REVIEW

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Evolution of vertebrates as viewed from the crest

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The origin of vertebrates was accompanied by the advent of a novel cell type: the neural crest. Emerging from the central nervous system, these cells migrate to diverse locations and differentiate into numerous derivatives. By coupling morphological and gene regulatory information from vertebrates and other chordates, we describe how addition of the neural-crest-specification program may have enabled cells at the neural plate border to acquire multipotency and migratory ability. Analysis of the topology of the neural crest gene regulatory network can serve as a useful template for understanding vertebrate evolution, including elaboration of neural crest derivatives.

The vertebrate body plan emerged in concert with extensive changes to anterior chordate morphology, including assembly of a craniofacial skeleton, expansion of the anterior neuroepithelium into a brain, reorganization of the pharynx and appearance of novel sensory systems[1–3]. The genesis of this vertebrate 'new head'[1] has been fundamentally linked to the emergence of two cell types, neural crest cells and ectodermal placodal cells. The neural crest is a transient vertebrate cell type, characterized by its site of origin within the central nervous system (CNS), multipotency, and its ability to migrate and differentiate into numerous derivatives, as diverse as cartilage, bone, melanocytes, peripheral neurons and glia[4]. Together with ectodermal placodes that give rise to the sense organs of the head (see refs 5, 6 for discussion of placode evolution), neural crest cells have contributed to the remarkable array of novel anatomies that make vertebrates unique.

Neural crest cells are unlike any other cell type, and the advent of this progenitor cell population affected chordate evolution in an unprecedented manner. Although cells with subsets of neural crest characteristics are present in invertebrate chordates, only vertebrates have a bona fide neural crest that gives rise to structural elements of the head, glia, pigment cells and neurons. Imbued with broad developmental potential and extensive migratory ability, neural crest cells have gained developmental roles at nearly all axial levels and extensively interact with many other tissues. For these reasons, the neural crest is often referred to as the fourth germ layer[7], associated with the emergence and elaboration of the vertebrate body plan[1,8,9].

In this Review, we examine the morphological and genetic features that distinguish vertebrates from other chordates, focusing on cells and tissues derived from the neural crest. We place special emphasis on contributions that resulted in the assembly of the vertebrate head, which has played a crucial part in establishment and diversification of vertebrates. We discuss the gene regulatory network (GRN) underlying the formation of the early neural crest cells that are common to all vertebrates. We then use this network, together with morphological criteria, to discuss how neural crest cells may have emerged from the putative homologues that are present in invertebrate chordates, highlighting how addition of the neural-crest-specification program may have enabled cells at the CNS border to acquire multipotency and migratory ability. In this context, we examine how studies of neural crest GRNs may clarify patterns of morphological evolution within vertebrates, including expansion of neural crest derivatives during diversification of vertebrate taxa. Taken together, the data paint a picture of the neural crest as a malleable population that has continued to imbue the vertebrate body with novel features.

Neural-crest-related innovations in early vertebrates

Emergence of the vertebrate lineage was accompanied by acquisition of the neural crest and its novel derivatives. All vertebrates have neural crest cells that arise from the dorsal portion of the CNS, exhibit multipotency by contributing to diverse derivatives, undergo an epithelial-to-mesenchymal transition (EMT), and have extensive migratory ability. 'Premigratory' neural crest cells initially reside in or adjacent to the dorsal neural tube, the newly formed CNS, of all vertebrates[10]. These cells undergo EMT to exit the CNS and migrate to numerous sites throughout the body, where they eventually contribute to their characteristic derivatives[4] (Fig. 1a). Cell-lineage analyses have shown that many individual neural crest precursors can contribute to multiple cell types *in vivo*[11–13] and *in vitro*[14,15], and are thus 'multipotent' stem or progenitor cells.

Comparisons between the two major groups of living vertebrates, the jawed vertebrates (gnathostomes) and their sister group the cyclostomes (agnathans)[16], identify many shared, derived traits likely to have been present in the neural crest of early vertebrates[17–20]. These include pigment cells, cellular pharyngeal cartilage and specialized pharyngeal musculature, an enteric nervous system, chromaffin cells, and perhaps cardiac valves[17,21]. Recent work has identified a new neural crest derivative, pillar cells[22], that support vertebrate gill epithelia (Box 1). Because neural crest cells interact with many other tissues, they have a broad impact by modifying neuroepithelial patterning, craniofacial patterning, and cranial musculoskeletal development (Box 2).

Many early vertebrate innovations are unique to jawed vertebrates and absent in cyclostomes. Some of these traits are likely to have arisen in stem gnathostomes, the early fishes that are ancestral to the jawed vertebrates. One of these innovations is the appearance of jaws, through modification of anterior pharyngeal arches. Other major gnathostome innovations include odontoblasts that produce dentine (Box 1), paravertebral sympathetic chain ganglia[23] (Box 3) and exoskeletal armour. Although exoskeletal armour might have arisen from neural crest at cranial levels, it is likely that trunk armour instead arose from mesoderm (Box 4).

