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Regulatory logic underlying diversification of the neural crest

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Abstract

The neural crest is a transient, multipotent population of cells that arises at the border of the developing nervous system. After closure of the neural tube, these cells undergo an epithelial to mesenchymal transition to delaminate and migrate, often to distinct locations in the embryo. Neural crest cells give rise to a diverse array of derivatives including neurons and glia of the peripheral nervous system, melanocytes, and bone and cartilage of the face. A gene regulatory network (GRN) controls specification, delamination, migration, and differentiation of this fascinating cell type. With increasing technological advances, direct linkages within the neural crest GRN are being uncovered. The underlying circuitry is useful for understanding important topics such as reprogramming, evolution, and disease.

Introduction to the Neural Crest

Neural crest formation occurs during early vertebrate development. This multipotent stem cell-like population forms within the future central nervous system (CNS) but then migrates away, undergoing some of the longest migrations of any embryonic cell type. These cells then contribute to a wide variety of derivatives including craniofacial cartilage and bone, neurons and glia of the peripheral nervous system, and melanocytes. As a vertebrate apomorphy, the neural crest is an innovation thought to be necessary for the origin, expansion, and acquisition of predation of vertebrates (the new head hypothesis) [1]. As a consequence, it has often been called “the fourth germ layer” [2,3]. Due to its developmental plasticity, migratory ability, and importance in vertebrate evolution, studies of the neural crest have had a profound impact on the fields of both embryology and evolutionary biology for over the last century.

Wilhelm His (1868) first identified the neural crest or “*Zwischenstrang” (*translation: intermediate cord) as a band of cells between the neural tube and non-neural ectoderm that gives rise to the spinal and cranial ganglia of chicken embryos. Later, grafting experiments revealed the developmental potential of neural crest cells in amphibians and birds, the latter using quail/chick chimera [4,5]. Tracking cells over time revealed distinct migratory pathways and a diverse array of neural crest derivatives. More recently, the neural crest field

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has expanded to reveal the cellular and molecular components underlying neural crest development.

With continuing advances in systems-level approaches and genomic analyses, Gene Regulatory Networks (GRNs) describing the formation and diversification of various stages of neural crest development are being uncovered at high-resolution (see Box 1). Here, we briefly describe how connections have been established within the neural crest GRN with emphasis on the most recent findings. Space limitation precludes description of the entire literature underlying building of each node, but please see Simoes-Costa and Bronner (2015) for further details [5].

Box 1

Increasing the resolution of GRN architecture with technological advances

Investigating gene regulatory networks requires tools for interrogating each node and regulatory linkage in detail. Most linkages in the GRN were initially established using simple perturbation analyses where a genetic knockdown was performed and gene expression changes were assayed by *in situ* hybridization. Cis-regulatory analyses to uncover transcription factor occupancy were done on a gene by gene basis and took years to examine each node. To achieve the goal of elucidating each node and establishing direct linkages in the GRN, high throughput examinations are a crucial. Genomic tools such as CRISPR/Cas9, RNA-seq, ATAC-seq, and ChIP-seq have become a standard for GRN analysis.

Tissue-specific transcriptomes have been used to uncover temporal and spatially specific regulatory states, even at the single cell level [62,95]. Fluorescent activated cell sorting (FACS) allows isolation of distinct cell populations even within an intermingled, heterogeneous population such as the neural crest. Using tissue-specific transgenic lines or tissue-specific enhancers driving fluorescent reporter expression, one can isolate a pure population of neural crest cells or derivatives at any time during their development. Establishing a comprehensive neural crest GRN depends upon establishing complete regulatory states at each event of neural crest development. Once the suite of genes involved in each regulatory module is uncovered, it is possible to establish direct regulatory linkages therein.

Recent advances in molecular biology enable uncovering the whole regulatory landscape such that direct linkages can be easily established computationally and tested *in vivo*. Tissues that were previously inaccessible to tissue-specific isolation can now be purified using a technique called “biotagging” which takes advantage of the strong non-covalent nature of in vivo biotinylation to specifically label cells in a tissue-specific manner, enabling study of their *in vivo* chromatin landscape [103]. Assay for transposase accessible chromatin using sequencing (ATAC-seq) surveys regions of open chromatin and transcription factor occupancy and has opened new opportunities for probing direct linkages within the GRN. Methods such as ChIP-seq, which investigates DNA-binding, and Hi-C, which uncovers chromatin conformation are also avenues for neural crest researchers to enhance each node of the GRN. Future studies will likely allow GRN

investigation at even higher resolution by more rapidly and efficiently establishing the whole hierarchy.

Gene Networks Overview

GRN circuitry provides a view of development from a logical perspective. Formulated via a systems-level integration of data accumulated in many vertebrate model systems, the neural crest GRN consists of sequential, separate regulatory modules, each comprised of a suite of transcription factors and signaling molecules (Figure 2) that explain the process of neural crest formation and differentiation into the vast array of derivatives. Underlying each regulatory event is a stable module embedded within a larger “neural crest gene regulatory network”, that combines signaling and transcriptional interactions. Through a series of feedback loops, each dynamic state within the network stabilizes, so that drivers of downstream gene batteries can be activated [6]. Also embedded within this GRN are circuits that confer multipotency and migratory ability.

