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Network architecture and regulatory logic in neural crest development

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Abstract

The neural crest is an ectodermal cell population that gives rise to over thirty cell types during vertebrate embryogenesis. These stem cells are formed at the border of the developing central nervous system and undergo extensive migration before differentiating into components of multiple tissues and organs. Neural crest formation and differentiation is a multi-step process, as these cells transition through sequential regulatory states before adopting their adult phenotype. Such changes are governed by a complex gene regulatory network (GRN) that integrates environmental and cell-intrinsic inputs to regulate cell identity. Studies of neural crest cells in a variety of vertebrate models have elucidated the function and regulation of dozens of the molecular players that are part of this network. The neural crest GRN has served as a platform to explore the molecular control of multipotency, cell differentiation, and the evolution of vertebrates. In this review, we employ this genetic program as a stepping-stone to explore the architecture and the regulatory principles of developmental GRNs. We also discuss how modern genomic approaches can further expand our understanding of genetic networks in this system and others.

Graphical Abstract.

Neural crest cell development can be represented by a gene regulatory network that drives their induction, specification, migration, and differentiation

Introduction

The development of multicellular life from a single cell is a laborious process that has fascinated scientists for decades. As embryonic development progresses, cells differentiate into a variety of complex tissues in a highly regulated manner. Differentiation requires extensive changes in gene expression profiles, and cells must respond to both environmental and intrinsic cues in order to produce the correct regulatory response. This information is integrated by gene regulatory networks (GRNs) which determine the genes that should be activated or repressed at each step of cell fate commitment (Britten & Davidson, 1969;

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Davidson & Levine, 2008; Levine & Davidson, 2005). To understand the control of cell state changes, scientists have assembled GRN models, compiling regulatory interactions which define cell types and regulate cell state changes (Peter & Davidson, 2015). This framework has been utilized by many developmental systems including the vertebrate neural tube, early drosophila patterning, the sea urchin endomesoderm, and the vertebrate neural crest (Dessaud, McMahon, & Briscoe, 2008; Jaeger et al., 2004; Peter & Davidson, 2011; Simoes-Costa & Bronner, 2015). Here we will discuss the regulatory principles of developmental GRNs by focusing on the neural crest: a well-studied, multipotent embryonic cell population capable of extensive differentiation and a wide range of cellular functions.

Neural crest (NC) cells have been used to study cell fate decisions due to their ability to give rise to diverse fates such as chromaffin cells (secretory), melanocytes (pigmentation), sensory neurons (neuronal), and chondrocytes (mesenchymal) (Le Douarin & Kalcheim, 1999). They are unique in their ability to contribute to both ectodermal and mesenchymal derivatives, earning them the nickname as the “the fourth germ layer” (Hall, 2000). Neural crest cells are a vertebrate-specific cell type and are hypothesized to have played a central role in the evolution of this clade. For example, their exclusive contribution to the anterior neurocranium represents a milestone in active predation and a divergence from invertebrate chordates (Gans & Northcutt, 1983). Various vertebrate model organisms have been used to examine neural crest development, with the most prominent being chicken, zebrafish, frog, and mouse. These studies were further fueled due to the relevance of neural crest cell types in human disease. Defects in neural crest specification, differentiation, or migration lead to a variety of congenital disabilities, termed neurocristopathies (Watt & Trainor, 2014). In fact, it was estimated that one third of all birth defects can be linked to abnormalities during neural crest development (Trainor, 2014). Thus, a thorough understanding of the genetic networks that control neural crest development may allow for better diagnosis and management of these conditions.

Neural crest formation and differentiation is a multi-step process in which multipotent progenitor cells transition through sequential regulatory states before committing to their adult phenotype (Figure 1) (Meulemans & Bronner-Fraser, 2004; Sauka-Spengler & Bronner-Fraser, 2008). These cell state changes are governed by a complex GRN, comprised of modules of regulatory factors that control neural crest identity and behavior throughout development (Table 1, Figure 2). Some important GRN regulators were first identified via gene expression analysis and subsequent loss- and gain-of-function experiments (Labosky & Kaestner, 1998; Nieto, Sargent, Wilkinson, & Cooke, 1994) while others were revealed through mouse mutant phenotypes or by their involvement in human disease (Mitchell, Timmons, Hebert, Rigby, & Tjian, 1991; Southard-Smith, Kos, & Pavan, 1998). After identifying a group of transcription factors required for neural crest development, researchers began interrogating how these genes regulated each other—resulting in the initial assembly of small regulatory circuits. This information was ultimately integrated in a hierarchical, comprehensive regulatory network—the first version of the neural crest GRN (Meulemans & Bronner-Fraser, 2004). Over time, there have been multiple updates to the network through integration of novel transcription factors. (Betancur, Bronner-Fraser, & Sauka-Spengler, 2010; Martik & Bronner, 2017; Sauka-Spengler & Bronner-Fraser, 2008; Simoes-Costa & Bronner, 2015). The GRN was constructed from experiments performed in

a variety of model organisms and thus represents the core interactions that are conserved amongst vertebrates (Simoes-Costa & Bronner, 2013). Here, we will further expand on the features of developmental GRNs, the overall architecture of the neural crest gene regulatory network, and the circuit-level logic embedded in this genomic program. We will also discuss recent genomic approaches and how they can be applied to construction of GRNs in developmental systems.