One central question in the early evolution of neural crest is the extent to which neural crest cell types are evolutionary novelties, rather than cell types (and regulatory programs) co-opted from other tissues. There are clearly some novel neural-crest-derived cell types, including

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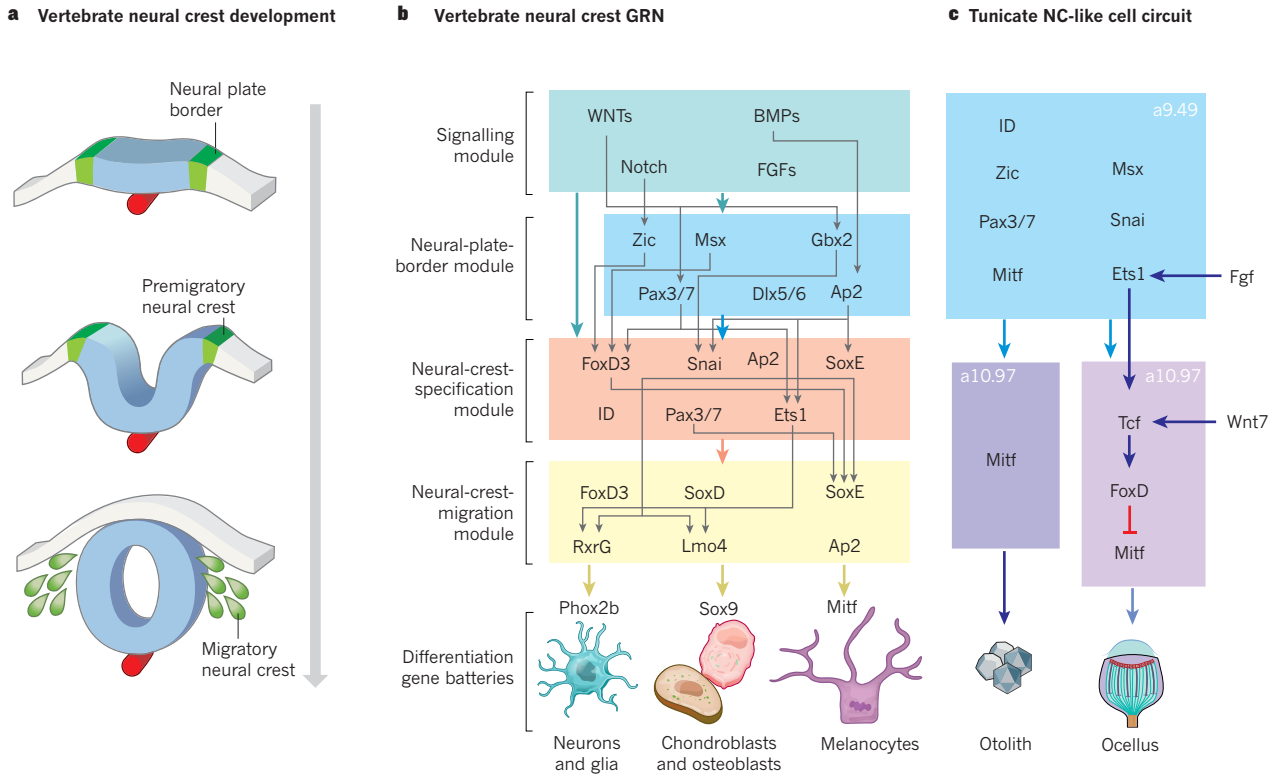

Figure 1 | Gene regulatory interactions controlling vertebrate neural crest formation and the tunicate a9.49 cell lineage. **a**, Different stages in neural crest formation. Neural crest cells are defined by their origin at the neural plate border, epithelial to mesenchymal transition, migratory capacity and multipotency. **b**, A neural crest gene regulatory network (GRN) endows this cell population with its unique features. This GRN is composed of different modules arranged hierarchically, which control each step of neural crest development[38]. The neural-crest-specification module seems to be missing from the neural plate border of invertebrate chordates. **c**, Regulatory circuit of a tunicate neural-crest (NC)-like pigmented cell precursor. Adapted from refs 38, 39 and based on the results from ref. 49.

pillar cells and odontoblasts, but many neural crest cell types are similar to cells in related chordates[24,25]. These cell types might either be homologous, representing a cell lineage that was co-opted and incorporated into the neural crest, or they might have arisen by convergent evolution. One example of co-option is the origin of pharyngeal cellular cartilage, probably accomplished by reuse of a program governing cellular cartilage formation in the oral region of invertebrate chordates[26]. Assessment of co-option or novelty can be aided by evaluation of GRNs that govern their formation.

A neural crest GRN is conserved across vertebrates

From a gene regulatory perspective, the body plan of all metazoans is encoded in the genome. During embryonic development, this code emerges as a complex GRN formed by transcription factors and *cis*-regulatory elements that co-operate with non-coding RNAs and epigenetic factors to pattern the body and drive development of individual elements and cell types[27]. According to this framework, the body-plan modifications observed during evolution are a direct consequence of changes in the developmental regulatory program[28].

Neural crest cells are characterized by site of origin, migratory behavior and multipotency. Importantly, they also share a molecular signature, expressing a suite of transcription factors, including *tfAP2* (ref. 29), *Snai1/2* (ref. 30), *FoxD3* (refs 31–33) and *SoxE* (refs 34, 35) genes. In particular, *FoxD3* and *SoxE* are characteristic of premigratory and early migratory neural crest cells and *SoxE* genes are crucial upstream regulators of all neural crest lineages. These transcription factors are part of the regulatory machinery that controls transcription of numerous effector genes, which together endow the neural crest with its unique properties. Interactions between transcription factors and their targets generate a GRN that controls neural crest formation, from induction at the neural plate border to differentiation into distinct cell types[36–39] (Fig. 1b).

The architecture of the neural crest GRN is thought to underlie the features observed in this cell population, such as multipotency and migratory capability. Functional experiments suggest that the neural crest GRN is comprised of distinct hierarchical levels[36,38]. First, signalling events (GRN signalling module) initiate the specification process, by inducing co-expression of transcription factors that comprise the 'neural-plate-border module'. This in turn leads to specification of bona fide neural crest cells (neural-crest-specification module), their migration from the CNS to diverse sites (neural-crest-migration module), and finally to diversification into different derivatives through the deployment of distinct differentiation gene batteries[36–39] (Fig. 1b). Each level of the neural crest GRN corresponds to a regulatory state that not only defines cell identity and behaviour at a given time point, but also drives transition to the next module of the network[40]. From an evolutionary perspective, assessing conservation of different levels of the neural crest GRN helps to identify the origin of each subcircuit and reconstruct the evolutionary history of neural crest cells[27,28]. As a result, the neural crest GRN provides a useful platform for understanding the molecular underpinnings of vertebrate evolution and how these cells may have participated in modifying vertebrate embryonic development. Neural-crest-GRN studies have indeed provided important clues regarding the establishment of the vertebrate lineage and its diversification[40–42].