Initial regulatory interactions within the neural crest GRN were largely established by perturbation analyses in mouse, zebrafish, frog, chick, and the sea lamprey, with focus on the role of transcription factors and signaling molecules. Recent advances in technology have increased the resolution of each interaction by enabling analysis of direct regulatory interactions (Box 1). Thus, we are now beginning to understand, in detail, the genetic control of the sequential events of neural crest formation from a multipotent progenitor to a differentiated state.

Neural Crest Formation along the Body Axis

The neural crest forms by a series of sequential regulatory steps: induction, specification, delamination, migration, and differentiation (Figure 1A). During gastrulation, many signaling events influence the region at the border between neural plate and non-neural ectoderm to promote neural crest formation. Initial specification of the neural crest from the neural plate border is complete when the neural folds become elevated during neurulation. Around the time of neural tube closure, premigratory neural crest cells initially reside within the neural folds/dorsal neural tube and then undergo an epithelial-to-mesenchymal transition (EMT) to emigrate. This occurs after neural tube closure in chick and zebrafish, but prior to tube closure in frog and mouse. Following EMT, neural crest cells then migrate extensively along distinct, stereotypical pathways often to distant locations where they differentiate into a variety of derivatives (Figure 1A, 1B).

Along the anterior-posterior body axis, there are distinct neural crest populations, (cranial, vagal, trunk, and sacral) with differential abilities to form different derivatives (Figure1B). Each is controlled by unique downstream gene batteries that are influenced by extrinsic environmental factors that direct migration and activate downstream differentiation genes. Whereas neural crest cells at all axial levels contribute to melanocytes, there are differences in neural crest derivatives from different axial levels: the cranial neural crest gives rise to craniofacial skeleton, connective tissue, and cranial ganglia [7]; vagal neural crest cells

contribute to the cardiac outflow tract and enteric nervous system [4,8]; trunk neural crest cells form dorsal root and sympathetic ganglia [9]; and sacral neural crest cells will give rise to components of the sympathetic and enteric nervous systems [10]. While each population is multipotent, only the cranial neural crest can give rise to cartilage [11–13] (see Box 2).

Box 2

Axial reprogramming by re-wiring GRNs

Along the body axis of the embryo, distinct neural crest populations have different developmental potentials and migratory behaviors. Whereas individual neural crest cells at all axial levels can contribute to multiple fates, the potential to form bone and cartilage is restricted to the cranial neural crest. When trunk neural crest is grafted *in vivo* to the head, it fails to give rise to cartilage. While the GRNs at different axial levels (cranial, trunk, vagal, and sacral) are similar in that they each drive neural crest formation, maintain multipotency, and bestow migratory ability, each axial level is endowed with slightly regulatory differences leading to formation of different derivatives. Axial level differences in cis-regulatory activity and regulatory circuitry confer differential ability to respond to environmental cues during later development and, ultimately, lead to differences in cell fate.

Different enhancers control the activity of neural crest specifier genes *Foxd3* and *Sox10* in the cranial neural crest versus the trunk neural crest [29,45,59]. For instance, two enhancers for *Foxd3*, NC1 and NC2, are activated differentially in the cranial and trunk crest, respectively. In the cranial crest, NC1 is activated by Ets1 along with Msx1 and Pax3/7 to drive expression; in the trunk, Msx1 and Pax3/7 are also important for activation of NC2 in the trunk, but work together with Zic1 rather than Ets1 [29]. Such axial level differences likely occur throughout the GRN, though these remain to be completely understood.

Recently, a cranial specific subcircuit reflecting an axial level specific regulatory modulate was show to be missing from the trunk population [13]. A tissue-specific transcriptomics approach, used to isolate pure populations of cranial versus trunk neural crest, led to identification of regulatory state differences in the cranial and trunk neural crest. Just 15 transcription factors were found to be differentially expressed within the cranial neural crest compared to the trunk GRN. Of those 15, only 6 were expressed throughout the cranial neural crest. Focusing on these six transcription factors enabled building of a regulatory circuit with direct linkages. Surprisingly, ectopically driving 3 downstream factors (Sox8, Tfap2b, and Ets1), of this small gene circuit gave the trunk neural crest the ability to form cartilage in an ectopic location. By rewiring the trunk NC GRN by the ectopic expression of this cranial-specific subcircuit, trunk neural crest cell identity was changed to cranial-like. The ability to rewire GRNs, *in vivo*, has powerful implications on therapeutic approaches.