An overview of the neural crest gene regulatory network

The neural crest GRN was assembled according to the framework originally employed by Davidson and colleagues to study the early development of the sea urchin embryo (Davidson, 2006; Davidson & Levine, 2008; Davidson, McClay, & Hood, 2003). These authors assembled genetic networks from epistatic interactions between the regulatory factors that control cell identity. GRNs have important features that distinguish them from other biological networks. First, they are constructed from regulatory interactions compiled from functional studies and are meant to serve as a causal (and not correlative) model of a genetic program. Thus, a developmental GRN should explain the emergence of new regulatory states in response to initial inputs. Second, GRNs are modular, with each module describing a discrete regulatory state during development (Peter & Davidson, 2015). Modules are organized in a hierarchal and sequential progression within the network, as to reflect the stepwise nature of cell fate commitment. Both of these properties are evident in the neural crest GRN: it was assembled from functional and biochemical experiments that identified functional interactions between genes, and it can be subdivided into regulatory modules that describe the processes of neural crest induction, specification, migration, and differentiation (Simoes-Costa & Bronner, 2015).

It is important to note that the neural crest resides along the entire anterior-posterior axis of the developing embryo, and that the neural crest GRN differs greatly between these axial levels. For example, a number of cranial- and trunk-specific transcriptional regulators have been characterized which are important for promoting different differentiation trajectories of these populations (Simoes-Costa & Bronner, 2016). Even so, the majority of studies that were used to assemble the current neural crest GRN have been conducted in the cranial neural crest. For the purpose of this review, we will focus on the regulatory interactions that occur within the cranial neural crest. A discussion on the differences in GRN architecture between distinct neural crest subpopulations can be found in our recent review on the topic (Rothstein, Bhattacharya, & Simoes-Costa, 2018).

The neural crest GRN modules are comprised of multiple genes organized in regulatory circuits (Table 1). These genes are represented as nodes within the network and are able to receive and propagate regulatory inputs to downstream genes (Table 1). Typically, nodes are transcription factors, but could also be signaling effectors, noncoding RNAs, or epigenetic remodelers. Nodes are connected by regulatory links representing functional interactions between genes. Depending on the biological function of the upstream node, links can be activating or repressive. The regulatory link between *Pax7* and *FoxD3*, which is represented by a solid line that connects these genes in the neural crest GRN (Figure 2A, B), is an example of the former. This link was established through a number of observations. First,

gene expression analysis has demonstrated that *Pax7* expression precedes the onset of *FoxD3* transcription (Basch, Bronner-Fraser, & Garcia-Castro, 2006). Second, *Pax7* directly activates expression of *FoxD3* in neural crest cells by binding to a *Foxd3* enhancer (Simoes-Costa, McKeown, Tan-Cabugao, Sauka-Spengler, & Bronner, 2012). This signifies a direct, activating interaction between *Pax7* and *Foxd3* and thus the link between these two nodes is represented as a solid line ending in an arrow. The complete neural crest GRN is composed of hundreds of such interactions, which take place within and between different modules. A defining insight of the GRN framework is that these interactions are not random, but instead form circuits that perform specific tasks within cells (Davidson, 2006; Davidson & Levine, 2008). Different combinations of activating or repressive links construct stereotypical circuit logic, which is conserved across many different developmental GRNs. Thus, the architecture (i.e. the arrangement of nodes and links and the circuits they form) of the neural crest GRN encodes the regulatory logic that underlies the potential and behavior of these cells.

