novel sequencing methods with classic experimental embryology and demonstrated how *in vitro* approaches enrich discoveries from *in vivo* models. Expanding this interdisciplinary approach to include paleontological evidence will be crucial for uncovering the mechanisms by which the NC is regionalized along the AP axis and understanding its contribution to the vertebrate body plan.

Acknowledgements

We thank Megan Martik and Marianne Bronner for their thoughtful comments on the manuscript, and Lily Tang and Marianne Bronner for sharing an unpublished draft of their accompanying article. We are grateful to Thomas Frith for his comments on the stem cell section of the manuscript, and to Michael Coates for sharing his deep knowledge of evolutionary biology with us. We also thank the reviewers of the article, both for their careful reading and for their helpful recommendations. Finally, this work benefitted from the resources of the ZFIN database (zfin.org).

Competing interests

The authors declare no competing or financial interests.

Funding

Related work in the Prince lab was funded by a National Science Foundation award (1528911) and by the Chicago Biomedical Consortium with support from the Searle Funds at Chicago Community Trust (C-070 to V.E.P. and Ankur Saxena). M.R. and A.B. were supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) of the National Institutes of Health (T32HD055164). This material is additionally based upon research supported by a grant from the NICHD (F31HD097957 to M.R.) and by the National Science Foundation Graduate Research Fellowship Program (DGE-1144082 to A.B.). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Institutes of Health or the National Science Foundation. Deposited in PMC for release after 12 months.

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