While different neural crest GRN regulatory programs are deployed at each axial level, most experiments to date have focused on the cranial neural crest. Due to the extensive literature on this cell type, here we focus on the cranial crest, but highlight axial level differences

where known. Interestingly, GRN subcircuits unique to one axial level can be ectopically expressed at other axial levels to change their identity; for example, transplantation of a cranial subcircuit comprised of three transcription factors is sufficient to reprogram trunk neural crest cells and imbue them with the ability to make cartilage, a derivative they normally cannot make [13] (Box 2).

Induction and Specification of the Neural Plate Border

During gastrulation, restrictive boundaries are established between the neural plate (CNS) and non-neural ectoderm (epidermis) to form the neural plate border [14] (Figure 1A). Coordinated signaling events initiate a cascade of transcriptional activation to give rise to neural crest and placodal progenitors within the neural plate border domain [14]. While these domains become refined throughout gastrulation, many neural plate border cells can contribute to neural, neural crest, and placodal ectodermal fates, such that the fates of each cell type are only established after neural tube closure [15].

Inductive signals from neighboring tissues activate a cascade of transcription factors that specify the neural plate border from which the neural crest will arise. Three major signaling pathways, BMP, WNT, and FGF, work combinatorially to drive expression of neural plate border specifier genes [14,16–18]. Crosstalk between signaling pathways result in the formation of the neural plate border; for example, Schille and colleagues in frog, found that WNT/PCP receptor, Ror2, upregulates Gdf6 at the neural plate border [19], thus, linking Wnt/PCP signaling to BMP signaling.

The initial signaling inputs that restrict the neural plate border also regulate activation of a transcriptional circuit, involving *Msx1, Dlx5/6, Pax3/7*, and *Gbx2,* to further refine the expression domain [20]. Also activated at the border are *Zic1*, *Tfap2, Gata2/3, Foxi1/2*, and *Hairy2* [14,21,22]. The components of the circuit are largely conserved across vertebrates though there are often paralogue switches between species. Positive feedback interactions within this hierarchical gene network, e.g. between Tfap2a and Msx1, ensure expression of important neural crest specifier genes *Msx1* and *Pax3/7*. These specifier genes are critical players in activation of the downstream specification module (see below). Also important at the neural plate border are epigenetic factors like DNA methyltransferase 3a (Dnmt3a), which directly represses neural genes [23].

While signaling events initially activate neural plate border specifier genes, understanding of how these genes are directly activated by each signaling pathway remains incomplete. In frog, *Gbx* is directly activated by β–catenin through a proximal enhancer [24]. In zebrafish, *Zic3* and *Pax3a* expression depend on multiple enhancers that respond differentially to BMP, WNT, and FGFs [25]. Sharpening of neural plate boundaries is achieved by reciprocal transcriptional repression, or mutual exclusion across regulatory states, which stabilizes each state (i.e. by excluding neural crest from placodal fate) (Figure 2).

Does neural crest induction occur via a gain of developmental potential or by maintenance of early embryonic stem cell properties? Recently, it has been proposed in frog that neural crest cells retain pluripotency from the blastula stage [26]. Accordingly, a parsimonious

explanation may be that neural crest multipotency occurs via maintenance of a pluripotent state from the early blastula rather than induction of a new regulatory state. Thus, the shared molecular underpinnings of neural crest and blastula cells may reflect the possibility that transcription factors like Pax7 and Snai1/2 are simply pluripotency maintenance factors. To gain insight into whether neural crest formation occurs via induction or pluripotency maintenance, it will be important to better resolve direct linkages in the neural plate border regulatory module. These circuits can then be compared with other pluripotency programs.

Neural Crest Specification

After formation of the neural plate border, signaling inputs are closely linked to upregulation of neural crest specifier genes. For example, Wnt signaling is upstream of *Snai2* and *Foxd3* in chick and *Zic1* and *Pax3* in frog neural crest specification [17] [27]. In chick, the initial neural crest specifier circuit is controlled by upstream neural plate border specifiers Pax3/7 and Msx1, which form a transcriptional complex with Wnt effector, Axud1, to directly bind to and activate neural crest specifier *Foxd3* [28,29], thus connecting Wnt signaling at the border with the downstream specification modules. Further integrating upstream Wnt signaling to downstream modules is a proximal enhancer of *Snai2*, which is directly activated by TCF/Lef and β–catenin [30].

In chick, Pax7 and Msx1 are also required for activation of neural crest specifier, *Ets1* [31], which also directly regulates *Foxd3* in the cranial neural crest; in the trunk, Zic1 works together with Msx1 and Pax3/7 to directly regulate *Foxd3* [29]. In frog, Zic1 and Pax3/7 bind directly to *Snai1/2* promoters [32,33]. Epigenetic modifier JmjD2A, a histone demethylase, is responsible for removing repressive methylation marks from the Snai2 promoter [34]. In turn, Snai1/2 in frog positively regulates *Twist, Foxd3,* and *Sox9* [35].