Extensive work in amniotes, frogs, teleosts and cyclostomes has revealed remarkable similarities in the overall structure of the neural crest GRN, demonstrating that it is virtually the same from amniotes to cyclostomes (Fig. 1b)[8,10,19,43]. Some important species-specific differences exist, but they are likely to reflect the continuous restructuring of the GRN in individual clades. Nevertheless, expression patterns and epistatic interactions between FoxD3, SoxE, Snai1/2 and Pax3/7 transcription factors point to a very conserved module of neural crest specification[38]. The overall conservation of the neural crest GRN correlates with conservation

BOX 1

Neural crest derivatives and the vertebrate pharynx

Changes in pharyngeal patterning are central to the evolution and diversification of vertebrate groups[1,98]. Vertebrate pharyngeal arches have a similar general structure, characterized as a bilaterally symmetric series of endodermal evaginations that, with ectoderm, enclose a region of neural crest cells surrounding paraxial mesoderm[99,100]. Neural crest cells and paraxial mesoderm give rise to pharyngeal skeletal elements and musculature, respectively.

Some aspects of vertebrate pharyngeal patterning are integrated within or modified from features common to many deuterostomes. Pharyngeal segmentation is a trait of ancestral deuterostomes[101], and unambiguous pharyngeal arch homologues with similar genetic controls are present in hemichordates, cephalochordates and adult urochordates[99,101], despite being secondarily lost in echinoderms[99,102]. Pharyngeal mesoderm also has a broad phylogenetic distribution, being present throughout chordates[103,104]. Neural-crest-derived cellular cartilage of vertebrates, rather than being a novelty of vertebrates[21], instead seems to have been co-opted from cellular cartilage homologous to that present within the oral cirri of cephalochordates[26].

Although some vertebrate pharyngeal patterning stems from ancestral conditions, many novel elements arise from vertebrate neural crest cells. Modification of early neural crest development was important for generating the diversity of pharyngeal structures observed throughout vertebrates. For example, in vertebrate gills, epithelial surfaces are supported by novel neural-crest-derived cells, pillar cells, which are ancestrally shared throughout vertebrates[22]. In addition, in the transition from agnathans to gnathostomes, modifications to the anterior-most pharyngeal arch cartilages and neural-crest-modified musculature resulted in the formation of the jaws, as well as the formation of neck muscles[18,105–107].

Another vertebrate novelty associated with the pharynx and its integuments are odontodes: dental elements composed of mineral material and associated cells. In living jawed vertebrates, their formation is mediated by conserved gene regulatory subcircuits, identified by coexpression of transcription factors, including runx2 and eda/edar, among others[108], and require the inductive influence of neural-crest-derived mesenchyme. Fossil evidence suggests that odontodes emerged during the evolution of stem gnathostomes, in external dermal armour[108–110], consistent with the 'outside–in' model, which suggests that odontodes emerged first as structural elements associated with external integument, and were later incorporated into the oral cavity and pharynx. Mineralized dental elements found in conodont fossils are considered non-homologous to gnathostome teeth[109]. Both groups of living cyclostomes, lampreys and hagfish, have keratinized dental elements, but these are morphologically distinct from gnathostome teeth and are probably not homologous. Continued analysis of cyclostome dental elements might clarify whether neural crest cells played a part in their ontogeny.

of morphology, migratory behaviour and differentiation into multiple derivatives, establishing the neural crest as an ancient vertebrate cell type. Superimposed on the conserved basic structure of the neural crest GRN is adaptability and flexibility. During the course of evolution, differentiation modules that encode for novel derivatives, such as jaws and sympathetic ganglia, have been added to the neural crest repertoire and thus must have been added as 'plug-ins' to the GRN.

Although the core elements are highly conserved, adaptations, additions and potentially losses have occurred between species. Indeed, it is clear that the specification module of the neural crest GRN is strongly conserved within vertebrates, but there are important gene regulatory differences between jawless and jawed vertebrates that might provide interesting hints regarding the molecular roots of vertebrate morphological diversification. Extensive analysis of the lamprey neural crest GRN has revealed the notable absence of transcription factors *Ets-1* and *Twist* in the premigratory neural crest[10]. This is intriguing since Ets-1 has been shown to be essential for cranial neural crest specification in gnathostomes[34]. Instead, in the lamprey, it is expressed much later in the neural-crest-derived portion of the branchial arches and dorsal root ganglia. One possibility is that Ets-1 was added to the gnathostome neural crest specification, representing an example of a transcription factor that was co-opted from a distal level of the network to a more proximal level. However, it is also possible that it may have been selectively lost in the lamprey neural crest. Examining expression of Ets-1 in other cyclostomes and functional experiments in lampreys may help to clarify this point. Other GRN components that have crucial functions in teleosts and amphibians may have been lost or replaced in amniotes. For example, although Snai1/2 and Twist seem to be crucial for neural crest formation in frogs[44,45], they are dispensable in mice[46], perhaps due to redundant functions with other EMT factors such as Sip1 (ref. 47).

Taken together, these studies reveal that the topology of the neural crest GRN, with cells progressing through successive regulatory states from induction to differentiation, forms a useful template for understanding vertebrate evolution[36]. This GRN can also be useful for assessing the likelihood that similar cell types in other animals might be homologous to the neural crest.

Do invertebrate chordates have neural crest cells?