The regulatory modules of the neural crest GRN

The life of a neural crest cell is characterized by several landmark events. First, immediately after gastrulation, the ectodermal germ layer is subdivided into two territories: the neural and the non-neural ectoderm (Le Douarin & Kalcheim, 1999). The non-neural ectoderm will give rise to the epidermis and cranial placodes, while the neural ectoderm will go on to form the central nervous system and the neural crest. Neural crest cells arise from a region at the junction of the neural and non-neural ectoderm, termed the neural plate border (NPB) (Figure 1A). These boundaries are established primarily through a number of extracellular signaling systems, which provide inductive cues during the process of **neural plate border induction** (Groves & LaBonne, 2014). Next, during neurulation, cells at the neural plate border elevate and, upon neural tube closure, reside at the dorsal aspect of the neural tube (Figure 1B). It is during this time that neural plate border cells become specified, in which they acquire characteristics of *bona fide* neural crest. This process is termed **neural crest specification** (Sauka-Spengler & Bronner-Fraser, 2008). Once neural crest cells have been specified, they begin **neural crest migration**, losing their epithelial connections and delaminating from the neural tube. These cells undergo a striking epithelial to mesenchymal transition and migrate throughout the embryo—relying on environmental cues for proper guidance (Figure 1C). Ultimately, at the completion of migration, neural crest cells differentiate into their terminal fates. As mentioned before, this terminal differentiation will lead to numerous and diverse derivatives, including cell types such as chondrocytes, neurons, and melanocytes (Martik & Bronner, 2017). Each of these successive processes (neural plate border induction, neural crest specification, epithelial to mesenchymal transition, and differentiation) is controlled by a specific GRN module, consisting of a unique combination of regulatory factors. Below, we highlight the key modules of the neural crest GRN, which allow for the formation and differentiation of this cell type (Figure 2).

Induction of neural crest from the neural plate border.—The earliest identification of presumptive neural crest cells can be traced to the formation of the neural plate border (NPB), a marginal region located between the neural plate and non-neural ectoderm (Figure 1A). The NPB is characterized by the combined expression of specific transcription factors. Studies in mice, chick, and frog have shown that neural tissue is marked by *Sox2/3* and *Otx2*

(Bally-Cuif, Gulisano, Broccoli, & Boncinelli, 1995; Papanayotou et al., 2008; Streit, Berliner, Papanayotou, Sirulnik, & Stern, 2000), while non-neural ectoderm is marked by *Dlx5/6*, *Gata2/3*, and *Foxi* (Matsuo-Takasaka, Matsumura, & Sasai, 2005; McLarren, Litsiou, & Streit, 2003; Sheng & Stern, 1999) (Figure 2A). In response to WNT, FGF, and intermediate BMP signaling, presumptive neural crest cells within the NPB induce the expression of *Pax7*, *Msx1*, *Tfap2a* and *Zic1* (Figure 2A, NPB) (Groves & LaBonne, 2014). This unique combination of factors initiates the neural crest GRN and distinguishes neural crest from another multipotent cell population induced at the NPB, the pre-placodal region (Figure 2, PPR). PPR cells are located more laterally and thus are subjected to different signaling system dynamics (Brugmann & Moody, 2005). In chicken and frog, these signaling dynamics induce the expression of *Eya1/2* and *Six1/4* (Christophorou, Bailey, Hanson, & Streit, 2009; David, Ahrens, Wedlich, & Schlosser, 2001; Esteve & Bovolenta, 1999; Ishihara, Ikeda, Sato, Yajima, & Kawakami, 2008). The boundaries between these regions are not absolute, as there have been examples of cells within the neural plate border region expressing both *Sox2* and *Pax7* in chicken (Roellig, Tan-Cabugao, Esaian, & Bronner, 2017). Furthermore, spatial transcriptomics have shown co-expression of neural and differentiation markers within avian neural crest cells (Lignell, Kerosuo, Streichan, Cai, & Bronner, 2017). Thus, it is likely that cells of the early neural plate border are competent to contribute multiple lineages until further specified.

Specification of neural crest cells and pre-migratory markers.—During neural crest induction and specification, the neural plate undergoes drastic morphological changes and folds upon itself to form the neural tube (neurulation, Figure 1B). During this folding, nodes within the NPB module activate members of the specification module, granting neural crest cells a unique molecular signature. This signature is bestowed by the expression of a number of neural crest-specific transcription factors that allow for the maintenance of multipotency and primes neural crest cells to undergo migration. As the neural tube begins to fold, neural crest cells begin to express specification markers such as *FoxD3*, *Ets1*, *Sox8/9/10* and *Snail/2* (Figure 2B) (Khudyakov & Bronner-Fraser, 2009). Furthermore, several markers first expressed in the NPB continue to be expressed in pre-migratory neural crest but play distinct, separate functions in later modules. For example, the transcription factor *Tfap2a* is essential for both NPB induction and neural crest specification across vertebrates. Work in *Xenopus* shows that *Tfap2a* first promotes NPB induction via *Msx1* and *Pax3*; it is subsequently redeployed during specification to drive expression of *Snai2*, *Foxd3*, and *Sox10* (de Croze, Maczkowiak, & Monsoro-Burq, 2011). This is consistent with findings in chicken and lamprey (W. Li & Cornell, 2007; Nikitina, Sauka-Spengler, & Bronner-Fraser, 2008). The neural crest specification module is characterized by a number of positive feedback and feed forward circuits that act to rapidly activate multiple genes—establishing a unique regulatory state in a subset of the neural plate border cells. This new regulatory state primes the nascent neural crest for the drastic molecular and morphological changes that occur during cell migration.