Transcription factor ap2a (Tfap2a) is required for neural crest specification in fish, frog, chick, and mouse [36–39] and has a role in differentiation of melanocytes and sympathetic ganglia (as discussed below) [37,40,41]. In addition, transcription factors Myb, Myc, and Prdm1 are also required for specification [42–44]. Myb directly interacts with Pax3/7 as well as Sox10 in chick, and Prdm1 directly interacts with Tfap2a and Foxd3 in fish [43–46].

In addition to specifying the neural crest, these transcription factors are also important for activating the downstream EMT programs and stabilizing the specification regulatory state through a series of coherent feed-forward and feedback loops (Figure 2). This robust circuitry will further delineate the neural crest domain at the neural plate border from the pre-placodal domain. Below, we will discuss how this stable state initiates downstream of EMT effector modules and how environmental cues then promote many differentiation gene batteries.

Transcriptional control of morphogenesis

EMT and delamination from the neural tube

Once specified, the neural crest undergoes an epithelial to mesenchymal transition and delaminates from the dorsal neural tube to migrate extensively throughout the embryo

(Figure 1A). EMT coordinates signaling and transcriptional regulation to trigger major structural changes, including de-adhesion, cytoskeletal rearrangements, and gain of motility. Comprehension of the molecular underpinnings of this complex morphogenesis is incomplete; our current understanding largely reflects the effector gene modules controlling de-adhesion.

Changes in adhesion involve dissolution of cadherin-mediated adherens junctions. In neural crest delamination, there is a "Cadherin switch" between type I Cadherins (including Ecad and Ncad) that are more important for epithelial adhesion, and more mesenchymal type II Cadherins (including Cad7 and Cad11). The transcriptional regulation of Cadherins is crucial for EMT. Snail transcription factors downregulate type I Cadherins by directly binding the Ecad promoter [47]. Snai1/2 also has been implicated, along with Lmo4, in downregulating Ncad (Type I) and Cad6b (a Type II Cadherin that is downregulated during delamination) [48]. Epigenetically, PHD12, an adapter protein, complexes Snai2 with Sin3A/HDAC to bind the Cad6b promoter that will, in turn, control repression of Snail2 on *Cad6b*[49]. Similarly, the Polycomb repressive complex 2 (PRC2) catalytic subunit EZH2 interacts directly with Snai2 to repress *Ecad* by binding its promoter[50].

Also important for downregulating *Ecad* are transcription factors Zeb2, FoxD3 [51,52] and Twist, which is downstream of Hif-1α [53] Transcription factor Sox9 also directly interacts with Snai1/2 to drive EMT [54,55]. While type I Cadherins (and Cad6b) are being repressed, type II Cadherins, such as *Cadherin-7*, are upregulated by upstream specifiers FoxD3 and Sox10 [52]. This switch in expression, along with activation of *Integrin-β1* initiates downstream dispersal and migratory mechanisms.

Wnt signaling also influences EMT by transcriptionally regulating *Snai1/2* [30,56]. However, Wnt/β–catenin signaling is transiently downregulated at the onset of delamination [57] by the scaffold protein DACT1/2 that regulates the subcellular distribution of β–catenin[57].

Acquisition of a Migratory ability

After delamination, neural crest cells acquire a regulatory state that enables long distance migration throughout the embryo in response to environmental cues. Migratory neural crest cells express Sox10, a critical regulator of neural crest migration and differentiation. Expression of Sox10 is controlled by multiple enhancers in chick, mice, and fish [45,58–61]. The chick cranial specific enhancer, *10E2*, is regulated by neural crest specifiers Myb, Ets1, Sox9 [45]. In the trunk, Sox5 and Sox8 directly interact with the trunk specific enhancer, *10E1* [59]. Enhancer analysis in the mouse revealed seven enhancer regions that were directly bound by Pax3/7, Tfap2a, FoxD3, TCF/Lef, Sox10, as well as other Sox proteins [60,61]. Furthermore, an intronic enhancer in fish was uncovered, with direct binding sites of FoxD3, TCF/Lef, and Sox proteins [58].

Recently, tissue-specific transcriptomes have identified numerous transcription factors upregulated in the chick migratory cranial neural crest [62]. Furthermore, perturbation analysis revealed putative regulatory linkages between the upstream premigratory module

and downstream transcription factors, including Lmo4, RxrG, Ltk [62], though direct connections and other linkages still need to be uncovered.

To maintain migratory ability yet remain plastic enough to respond to differential cues, the migration regulatory state must be stable. How differential migratory paths change regulatory states while both temporally and spatially responding to different cues remains unknown. As discussed below, distinct environmental signals activate downstream differentiation gene batteries to give rise to diverse array of derivatives.

Differentiation GRN circuits

Differentiation gene batteries interpret and differentially respond to a complex set of environmental signals. The gene batteries operate under a positive feed forward circuitry where initial neural crest regulators function together with locally activated differentiation effector genes. Below, we outline the most characterized terminal differentiation gene modules, which include five representative neural crest derivatives: melanocytes, chondrocytes, Schwann cells, the enteric nervous system, and the sympathetic nervous system (Figure 2).