Deciphering how the neural crest arose as a cell type is crucial for furthering our understanding of vertebrate evolution. Tackling this problem requires deeper knowledge of deuterostome embryonic development in multiple species, with particular attention to neural-crest-like cell types in other chordates. Recent studies have described intriguing embryonic cell populations in ascidians that have some, but not all, neural crest characteristics. For example, the trunk lateral cells in the colonial tunicate *Ecteinascidia turbinata* are derived from the A7.6 lineage, which originates in the vicinity of the neural tube, undergoes migration and gives rise to pigmented cell types[48]. Similarly, in *Ciona intestinalis*, results show that the a9.49 cell lineage originates from the neural plate border and gives rise to the pigmented sensory cells of the otolith and the ocellus[49]. These cells normally translocate only a few cell diameters, whereas misexpression of Twist in this lineage results in acquisition of mesenchymal morphology and long-range migration[49]. In cephalochordates, there have been many proposed homologues of neural crest (see ref. 50 for a discussion), including a bipotential neuroepithelial precursor to pigment cells of the ocellus[50]. Further assessment of this homology will require additional analyses of amphioxus ocellus development. Cephalochordates also have an ependymal cell in the neural tube that expresses *Snail*, a homologue of *Snai1* and a neural-crest-specifier gene in vertebrates, but this cell seems to be non-migratory[51,52].

The neural crest GRN is particularly useful for understanding assessment of GRN conservation outside of vertebrates. The available molecular data obtained from embryonic cell types in tunicates and cephalochordates suggest that gene regulatory interactions that specify the neural plate border (neural-plate-border module) are deeply conserved throughout chordates[24,51] (Fig. 1c), and data from annelids suggest that this genetic program might be shared with protostomes, originating in stem bilaterians[53,54]. Similarly, the terminal differentiation programs (differentiation gene batteries) that drive the neural crest to assume definitive fates are

conserved, as exemplified by control of pigment-cell differentiation. This is expected because most of the differentiation batteries are thought to be ancient subcircuits that were co-opted by different cell types[27]. Although they are integral parts of the neural crest GRN, these neural-plate-border and differentiation subcircuits do not fully define neural crest identity in vertebrates. Proximally in the program, the neural plate border contains other cell types (neural tube and placode) in addition to neural crest, and is important for the delimitation of the neural plate. Distally, other deuterostomes have some differentiated cell types that in vertebrates can arise from neural crest: melanocytes, ectomesenchyme, autonomic neurons and glia. It has been proposed that during early vertebrate evolution, the neural-crest-specification module may have been assembled within the neural-plate-border cell lineage, interposed between the neural plate border and the distal differentiation modules of the network, to endow these cells with a full 'neural crest' phenotype.

Importantly, neural crest identity in all vertebrates is intrinsically linked to the neural-crest-specification kernel of the GRN, which endows these cells with its defining features such as multipotency, the ability to undergo EMT and migratory capacity[40]. Important genes in the specification subcircuit include *SoxE*, *FoxD* and *Snai1/2*, homologues of which are present in the genomes of invertebrate chordates[51,55]. For example, the amphioxus genome has all the transcription factors identified in the neural-crest-specifier module of the vertebrate neural crest GRN. However, only *AmphiSnail* is expressed in the putative neural crest domain[56]. Therefore, a key question is whether the neural-crest-like cells from tunicates possess this particular subcircuit. Molecular analyses suggest that tunicates and amphioxus have the neural-plate-border subcircuit[24], and thus invertebrate neural-crest-like cells may be homologous to neural-plate-border cells of vertebrates. However, although some neural-plate-specifier genes are expressed in these cells (for example, *FoxD*[49]) other crucial transcription-factor genes, notably *SoxE* genes, seem to be absent. In ascidians, it is not yet clear whether epistatic interactions between the transcription factors expressed in putative neural crest cells are similar to those observed in the vertebrate neural crest GRN (Fig. 1c). This, together with the fact that cells of the a9.49 lineage have not yet been shown to be multipotent, or to have extensive migratory capabilities, makes it more difficult to determine whether they are true neural crest homologues. Further gene-regulatory studies will be necessary to establish the relationship between these cells and the vertebrate neural crest.

As a cautionary note, there is inherent danger in assigning evolutionary relationships among cell types on the basis of molecular similarity alone, because transcription factors are reused throughout development, and are neither lineage- nor cell-type-specific. For instance, many bona fide neural crest transcription factors are expressed at the neural plate border, in later differentiation programs and in other lineages. Thus, one cannot attribute homology or lineage relationships on the basis of a few molecular markers. A more inclusive argument that includes morphological and behavioural information, expression data and, ideally, *cis*-regulatory studies[57] perhaps provides the most reliable means to establish conservation of developmental mechanisms and ascribe homology between cell populations.

Gene regulatory changes behind neural crest emergence

Radical changes of body plan, such as those that took place in early vertebrate evolution, require substantial rearrangements in the structure of developmental GRNs[27]. The emergence of the neural crest was dependent on the assembly of a specification subcircuit that allowed this cell population not only to exhibit its stereotypical behaviour, but also to drive multiple differentiation programs, resulting in its multipotent state. Understanding how a novel, complex specification subcircuit emerged during chordate evolution is a daunting task. However, observation of the neural crest GRN can provide important clues about vertebrate evolution and suggest likely scenarios for the creation of a novel cell type.