Epithelial-to-mesenchymal transition and migration—During neurulation, neural crest cells begin to lose their epithelial identity, delaminate, and migrate throughout the embryo (Figure 1C). This epithelial-to-mesenchymal transition (EMT) is coordinated by

transcription factors that modulate genes involved in cell adhesion and extracellular matrix interactions. Several factors required for neural crest specification also promote EMT, further highlighting the reiterative use of GRN components in neural crest development (Figure 2C). The specification genes, *Sox10*, *Foxd3*, and *Snai2* are involved in the regulation of multiple members of the cadherin superfamily of cell adhesion molecules. In chicken embryos, *Snai2* inhibits the expression of epithelial cadherins (*Cdh1/2/6B*) in conjunction with *Lmo4* (Ferronha et al., 2013; Taneyhill, Coles, & Bronner-Fraser, 2007), while *Foxd3* and *Sox10* promote the expression of mesenchymal cadherins (*Cdh7/11*) (Cheung et al., 2005; Dottori, Gross, Labosky, & Goulding, 2001). The EMT module of the neural crest GRN also receives inputs from extracellular signaling systems. Most notably, Wnt directly activates *Snai1/2* expression to promote EMT in *Xenopus* (Vallin et al., 2001). Furthermore, the timing of EMT may be controlled by the expression of the *Wnt* inhibitor *Draxin*, which is expressed in pre-migratory chicken neural crest prior to neural crest delamination (Hutchins & Bronner, 2018).

After neural crest cells delaminate and undergo EMT, they begin migration in stereotypical and organized paths to their final destinations in the vertebrate embryo. The paths and signals that guide neural crest migration vary across different axial levels (Kuo & Erickson, 2010; Minoux & Rijli, 2010; Shellard & Mayor, 2016). Cranial neural crest cells at the level of the midbrain migrate collectively in an unsegmented sheet between the ectoderm and underlying paraxial mesoderm in the head. This dorsolateral migratory pathway is also observed in cranial neural crest emerging from the hindbrain, which are patterned into three distinct streams directed towards individual branchial arches. In chick, mouse, and zebrafish, repulsive interactions between neuropilin receptors (*Nrp1/2*), expressed in migratory neural crest, and their semaphorin ligands (*Sema3a/f*), expressed in neural crest-free regions in rhombomeres 3 and 5, help create these streams (Gammill, Gonzalez, & Bronner-Fraser, 2007; Osborne, Begbie, Chilton, Schmidt, & Eickholt, 2005; Schwarz, Vieira, Howard, Eickholt, & Ruhrberg, 2008; H. H. Yu & Moens, 2005). In chick, mouse, and frog, repulsive interactions between Ephrin ligands and their Eph receptors help to maintain separation of migratory neural crest streams into the branchial arches (Davy, 2004; Golding et al., 2004; Smith, Robinson, Patel, & Wilkinson, 1997). The progression of neural crest migration is driven by contact inhibition of locomotion and the PCP signaling pathway; however, the mechanisms controlling when neural crest should stop migration are not clear (Carmona-Fontaine et al., 2008). As classical transplantation experiments have shown, the location of neural crest migration and the environment is essential for proper differentiation.

Differentiation—Once neural crest cells have migrated to various regions throughout the body, they undergo differentiation. Here, we discuss three major terminal cell types of cranial neural crest cells: chondrocytes, autonomic neurons, and melanocytes (Figure 2D). Neural crest have the potential to differentiate into chondrocytes that are responsible for generation of the craniofacial skeleton (Rothstein et al., 2018). Disruption of chondrocyte maturation leads to craniofacial abnormalities, which make up a large proportion of birth defects (Watt & Trainor, 2014). The core regulatory circuit driving chondrocyte development involves *Sox5/6* and *Sox9*, which directly activate the cartilage differentiation markers *Col2a1* and *Agc1* (Figure 2D, Chondrocytes) (Bell et al., 1997; Lefebvre, Li, & de

Crombrughe, 1998). Studies in mice and cell cultures have shown that Smad2/3-mediated TGF- β signaling plays a crucial role in regulation of chondrocyte development (Furumatsu, Tsuda, Taniguchi, Tajima, & Asahara, 2005; Ito et al., 2002). Neural crest cells also differentiate into autonomic nerves located throughout the body. Studies in cell culture, mice, and zebrafish have been used to build a simplified differentiation module for this fate (Figure 2D, Neurons). In this module, *Sox10* promotes expression of *Ascl1* and *Phox2b*. In turn, *Phox2b* in concert with *Tfap2a*, activates the neuronal differentiation gene *Dbh* (Kim, Hong, LeDoux, & Kim, 2001; Morrison, Zimmerman, Look, & Stewart, 2016). Additionally, *Sox10* directly activates the master regulator of melanocyte development, *Mitf* (Elworthy, Lister, Carney, Raible, & Kelsh, 2003). *Mitf*, along with *Sox10* and *Tfap2* factors, directly stimulates the expression of the melanin synthesizing enzymes *Dct* and *Tyr* (Figure 2D, Melanocytes) (Potterf et al., 2001; Seberg et al., 2017).