Melanocytes

Melanocytes, which produce pigmentation of the skin and hair follicles, arise from neural crest cells at all axial levels [63]. Transcriptionally, Sox10 acts synergistically with Pax3/7 to bind and activate a proximal promoter of the critical pigmentation transcription factor *Mitf* [63–65]. Sox10 also directly activates *Mef2c* in mice, while Mef2c autoregulates itself [66]. In melanocytes, Wnt signaling induces expression of *Mitf* [67,68]. Melanocyte function depends on Mitf expression which, along with Sox10, directly controls the production of the melanin synthesis enzymes *Dopachrome tautomerase (Dct), tyrosinase (Tyr)*, and *Premelanosome protein (Pmel)* [69,70]. Tfap2a is a pleiotropic transcription factor that has important roles in early neural crest specification as well as many differentiation gene batteries. Recently, Tfap2a will often co-occupy active regulatory elements of melanin synthesis genes with Mitf, including super-enhancers of melanin synthesis genes *Dct* and *Pmel* [40].

On the epigenetic level, HDAC1 is important for repressing *Foxd3* and other neural crest fates as well as *Sox10* to modulate its activity, allowing for melanocyte differentiation [65]. Taken together, these studies are building a detailed understanding of melanocytic differentiation at the gene regulatory level. These data will have an important role in understanding how melanocyte differentiation may be disrupted in diseases such as melanoma (Box 3).

Box 3

Using GRNs as tools to understand disease

With the current state of the neural crest GRN, we can begin to extract information from and utilize it as a tool to understand neural crest derived diseases, neurocristopathies. Neurocristopathies are diseases, craniofacial malformations, and tumors of the neural

crest. They include but are not limited to: melanoma, DiGeorge syndrome (craniofacial and heart defects), Treacher-Collins syndrome (craniofacial defects), Hirschsprung disease (aganglionic megacolon), pheochromocytoma, Schwannomas, and neuroblastoma. Neurocristopathies arise at various stages of neural crest development. Many mutations have been uncovered in numerous human disease. It is likely that underlying these diseases are mutations that cause a mis-wiring of GRN architecture. For instance, a nonpolyalanine repeat expansion mutation in *PHOX2B* alters a wildtype repression of a *Sox10* enhancer to a transactivation of *Sox10* leading to an imbalanced differentiation into glial versus neuronal lineages. This GRN mis-wiring event results in a higher incidence of Hirschsprung and neuroblastoma [88].

It will be critical to ascertain the underlying genetic circuitry of developing neural crest lineages at the onset of neurocristopathies. The role of individual genes necessary for onset of different neurocristopathies is an important first step for developing treatments; however, these genes regulate one another and interact within a network of many genes. Understanding the whole circuit can provide a new perspective on treatment options as well as disease ontogeny. Any aberrations within the regulatory network can confer downstream effects on other potential disease targets. Furthermore, having the potential to re-program/re-wire nonfunctioning gene networks using gene-editing techniques such as CRISPR/Cas9 could deliver a new avenue for ascertaining and treating with novel therapeutic targets.

Chondrocytes

Cranial neural crest-derived chondrocytes give rise to different elements of the craniofacial skeleton by forming the scaffold for facial bone. Sox9 in concert with Sox5/6 provide direct inputs into collagen gene, *Col2a1* and cartilage differentiation marker, *Agc1* [71,72]. A role for TGFβ pathway, through Smad3, in chondrogenic differentiation recruits histone acetyltransferase creb-binding protein (CBP)/p300 to allow for Sox9 activation of regulatory genes [73,74].

Schwann cells

Schwann cells are neuronal support cells that produce an insulating myelin sheath that surrounds axons of the peripheral nervous system and promote neuronal conduction by propagating electrical signals through often long peripheral nerves. Regulatory interactions important for Schwann cell development involve transcription factors that activate genes encoding myelin proteins and lipids as well as a repressor, Zeb2, which maintains a differentiated state [75,76].

The SoxE transcription factor, Sox10, activates POU domain transcription factor, *Oct6,* by directly binding to a Schwann cell specific enhancer (SCE) that is 10kb downstream of the *Oct6* coding sequence [77–80]. Sox10 bound *Oct6* recruits Brg1-containing BAF remodeling complexes and histone deacetylases HDAC1 and HDAC2 for proper enhancer activity [81,82]. The direct interactions of Sox10 and Oct6 to activate *Krox20* also require BAF and HDAC1/2 for local remodeling and changes to the chromatin structure. Upon activation, this forms a coherent feed-forward circuit stabilizing the regulatory state for

terminal Schwann cell differentiation. The combination of Krox20 and Sox10 controls terminal differentiation by co-occupying myelin gene regulatory regions for *myelin basic protein (MBP), myelin protein zero (MPZ), proteolipid protein (PLP)*, and gap junction proteins *Connexin32* and *Connexin47* [76,83]. Furthermore, dysregulation of this gene battery leads to the formation of schwannomas, which are malignant tumors that form from abnormally differentiated Schwann cells (Box 3).