Given the deep conservation of the neural-plate-border-specification program[24], it seems reasonable to assume that this circuit was crucial for assembly of the vertebrate neural crest GRN. Because all of the neural-crest-specifier genes are present in the genomes of invertebrate chordates[58,59], it is likely that they were added to the GRN by deployment or co-option of transcription factors that were originally part of other developmental GRNs, such as the neural-plate-border subcircuit, mesodermal programs and terminal differentiation modules. According to this view, changes in their *cis*-regulatory apparatus placed the neural-crest-specifier genes downstream of the neural-plate-border program and signalling systems. Such *cis*-regulatory changes might have facilitated redeployment of neural-plate-border (*Pax3/7* and *TFAP2*) and stem-cell genes (*FoxD3*) in the specification module. For example, an amphioxus *FoxD* enhancer that recapitulates endogenous amphioxus *FoxD* expression

BOX 2

Role of the neural crest in signalling

Brain and facial patterning. Increased complexity in vertebrate neuroanatomy might partly stem from interactions between neural crest cells and other cell types. An example of the important role of the neural crest in expansion of the head comes from recent experiments in amniotes[111]. Surgical removal of the neural crest at forebrain to rostral hindbrain levels results in the absence of facial and skull cartilages and bones, as well as severe brain defects including anencephaly[112]. These defects can be rescued by grafting small populations of premigratory neural crest from the same axial level, but not from more caudal regions with Hox gene expression. At a molecular level, this results from production of BMP inhibitors, Gremlin and Noggin, by the rostral neural crest that in turn lead to regulation of expression of FGF8 in the anterior neural ridge (ANR). Consistent with this, implantation of FGF8 beads after neural crest ablation rescues this phenotype to restore subsequent downstream signalling events and proper head development[100,113]. FGF signalling associated with an ANR-like signalling centre is potentially present throughout deuterostomes[114,115], suggesting that neural crest cells have adopted or co-opted roles in the regulation of neural or craniofacial patterning, at least in amniotes. Examination of additional vertebrate groups might clarify when this might have arisen.

Cranial muscles and the neural crest. The vertebrate head includes muscles that control the movement of the eyes (extraocular muscles), face, jaws, throat, larynx and tongue, collectively called branchiomeric muscles[116]. Derived from unsegmented paraxial mesoderm anterior to the otic vesicle, they form under the control of a Pitx2c and Tcf21/MyoR regulatory subcircuit that seems to be conserved at least throughout the bony fishes[117,118] (Fig. 2). The neural crest is crucial for multiple stages of cranial mesoderm development, including defining the location, orientation, patterning and differentiation state of muscle precursor cells[57,106,107,116]. Mesoderm cells follow migrating neural crest cells into the pharyngeal arches[86,116]. Branchiomeric muscles initially remain in a precursor state, repressed by signals emanating from the nearby neural tube and ectoderm. Neural crest cells secrete signals that derepress myogenesis, allowing the formation of cranial myofibres[119]. These distinct myogenic regulatory sub-networks are thought to have arisen in early vertebrates concurrent with other cephalic modifications[117,119], but have also been compared with muscle precursors in the amphioxus atrium[104] and potentially with visceral musculature of protostomes[120]. Vertebrate cranial muscle patterning, differentiation and organization might require regulatory control that arose from novel interactions with the neural crest (Fig. 2).

BOX 3

Peripheral nervous system

A peripheral nervous system, including the sympathetic chain ganglia, is a common feature of all jawed vertebrates. Sympathetic ganglion cells are responsible for regulating homeostatic functions of peripheral organs. They arise from neural crest cells that migrate ventrally from the trunk neural tube to positions adjacent to the dorsal aorta, and form under the control of a gene regulatory circuit including *Phox2*, *Hand2* and *Ascl1*. These genes collaborate to promote the construction of a sympathetic neural phenotype, including production of noradrenaline. In bony fishes and tetrapods, sympathetic ganglia are connected along the anteroposterior axis through chains, but in extant chondrichyans (sharks, rays and skates) ganglia are largely separate. Cyclostomes do not seem to have a comparably organized sympathetic system, but very rare ganglion-like cells of unknown function have been identified[121]. In general, autonomic function in cyclostomes seems to be controlled directly by spinal neurons of the central nervous system[121], which is similar to the peripheral organization of amphioxus, and thus is likely to represent a primitive condition for chordates. Taken together, these data suggest that sympathetic ganglia probably evolved in stem gnathostomes, and were further elaborated in stem osteichthyes.

in somites and notochord[60] was able to drive similar expression when electroporated into chick embryos[51]. However, this enhancer failed to drive expression in the neural crest, suggesting that the novel neural crest expression domains rely on distinct gene regulatory processes that are absent in amphioxus[51]. Similarly, co-option of EMT driver genes such as *Snai2* (ref. 30) and *Sip1* (ref. 47) may have allowed the neural crest to leave the neural plate border domain. This was probably accompanied by co-option of mesenchymal gene circuits that allowed these cells to exhibit migratory behaviour.

A key feature of the neural crest is its ability to form numerous derivatives (multipotency). Mechanistically, this implies that neural crest cells are capable of deploying a variety of differentiation gene batteries depending on signalling interactions during migration and once at their final sites. Neural-crest-specifier genes from the SoxE family play a crucial part in activating differentiation programs that lead to multiple derivatives, as diverse as neurons, Schwann cells, pigment cells and cartilage[38]. Thus, a likely scenario was that a variety of differentiation gene batteries were placed downstream of the neural-crest-specification module by gain of function *cis*-regulatory changes, which placed differentiation driver genes (for example, *Mitf*, *Ascl1* or *Phox2b*) under the control of neural-crest-specifier genes. Again, examples of redeployment of such ancient differentiation gene batteries by different cell types have been described in different contexts, and are thought to be a common feature in GRN evolution[27,61]. Indeed, a recent study[26] suggests that *cis*-regulatory changes in ancestral pro-chondrocytic genes allowed for their activation in the neural crest by factors such as SoxE and Tfap2, allowing for the establishment of the vertebrate head skeleton. Thus, it is possible that the emergence of the neural-crest-specifier module served as a platform for the redeployment of multiple, pre-existing genetic subcircuits that endowed the neural crest with its defining features.

Although *cis*-regulatory changes were probably the most important events in the emergence of the neural-crest-specification module, it is also likely that changes in protein sequence had an important role therein. Neural crest cells employ a large repertoire of adhesion molecules, receptors and signalling molecules, and gene diversification and neofunctionalization might have enabled acquisition of the complex cell behaviours exhibited by the neural crest. Furthermore, recent data suggest that neofunctionalization of neural-crest-specifier genes such as *FoxD3* was important for the emergence of this cell type[62], perhaps by mediating new protein–protein interactions and allowing for the assembly of novel, vertebrate-specific transcriptional complexes.