Much like in the case of neural crest induction, the differentiation GRN modules are highly dependent upon extracellular signaling systems. While chondrocyte differentiation requires TGF- β signaling (Furumatsu et al., 2005) autonomic neurons are reliant on cues from the BMP pathway (Morikawa et al., 2009). Finally, melanocyte differentiation is governed by Wnt signaling inputs (Jin, Erickson, Takada, & Burrus, 2001; Takeda et al., 2000)(Jin, Erickson, Takada, & Burrus, 2001; Takeda et al., 2000). These environmental inputs result in the activation of effector genes that impact genetic sub-circuits, changing the transcriptional output of the cells (Simoes-Costa & Bronner, 2015). Thus, neural crest cells that have migrated to different regions of the body are subjected to different spatial signaling cues, which ultimately dictate their terminal fate.

Gene regulatory logic in neural crest development

Thus far, we have delineated the genetic interactions that characterize each module of the neural crest GRN. The large number of connections between the genes results in a complex network that may seem impenetrable at first (Figure 2). However, careful analysis of the architecture of development GRNs has revealed that network modules are composed of functional units called *sub-circuits* (Table 1) (Peter & Davidson, 2015). Sub-circuits are groups of genes wired following well-defined topologies that are deployed to perform distinct tasks during development. These tasks may include the activation of entire GRN modules, repression of an opposing regulatory state or refinement of spatial expression domains (Groves & LaBonne, 2014; Laslo et al., 2006; E. Li, Materna, & Davidson, 2012). Sub-circuits are able to exert these functions because they contain logic that allows for processing of gene regulatory information. In this section, we will highlight different types of logic encoded within the neural crest GRN and exemplify how regulatory sub-circuits may control changes in gene expression and cell identity.

One of the most common regulatory sub-circuits in developmental GRNs is the *coherent feed-forward loop*. In this type of sub-circuit, one network node activates another, and they are both required for the activation of other downstream targets. Feed-forward loops are required to generate the stepwise activation of gene expression observed in the process of cell fate commitment. In neural crest development, this can be observed in the action of *Tfap2a* during neural crest specification (Figure 2A, NPB). *Tfap2a* is part of a coherent feed-

forward sub-circuit that activates *Zic1*, *Pax7*, and finally *Snai1/2* (Figure 3A) (de Croze et al., 2011; W. Li & Cornell, 2007; Nikitina et al., 2008). The way these genes are connected ensures the orderly activation of network components, with transcription of *Snai1/2* only beginning after the three factors are already present in neural crest progenitors. This is because feed-forward loops often contain “AND logic” (in this example *Tfap2a*, *Zic1* AND *Pax7* are all required for *Snai1/2* expression) (Istrail & Davidson, 2005). These types of feed-forward loops also allow transcriptional regulators to be reiteratively used during development, since they establish the requirement of specific combinations of transcription factors for the activation of downstream genes. Indeed, *Tfap2a* acts during different moments of neural crest specification, first by cooperating with *Zic1* and *Pax7*, and subsequently participating in the regulation of *Sox10* with *Ets1* and *FoxD3* (Figure 3A) (de Croze et al., 2011).

Sub-circuits are also required for the establishment of discrete spatial domains of gene expression. One example of this can be observed in the action of *cross-repressive sub-circuits*, which are composed of nodes that directly or indirectly inhibit each other (Peter & Davidson, 2015). This mutual inhibition prevents circuit components from being co-expressed by the same cells, promoting the establishment of spatially segregated regulatory states. This allows the neural crest to become transcriptionally distinct from the neighboring neural plate and non-neural ectoderm (Figure 1A). A mutual cross-repression circuit that aids in this task is centered on the transcription factor *Pax7*, which acts to promote neural crest identity by activating multiple specification genes (Figure 3B). In neural crest progenitors, this transcription factor also inhibits neural identity by repressing the expression of *Sox2* (Feledy et al., 1999; Pieper, Ahrens, Rink, Peter, & Schlosser, 2012; Roellig et al., 2017). Conversely, *Sox2* from the neural plate represses *Snai2*, a neural crest specifier gene downstream of *Pax7* (Wakamatsu, Endo, Osumi, & Weston, 2004). In the adjacent pre-placodal region, the transcription factors *Eya1/2* and *Six1/4* promote the placodal fate while repressing neural crest identity via inhibition of *Pax7* (Brugmann, Pandur, Kenyon, Pignoni, & Moody, 2004). The combination of these cross-repressive interactions forms a stable switch in which three spatially separated domains and the corresponding cellular identities are reinforced (Figure 3B).