Enteric nervous system

The enteric nervous system (ENS) is comprised of a complex network of neurons and glia organized into ganglionic plexuses. The ENS regulates transport of food, digestion and absorption of nutrients, as well as other functions of the gastrointestinal tract [84]. Enteric neurons and glia arise from the vagal neural crest cells which migrate to the foregut mesenchyme. The receptor tyrosine kinase Ret signaling is responsible for the migration of enteric precursors to populate the entire gut. Transcription factor Sox10 is expressed in the developing ENS and interacts with Pax3 to synergistically activate a *Ret* enhancer [84,85]. *Ret* expression is also regulated by paired homeobox transcription factor, Phox2b[86]. Glial-derived neurotrophic factor (GDNF) activates Ret and co-receptor GDRα1 in the migrating enteric neural crest cells to activate a cascade of downstream pathways [87].

Within the ENS circuit, there is mutual repression of Sox10 and Phox2b. Whereas cells with upregulated Sox10 assume a glial fate, Phox2b+ cells form neuronal lineages. This reciprocal repression subcircuit within the ENS circuit is responsible for irreversibly tipping cell fate in one direction and ensuring a balance of glial and neuronal fates along the gut [88].

Also important within the ENS circuitry are bHLH transcription factors Achaete-scute (Ascl1) and Hand2 [89,90]. Ascl1 deletion in mice results in downregulation of *calbindin*, *tyrosine hydroxylase*, and *vasoactive intestinal peptide (VIP)*, all of which have an important role in development of neuronal subtypes [91]. Ascl1 also has been shown to downregulate *Sox10*, which is important for ensuring production of neurons [92]. Mutations in Hand2 cause downregulation of neuronal differentiation leading to a loss of of nNOS, calretinin, and VIP neuronal subtypes in mice [89,93,94]. Recently, an elegant paper has characterized the neuronal and glial profile of the mammalian gut [95]. Another recent study performed a transcriptome analysis of sorted enteric neurons in zebrafish to uncover novel genes involved in ENS development [96]. Understanding all of the neuronal subtypes and the transcriptional and regulatory landscapes within the gut will further delineate neural gene regulatory circuits underlying the complexity of the enteric nervous system.

Sympathetic nervous system

The sympathetic nervous system is the branch of the autonomic nervous system responsible for the flight-or-fight response and needed to maintain organ homeostasis, heart rate, and many other functions in response to an external challenge. Sympathetic neurons arise primarily from trunk neural crest. In response to BMP signaling at the dorsal aorta, a cascade of transcription factors is activated within a differentiation gene battery [97]. *Smad4-dependent* and *–independent* BMP signaling activates *Ascl1* and *Phox2b*. Through a

positive feedback mechanism, Phox2b is required for maintenance of *Ascl1* expression [97,98]. Downstream of the initial activation of *Phox2b* and *Ascl1* is the activation of *Phox2a, Hand2,* and zinc finger proteins *Gata2* and *Gata3,* and *Insm1* [97,99,100].

Sympathetic neurons are primarily adrenergic, producing norepinephrine via enzymes tyrosine hydroxylase (TH) and dopamine beta hydroxylase (DBH), which are directly bound by transcription factors Tfap2a and Phox2a to activate their transcription [99,101]. The sympathetic nervous system differentiation circuit is laced with positive feedback loops that result in expression of norepinephrine and neurogenesis regulators. Understanding the genetic program controlling normal development of the sympathoadrenal lineage may also inform upon the aberrant regulatory network that leads to onset of neuroblastoma, thus helping identify novel therapeutic targets.

Concluding Remarks

This overview of the gene regulatory network underlying neural crest development highlights recent advances, from initial induction to differentiation into a subset of derivatives. This combination of studies in numerous vertebrate models allows us to infer and update a GRN as viewed in a Biotapestry model (Figure 2) [102]. As more linkages are validated, regulatory interactions within this robust GRN will become increasingly resolved. This neural crest GRN provides a useful tool to understand developmental processes, disease, cancer (Box 3), and evolution (Box 4) at a systems-level.

Box 4

Using GRNs to understand morphological novelty

Morphological novelties arise due to changes in the regulatory program. The neural crest is a cell type that is unique to vertebrates, raising the question of whether evolution of neural crest was preceded by co-opted regulatory programs from other tissues into the neural crest lineage. Questions such as these can be answered by GRN comparisons to interrogate which nodes underlie trait diversity. For example, comparisons between jawed and jawless like the sea lamprey may lead to identification of traits as well as GRN states that were present in early vertebrates. Here, we focus on regulatory architecture comparison between vertebrates. For a discussion on invertebrate chordates, see Green et al 2015 [104].