A role for gene duplications in early neural crest evolution

The extensive changes in gene regulation required for the evolution of the neural crest as a cell type might have been facilitated by large-scale genome duplications that took place early in the vertebrate lineage. It has long been suspected that rare, large-scale genomic rearrangements and genome-wide duplications in stem vertebrates had a key role in elaborating the vertebrate body plan[54,63–65] and increasing vertebrate complexity[66,67]. The presence of multiple homologous Hox clusters and conserved syntenic paralogy regions among jawed vertebrate chromosomes are usually taken to support the contention that there were two rounds of genome duplication during early vertebrate evolution[66]. Recent analysis of the genome of the sea lamprey (*Petromyzon marinus*) suggested that ancestors of the lamprey (and hagfish) diverged from vertebrates after these two rounds of duplication[68–70], but this is still controversial, and an alternative model suggests that there was only a single round of duplication in stem vertebrates, followed by lineage-specific segmental duplications in jawed vertebrates and cyclostomes[71]. Regardless of the precise number and timing of genome duplications, vertebrates have certainly undergone additional gene duplications relative to invertebrates, and these increases in gene number may have facilitated the evolution of vertebrate regulatory and anatomic complexity[63], potentially affecting the formation of the many novel cell types in vertebrates.

A full assessment of the extent to which gene and genome duplications have affected early vertebrate evolution remains incomplete, and is somewhat controversial[72]. One way to approach this question is to determine whether the timing of the acquisition of particular traits compares with the inferred timing of gene duplications. Many traits were thought to arise in the vertebrate stem: these include key innovations such as the addition of neural-crest-derived pharyngeal cartilages, modification of cranial muscles, the development of segmented and Hox-patterned hindbrain[57], and perhaps the beginnings of peripheral nervous organization (Fig. 2). These distinct vertebrate characters are rooted in invertebrate chordates, but seem to have been fundamentally transformed by the innovation of neural crest cells and their interactions with other cell types. Thus, the timing of the acquisition of these traits correlates nicely with inferred instances of genome duplication, although one cannot distinguish cause from effect.

Ultimately, the fundamental question is how genomic duplications affected the organization of developmental GRNs. As has been discussed[54], such duplications may cause important shifts in gene regulatory mechanisms during vertebrate evolution. Indeed, it is possible that large-scale genome duplications may have facilitated extensive changes in the *cis*-regulatory apparatus controlling the transcription of neural crest genes[73], leading to their co-option and assembly into the neural-crest-specification module. Such events might have enabled the deployment of genes, such as those that encode SoxE transcription factors, in the neural-crest-specification module. Depending on the species, *Sox8*, *Sox9* and *Sox10* have early and sometimes overlapping functions in neural crest specification, with different paralogues deployed at different times depending on the species. However, expressing at least one of the *SoxE* paralogues seems crucial for the maintenance of neural crest identity. Interestingly, it has recently been shown that *Sox10* alone is sufficient to reprogram fibroblast cells to a neural crest fate, highlighting the importance of *SoxE* genes in neural crest specification[74]. Furthermore, acquisition of migratory ability by the neural crest may have been fostered by diversification of receptors and ligands that enabled chemotactic behaviour. Genome-wide analysis shows that vertebrates have a much more complex arsenal of such molecules than do invertebrate chordates[58,75]. Thus, although the role of whole-genome duplications in neural crest evolution is still not fully understood, it

is likely that these duplications provided the neural crest with the molecular toolkit necessary for its complex behaviour.

Evolution of crest populations along the rostrocaudal axis

Neural crest cells arising from different levels of the neural axis are endowed with distinct developmental potentials and behaviour. For example, the cranial neural crest of gnathostomes gives rise to ectomesenchymal derivatives (for example, the bone and cartilage of the face) in addition to melanocytes, glia and a subset of cranial sensory neurons. By contrast, the trunk neural crest is not able to contribute to cartilage or bone *in vivo*. Rather, these cells form melanocytes, dorsal root and sympathetic ganglia and chromaffin cells. Although the gene regulatory interactions underlying these differences remain unknown, they probably reflect disparities in the mechanisms of specification observed among neural crest subpopulations[33].

Classic heterotopic grafting experiments in the chick demonstrate that the trunk neural crest has restricted developmental potential compared with the cranial population (reviewed in ref. 4). Cranial neural crest cells transplanted to the trunk can not only give rise to all trunk neural crest derivatives, but also form ectopic cartilage nodules that are characteristic of their site of origin[76,77]. By contrast, trunk neural crest transplanted to the head fail to contribute to facial bone and cartilage, although they can form sensory neurons and glia[78]. These results indicate that there are cell-autonomous differences between neural crest subpopulations established during specification. This is consistent with *cis*-regulatory analysis of neural-crest-specifier genes, which shows that expression of both *FoxD3* and *Sox10* in the neural crest is controlled by separate enhancers in the head compared with the trunk[33,34]. Furthermore, activity of these enhancers depends on axial-specific inputs, suggesting that specification of the cranial and trunk neural crest cells relies on different genetic programs[33,38].