Another example of a regulatory sub-circuit that is abundant in the neural crest GRN are *positive feedback loops*. Positive feedback logic results from a gene directly or indirectly activating itself, stabilizing the regulatory state and mitigating upstream signaling variation (Peter & Davidson, 2015). As neural crest cells migrate, they maintain their regulatory identity despite being exposed to a myriad of extracellular signals and interacting with multiple cells. One factor that is robustly expressed in migratory neural crest is *Sox10*, and its continuous expression is important to maintain these cells in a multipotent state (Wahlbuhl, Reiprich, Vogl, Bosl, & Wegner, 2012). This gene forms a positive feedback loop by directly regulating its own expression while simultaneously receiving input from multiple specification genes (Figure 3C) (Honoré, Aybar, & Mayor, 2003; Mead et al., 2013). This positive feedback circuit results in the strong and stable expression of *Sox10* beginning early in migration, which is maintained until differentiation.

Above we described three examples of GRN sub-circuits that employ regulatory logic to perform well-defined tasks during the formation and migration of neural crest cells. Similar topologies have been described in multiple developmental GRNs, which support the idea that sub-circuits follow conserved principles in despite differences in cell type and transcriptional regulators (Peter & Davidson, 2015, 2017). Thus, an important premise of the theoretical framework of developmental GRNs is that the arrangement of nodes in the network encodes the logic of developmental processes.

The evolution of the neural crest GRN

Neural crest cells are thought to have played a central role in chordate evolution, as they give rise to many of the defining features of vertebrates, including the frontal skull. Moreover, teeth and jaws are neural crest derivatives, and it is postulated that the emergence of this cell type may have allowed vertebrates to engage in predatory behavior (Gans & Northcutt, 1983). According to the “New Head” hypothesis this resulted in an increase of body size and allowed these animals to explore and occupy new environments. Thus, understanding the phylogeny of the neural crest GRN is an important question in evolutionary biology. To address this, comparative studies have examined the expression and function of neural crest genes in multiple chordates species. For instance, a comprehensive characterization of the neural crest GRN in the lamprey has revealed a deep conservation of this regulatory program in basal vertebrates (Sauka-Spengler, Meulemans, Jones, & Bronner-Fraser, 2007). Furthermore, studies focused on invertebrate chordates identified cell types that may be evolutionarily linked to the neural crest.

Though the neural crest has largely been deemed a “vertebrate-specific” evolutionary novelty, evidence from several non-vertebrate chordate species has revealed the existence of neural crest-like cells, which are likely precursors of this cell type. For example, the ascidian *Ciona intestinalis* belongs to the subphylum Tunicata, the sister clade to vertebrates (Delsuc, Brinkmann, Chourrout, & Philippe, 2006). This species possesses multiple migratory cell types which are positioned at the neural plate border and give rise to neuronal and melanocytic derivatives (Abitua, Wagner, Navarrete, & Levine, 2012; Stolfi, Ryan, Meinertzhagen, & Christiaen, 2015). Notably, these cells express key transcription factors contained within the neural crest GRN, including *Foxd3*, *Msx*, *Pax3/7*, and *Snail*. Furthermore, these factors participate in conserved regulatory circuitry, such as mutual repression by neural crest and placodal progenitors (Horie et al., 2018). This data suggests that the neural crest GRN arose in part from the co-option of ancestral regulatory circuits.

This idea is further supported by work conducted in the cephalochordate, amphioxus (*Branchiostoma floridae*). A survey of neural crest gene expression patterns in amphioxus development revealed many factors, including those involved in neural plate border induction, neural crest specification, and melanocyte differentiation, are conserved (J. K. Yu, Meulemans, McKeown, & Bronner-Fraser, 2008). Even so, many *bona fide* neural crest genes display divergent expression patterns in amphioxus, suggesting these genes may have acquired new roles upon their co-option into the neural crest GRN. Furthermore, early during vertebrate evolution many of these genes underwent duplication events (Tai et al., 2016; J. K. Yu et al., 2008). This likely led to neofunctionalization of key regulatory factors

that were important for the establishment of the vertebrate clade. Thus, the regulatory circuitry present in non-vertebrate chordates likely served as the blueprint for the emergence of the *bona fide* neural crest, although the ancestral cell type may not have displayed some of the defining features of this cell type, namely its characteristic multipotency and migratory capacity.