Jaws are unique to jawed vertebrates, arising by modification of the anterior pharyngeal arches. Other features unique to jawed vertebrates include ondontoblasts that produce dentine, sympathetic neurons, and vagal-derived enteric neural crest [104,105]. While there are astonishing similarities between the early specification neural crest GRNs of jawed and jawless vertebrates [106], tracing regulatory differences between jawed and jawless vertebrates may have lead to formation of new cells types, thus correlating with the divergence of these cell fates.

Conserved in the early specification network are the importance of transcription factors Pax7, Msx1, and Zic1 in the sea lamprey [106]. Similar to the jawed vertebrate GRN (Figure 2), knockdown of these neural plate border transcription factors results in a

downregulation of *foxd3* and *soxE1* [106]. A key difference in the early gene networks of lamprey is a lack of cranial specific transcription factors, Ets1 and Twist. Could a lack of these transcription factors in early regulatory modules cause distinct traits to be missing in lamprey? Or could a heterochronic shift in gene expression lead to novel regulatory interactions and circuitry in jawed vertebrates? Further investigation of the jawless vertebrate GRN is sure to uncover such discrepancies.

Throughout evolution, differentiation gene batteries arose to give rise to novel cell types. It is likely that differentiation gene batteries were "plugged in" to the upstream GRN hierarchy by gain of function cis-regulatory changes in differentiation genes, which led to their control by upstream neural crest specifiers. Regulatory analyses such as ATAC-seq will help to uncover differences between lamprey and jawed vertebrates in regulatory DNA at these time points.

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Glossary

Apomorphy
the neural crest is an apomorphy in that it is a derived trait that is unique to vertebrates and present in their last common ancestor

Enhancer
short, regulatory regions of DNA that is bound by transcription factors to control the activation and transcription of a particular gene

Epithelial-Mesenchymal-Transition
a series of complex cell state changes (ie. structural remodeling, de-adhesion, delamination) that transforms an epithelial cell to a mesenchymal cell

Multipotent
a cell that can develop into many cell types

Neurocristopathy
syndromes that involve the dysregulation of one or more neural crest cell populations

Regulatory state
the combination of transcription factors and signaling molecules expressed in a given cell type at a specific time

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Trends

The neural crest GRN provides a unique look at regulatory hierarchies underlying multipotency and such developmental events as specification, delamination, migration, and differentiation into various derivatives.

Interrogating direct connections is now possible on a larger scale with a variety of new technological advances.

Rewiring of GRN architecture has been shown to reprogram trunk neural crest into a cranial identity, which delivers a powerful route for novel therapeutic approaches.

Gene regulatory networks provide a foundation from which to understand onset of neurocristopathies.

Regulatory changes within the neural crest GRN lead to hypotheses concerning the evolution of morphological novelties such as jaws.

Outstanding Questions Box

- What are the axial level gene regulatory network differences between cranial, vagal, trunk, and sacral neural crest cells, and are those differences responsible for the suite of derivatives each axial level is fated to become?
- Does neural crest induction occur via a gain of developmental potential at the neural plate border or by maintenance of early embryonic stem cell properties?
- How does the regulatory state of the migratory neural crest transition to initiate differential downstream gene batteries?
- How can bioinformatics tools and advanced technologies such as RNA-seq and ATAC-seq be exploited to uncover the entirety of the neural crest GRN complete with direct linkages?
- At what hierarchical level do gene regulatory network circuits change to give rise to morphological novelties such as jaws, sympathetic ganglia, and the vagal-derived enteric nervous system?

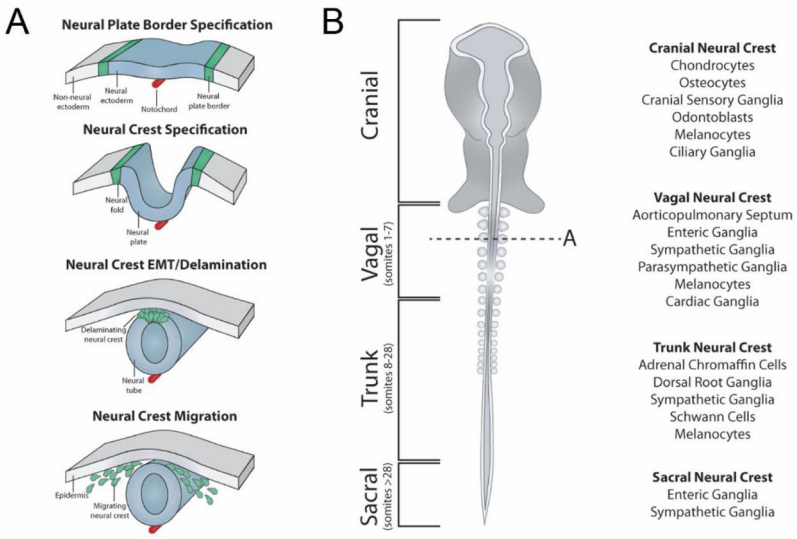

Figure 1.