The potential of the trunk neural crest has important implications for vertebrate evolution. For instance, it has been suggested that the neural crest played a central part in gnathostome evolution by giving rise to the exoskeleton of early vertebrates such as ostracoderms (armoured fishes)[41]. According to this scenario, at some point during vertebrate evolution the trunk neural crest was endowed with ectomesenchymal potential, which was subsequently lost in extant vertebrates. This hypothesis is based mainly on the fact that the skeletal plates that form the exoskeleton in armoured fishes were composed of dentine, a bona fide neural crest derivative[79,80]. Furthermore, studies in different model organisms suggest that the trunk neural crest exhibits at least some ectomesenchymal potential. For example, fate-map studies in zebrafish and frogs using vital dyes indicate that trunk neural crest contributes to the mesenchyme of the fins[80,81]. Finally, *in vitro* clonal analysis of avian trunk neural crest cells has shown that some clones exhibit gene expression that is characteristic of cartilage and bone[82], suggesting that these cells might possess a latent ectomesenchymal potential, which can be unlocked by environmental signals[83]. These studies suggest that the trunk neural crest might have some residual capacity to form ectomesenchyme, consistent with the hypothesis that the trunk neural crest gave rise to the exoskeleton of basal gnathostomes.

Recently, however, this view has been challenged by a number of studies that employ genetic fate mapping and cell-transplantation analysis to define neural crest contributions in teleost fishes (Box 4). These data show that mesenchyme-derived structures formerly attributed to the trunk neural crest lineage, such as the fin osteoblast, fin mesenchyme and mineral-forming cells of the scales, are in fact of mesodermal origin[84–87]. Taken together, these studies suggest that the trunk neural crest of teleosts has the same developmental restrictions observed in amniotes, calling into question the neural crest origin of the exoskeleton in armoured fishes. Although further studies in other model organisms are necessary for a pan-vertebrate view of trunk neural crest potential, these results indicate that trunk neural crest has been devoid of skeletogenic potential throughout its evolutionary history. These findings suggest that alternative hypotheses for the evolution of the neural crest subpopulations require consideration.

A second scenario is that the cranial neural crest was endowed with gene regulatory mechanisms that are absent from the trunk and may have been 'added on' early in vertebrate evolution. So far, a few developmentally important cranial-specific regulators have been identified. In gnathostomes, for example, *Ets1* (ref. 88) and *Id2* (ref. 89) are enriched in cranial crest cells and are crucial neural-crest-specifier genes for this subpopulation, but their expression is absent from the trunk. This raises the intriguing possibility that the genetic circuits underlying ectomesenchymal potential were added to an ancestral, trunk-like neural crest GRN. According to this view, the ectomesenchymal machinery was either co-opted from the mesoderm[26] or assembled *de novo* in the cranial region. This scenario implies that trunk neural crest cells have a simpler GRN topology than cranial neural crest, an experimentally tractable hypothesis that can be addressed by comparative studies. This view is consistent with the large number of transcriptional regulators that are shared among all neural crest populations, consistent with a common origin.

However, a complication is that transcription of genes such as *Sox10* and *FoxD3* is activated uniformly along the entire neural axis, but by distinct enhancers with differential inputs in the trunk compared with cranial regions[33,34]. A third scenario is that neural crest subpopulations may have segregated early in vertebrate evolution and possess different GRN topology. Consistent with enhancer analysis, this hypothesis suggests that many ancestral neural crest GRN connections have been rewired during evolution and that these changes in topology resulted in two populations that have multiple differences in potential and behaviour, despite sharing a similar genetic toolbox. This scenario implies that the trunk and cranial neural crest GRNs have substantial differences, and predicts that pan-neural crest genes are generally controlled by distinct, axial-specific enhancers. Importantly, the hypotheses already discussed can be tested by in-depth analysis of the genetic pathways controlling neural crest formation at different axial levels. In particular, elucidating the circuits controlling ectomesenchymal differentiation of the neural crest will have a great effect on how we interpret the evolution of this cell population. Furthermore, additional neural crest subpopulations exist, including vagal and sacral subtypes, which have distinct migratory pathways and contribute to different derivatives. A more inclusive gene regulatory view of these subpopulations might clarify how the developmental potential of the neural crest

BOX 4

Dermal skeleton

A dermal skeleton derived from odontodes is present in many vertebrates, both fossil and living. Dermal skeletal elements among living vertebrates include fin rays (lepidotrichia) of ray-finned (actinoptyerygian) fishes and scales, with multiple subtypes including placoid, ganoid and elasmoid scales in various taxa. Dermal skeletal elements have been proposed to be neural-crest-derived[122] at both cranial and trunk levels. However, recent analyses indicate that osteoblasts responsible for the elasmoid integumentary scales and fin rays of zebrafish derive from mesenchyme of mesodermal origin[87] rather than neural crest[80,123]. Similarly, ossified turtle shells that had been suggested to originate from both mesoderm-derived (endochondral rib) and neural-crest-derived (dermal) osteocytes, instead seem to develop only from mesoderm[124]. These data raise the question of whether the extensive dermal armour of stem gnathostomes originated from mesoderm or neural crest. At trunk levels, these dermal plates may have originated from mesoderm rather than neural crest, although they do arise from neural crest at cranial levels. However, it remains possible that neural crest cells contribute to other scale types, including the placoid scales of cartilaginous fishes that some have argued are more similar to dermal armour of early fishes[87].