Perspectives

While the neural crest GRN summarizes decades of studies from multiple laboratories, it is still a work in progress and contains significant limitations. First, the current version of the GRN compiles data obtained from multiple model organisms and likely overestimates the degree of evolutionary conservation of genes within modules of the network (Simoes-Costa & Bronner, 2013). Second, the majority of the experiments that allowed for circuitry building have been performed in the cranial neural crest, and other axial subpopulations have not been as extensively studied (Rothstein et al., 2018). Finally, the majority of the GRN was built utilizing a candidate-gene approach, and it has yet to be tested in an unbiased manner. Current genomic approaches can address these issues by providing tools that allow for unprecedented analysis of gene expression and identification of gene regulatory interactions.

The identification of GRN components has greatly benefitted from high-throughput screens, which have identified genes that are critical for neural crest development. A pioneering study by Gammill and Bronner-Fraser performed a macroarray screen that identified several new genes enriched neural crest cells (Gammill & Bronner-Fraser, 2002). Subsequently, sequencing (RNA-Seq) of purified cell populations allowed for the identification of hundreds of novel factors expressed in this cell population (Simoes-Costa & Bronner, 2016; Simoes-Costa, Tan-Cabugao, Antoshechkin, Sauka-Spengler, & Bronner, 2014). RNA-seq has also been employed to examine the transcriptional profile of neural crest cells from distinct axial subpopulations, which has allowed for the assembly of axial-specific regulatory circuits (Murko, Viecele, & Bronner, 2018; Simoes-Costa & Bronner, 2016; Tani-Matsuhana, Viecele, Gandhi, Inoue, & Bronner, 2018). However, the function of many of the neural crest factors identified via transcriptomics remains untested, and thus further functional and cis-regulatory analysis will be necessary to place them within the GRN.

While gene expression data is useful for identifying novel network components, it is not sufficient for the assembly of regulatory modules and sub-circuits. How can regulatory interactions be predicted in a high-throughput manner? Understanding the cis-regulatory elements that control individual nodes is crucial for identifying network links. One approach to identify these elements is to assay accessible chromatin using ATAC-Seq (Buenrostro, Wu, Chang, & Greenleaf, 2015). By performing this assay in different cell types or over time, clustering approaches can reveal cell type specific or dynamic groups of regulatory regions. These locations can then be further interrogated to identify enriched transcription factor binding motifs, linking downstream targets to their upstream regulators. To further refine direct regulation of a single transcription factor, it is possible to profile genomic occupancy using methods such as Chip-Seq or CUT&RUN (Johnson, Mortazavi, Myers, & Wold, 2007; Skene & Henikoff, 2017). Therefore, unbiased genomic methods can be used to identify putative links between GRN nodes. These links can then be validated *in vivo* by

perturbation (CRISPR/Cas9, morpholino, or dominant negative proteins) followed by a transcriptional readout (qRT-PCR, RNA-Seq, or *in situ* hybridization).

Recently, two studies in zebrafish have employed genomic methods on isolated neural crest populations at multiple timepoints in an effort to better characterize players within the neural crest GRN, focused upon the key nodes *Tfap2a* and *FoxD3* (Dooley et al., 2019; Lukoseviciute et al., 2018). Dooley *et al.* performed RNA-Seq on isolated migratory neural crest cells and identified around 5,000 neural crest-enriched genes. This study also employed transcriptomic analysis in mutant fish to identify genes downstream of *Tfap2a/c*. Lukoseviciute *et al.* performed RNA-Seq on isolated neural crest at multiple timepoints and also performed ATAC-Seq and Biotin-ChIP-Seq to profile the complicated role of *FoxD3* in neural crest development. Through a series of genomic experiments, Lukoseviciute *et al.* were able to demonstrate that *FoxD3* acts in a bimodal fashion as a pioneer factor during neural crest induction and a repressor of premature neural crest differentiation by modulating the epigenetic landscape of defined regulatory regions. These studies are useful examples of how the combination of functional analysis and gene expression profiling can identify a large number of putative interactions between components of the neural crest GRN.