Neural crest formation and its derivatives. (A) The neural crest forms by a series of important regulatory events: induction at the neural plate border, specification, delamination from the neural tube, migration throughout the embryo, and differentiation into many derivatives. During gastrulation, coordinated signaling events specify the region at the border between neural plate and non-neural ectoderm to promote neural crest formation. Initial specification of the neural crest from the neural plate border is complete when the neural folds become elevated during neurulation. In the chick, premigratory neural crest cells undergo an epithelial-to-mesenchymal transition (EMT) after neural tube closure to migrate extensively along distinct, stereotypical pathways to differentiate into a variety of derivatives. (B) Along the anterior-posterior length of the developing vertebrate embryo (left), different derivatives will form depending on the axial level (cranial, vagal, trunk, or sacral) from which they have originated. Neural crest cells at all axial levels contribute to melanocytes, the cranial neural crest gives rise to craniofacial skeleton, connective tissue, and cranial ganglia. The vagal neural crest cells contribute to the cardiac outflow tract and enteric nervous system. Trunk neural crest cells form dorsal root and sympathetic ganglia. Sacral neural crest cells will give rise to parts of the enteric and sympathetic nervous systems.

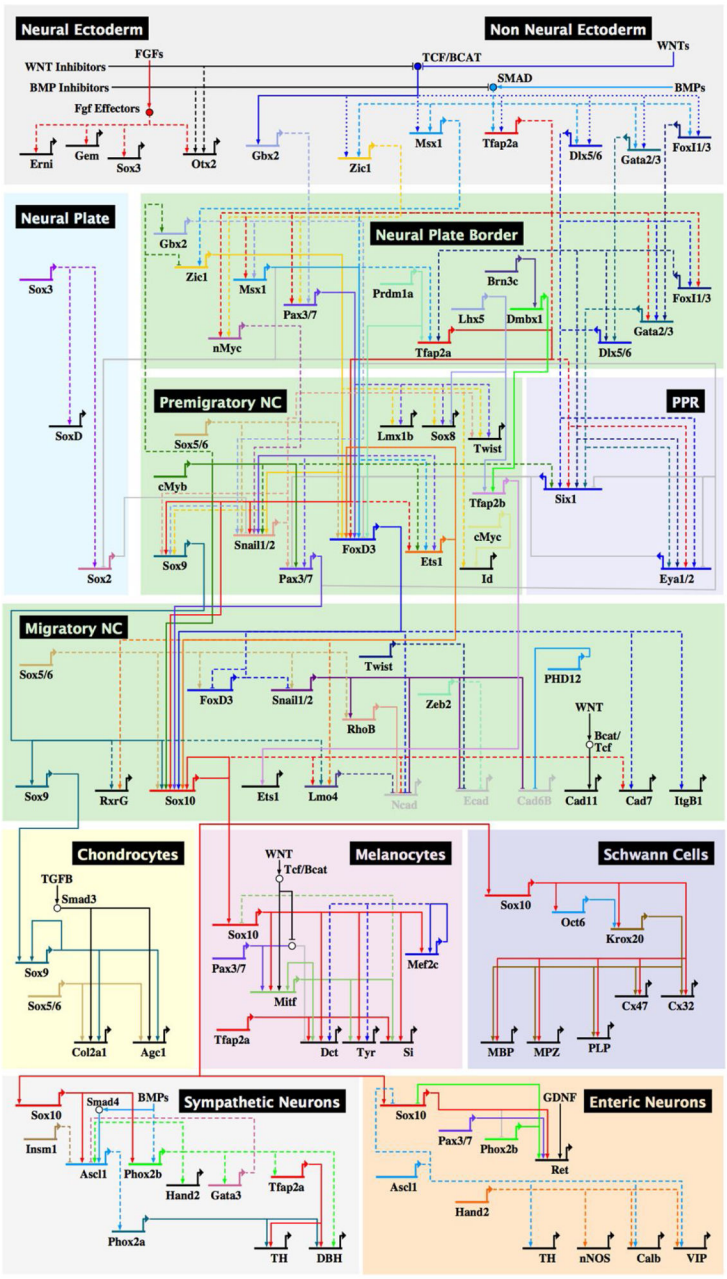

Figure 2.

The GRN underlying neural crest development. Constructed from a systems-level amalgamation of literature in many vertebrate model systems, the neural crest GRN is comprised of sequential regulatory modules, focusing on transcription factors and signaling molecules for each stage of neural crest formation: induction, specification, delamination, migration, and differentiation. Differentiation gene batteries for five representative neural crest derivatives are placed downstream in the GRN hierarchy. Direct regulatory interactions within the neural crest GRN are represented by a solid line, while interactions that have yet to be verified as direct are represented with a dashed line. The GRN is largely conserved

across vertebrates with some variation between species. For example, Twist1 is part of the neural crest specification module in frog and zebrafish, but missing in amniotes.