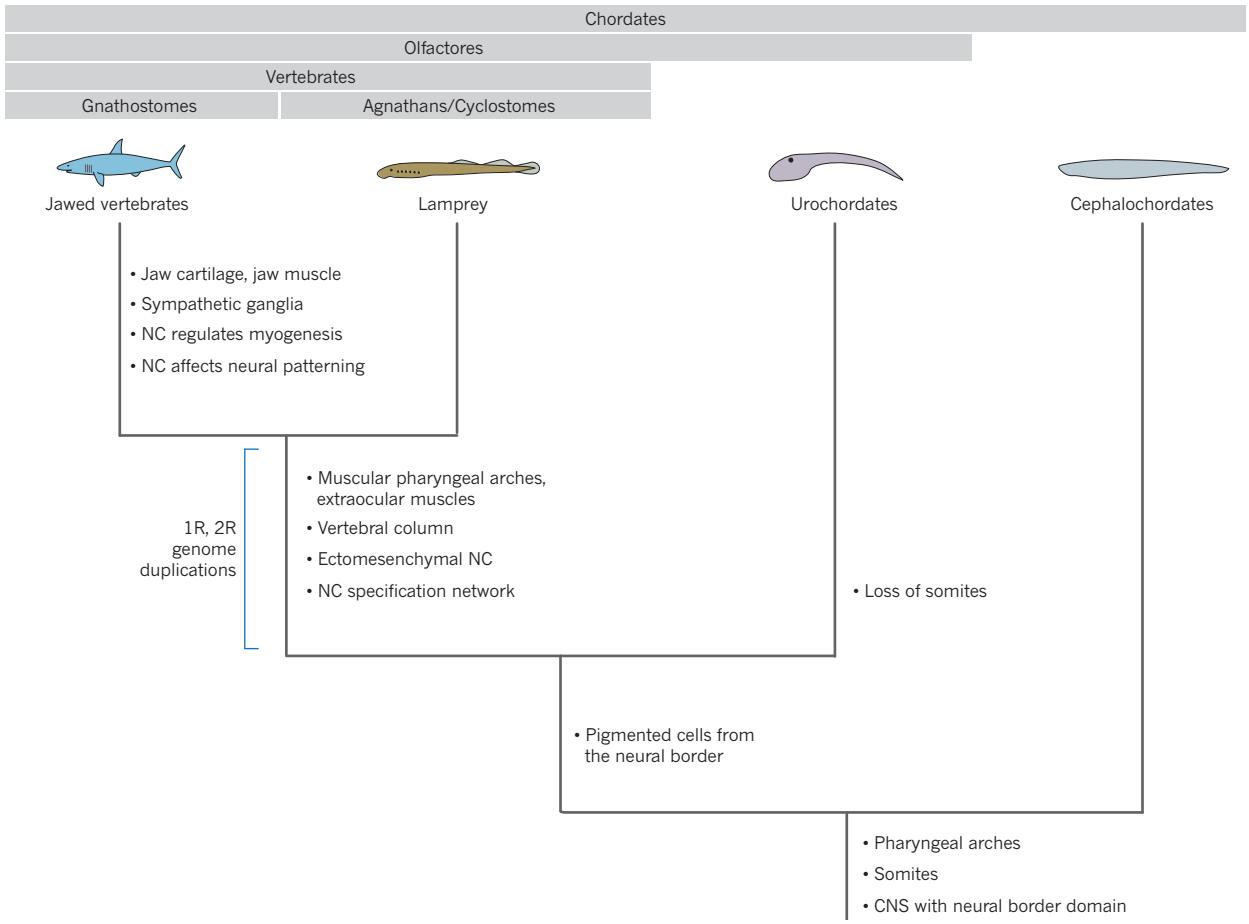

Figure 2 | Schematic cladogram of chordate features associated with neural crest cells or their derivatives. Labels at top indicate names of monophyletic groupings below. The timing of duplications is indicated in blue, whereas character changes are indicated by the bullet points. The order of character changes within a stem group is arbitrary. Adapted from ref. 97. CNS, central nervous system; NC, neural crest.

is established at the regulatory level, and have an impact on our views of the evolution of the vertebrate body plan.

Adult neural crest stem cells and post-embryonic growth

Many fossils suggest that the body size of the earliest vertebrates was, like many living invertebrates, quite small[90]. Only later did vertebrates begin to attain larger sizes, presumably through a process that involved extending the duration of post-embryonic growth. Extended growth requires coordinated development of many cell types, possibly including the establishment of stem-cell niches that govern the growth and regeneration of novel tissues.

Until recently, there was little indication of how adult neural crest cell populations were maintained. Recent evidence suggests that amniotes have adult neural crest stem-cell populations that maintain multipotency into adulthood, and which might enable the continuous replenishment of neural-crest-derived tissues[91,92], thus facilitating post-embryonic growth in concert with other tissues. These cells, called Schwann-cell precursors, reside on peripheral nerves and can produce multiple derivatives, including pigment cells and parasympathetic ganglia[93–96]. Whether the GRN underlying differentiation of these neural crest stem cells mirrors that of embryonic progenitor cells is an open and intriguing question that warrants further study. So far, these cells have only been identified in amniotes (in mammals and avians), but there is an obvious need for cells that fill this requirement in other vertebrates, and it is likely that cells such as these originated in early vertebrates.

These studies suggest that the influence of the neural crest in moulding the vertebrate body plan may extend beyond embryonic development, perhaps influencing the increase in size observed in several vertebrate clades. As vertebrates continued to grow post-embryonically, they may have required the setting aside of a population of neural crest stem cells, in the form of Schwann-cell precursors, that were retained to later stages. The relative proportion of adult tissues that these crest-derived stem cells contribute to is not yet known. Emerging data suggest that this cell population may form many derivatives classically attributed to the embryonic neural crest. Equally, they may represent the key to post-embryonic growth of the vertebrate body and therefore play a heretofore unknown part in promoting vertebrate evolution.

Expansion of neural crest cell types

Development of the neural crest sets vertebrates apart from invertebrate chordates. Formation of this novel cell type was probably facilitated by the addition of a new and uniquely vertebrate 'specification' kernel to the GRN, which in turn conferred multipotency and migratory ability to cells at the neural plate border. During the course of vertebrate evolution, even more derivatives have emerged under the umbrella of the neural crest (for example, additional elements to the peripheral nervous system, elaboration of the jaw or formation of the middle ear). Consolidation of key neural crest specifier genes such as *FoxD3*, *SoxE* and *TFAP2* in the neural-crest-specification module of its GRN may have facilitated evolution of this cell type, by allowing co-option of additional differentiation batteries under the control of neural crest regulators. Arguably, this has made the neural crest one of the most rapidly changing cell types in the vertebrate embryo and has perhaps contributed to the maintenance of neural crest stem cells in adults. ■

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