In the last few years, many studies have employed RNA-seq at the single-cell level to examine cellular heterogeneity in embryos, tissues and organs (Farrell et al., 2018; Wagner et al., 2018). This technology will surely impact the way that GRNs are assembled and tested. Analysis of this data using dimensionality reduction and clustering, followed by trajectory-learning algorithms, can generate extensive lineage maps which may inform upon GRN architecture and cell fate. Ordering cells using computational methods such as pseudotime approaches that can reveal key drivers of transitions between regulatory modules (Qiu et al., 2017). Soldatov and colleagues performed single-cell RNA-Seq (scRNA-Seq) on both cranial and trunk murine neural crest cells isolated from three time points across development, revealing a large amount of heterogeneity (Soldatov et al., 2019). By combining these datasets, the authors were able to identify co-expression of both autonomic and sensory neuron signatures in early migrating trunk neural crest cells. In later migrating cells, they observed co-expression of autonomic and mesenchymal signatures. This single-cell analysis led them to propose a fate tree in which neural crest cells displayed diverging transcriptional signatures that progressed from multipotent progenitors into more restricted autonomic-mesenchymal progenitors and finally fate-restricted mesenchymal progenitors. To validate the proposed bifurcation of fates, they utilized lineage tracing of *Phox2b* in mice, an autonomic neuron transcription factor identified to be active after the bifurcation between autonomic and sensory fates. They observed contribution of *Phox2b*-lineage traced cells to both autonomic and mesenchymal fates, but never sensory, indicating bipotential restriction of these cells. Furthermore, Soldatov and colleagues utilized lineage tracing of the mesenchymal-specific transcription factor *Prrx1* to show that, while *Phox2b*⁺ bipotent precursors can generate both autonomic and mesenchymal cells, *Prrx1*-lineage traced cells are exclusively restricted to the mesenchymal lineage, demonstrating progressive restriction of neural crest through sensory, autonomic, and finally mesenchymal states. It remains to be demonstrated that there is a fate restriction between sensory and autonomic/mesenchymal progenitors and if a similar fate restriction is conserved in other species. Additional studies

of neural crest transcription using scRNA-Seq, regulation using scATAC-Seq, and lineage using other single-cell technologies will help clarify how individual neural crest integrate environmental cues in different GRN modules to drive cell fate commitment and the characteristics of restricted progenitor cells.

Genomic methods generate huge amounts of data, and even if this data can be used to build circuits, the integration of all of this information into a comprehensive GRN presents another challenge. Currently, most GRNs are displayed in a topological map (Figure 2). The most popular tool to generate the topological maps is BioTapestry, initiated by the Davidson lab at Caltech (Longabaugh, Davidson, & Bolouri, 2005). This tool allows for organization and visualization of networks by input of nodes and their activating or repressing interactions with other nodes. Additional features include the ability to provide additional perturbation and temporal information and automatic reorganization as new nodes are added to the network. BioTapestry has been updated with significant improvements to the visualization and use through a web-based viewer, which is better able to display dynamic and large networks (Paquette, Leinonen, & Longabaugh, 2016). Future improvements may be necessary to allow for the visualization of complex, multi-dimensional GRNs assembled from genomic analyses.

Conclusions

The framework of developmental gene regulatory networks has allowed researchers to extract logic from molecular programs of remarkable complexity. In a cell population such as the neural crest, assembly of a GRN has shed light into cell fate specification, the regulation of EMT and migration, and the cell state changes that take place during differentiation. Studies of the evolutionary process have also benefitted from this framework since it allows detailed comparisons between the molecular networks operating in different organisms. GRNs are particularly useful at a time when there is an abundance of genomic data. As we grasp with the rapidly increasing amount of information extracted from cellular processes, GRNs provide a practical system to compile, organize and present the molecular programs that control embryonic development.

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Figure 1. Stages of cranial neural crest development in the chicken embryo.

A. During gastrulation (Hamburger and Hamilton stage 5, HH5), the avian ectoderm is divided in three spatial domains: the neural plate, the non-neural ectoderm (NNE) and the neural plate border (NPB). The neural crest progenitor cells reside within the NPB, which elevate during the closure of the neural tube. **B.** During neurulation (HH8), the neural crest cells are already specified and are positioned at the dorsal aspect of the neural tube. **C.** At HH9-10, cranial neural crest cells delaminate from the neural tube and begin migration along the dorsoventral pathway.

Figure 2. A gene regulatory network controls cranial neural crest development.

This diagram depicts a simplified version of the cranial neural crest GRN across vertebrates. Gray boxes denote different regulatory modules. Nodes are represented as horizontal arrows, which are linked together by direct (solid) or indirect (dashed) regulatory links that are activating (ending in arrows) or repressive (ending in a T). **A.** The neural plate border (NPB) is induced by signaling systems such as Wnts and BMPs. Adjacent neural and pre-placodal region (PPR) cells express a distinct set of transcription factors, some of which form cross-repressive circuitry with neural crest modules. Nodes within the NPB module activate the

Specification module (**B**), which subsequently activates the genes that are part of the Migration module (**C**) of the network. **D**. Different signals (TGF- β , BMP, and Wnt) influence differentiation of neural crest cells into chondrocytes, sympathetic neurons, and melanocytes.

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Figure 3. Regulatory sub-circuits within the neural crest GRN.

A. *Tfap2a*, a key node within both the neural plate border (NPB) and neural crest specification modules, uses a coherent feed-forward mechanism to activate other neural crest-specific transcription factors **B.** A mutual cross-repression circuit within the NPB. In this circuit, *Pax7* promotes neural crest fate by activating *Snai1/2* and repressing *Sox2/3*. In adjacent neural tissue, *Sox2/3* represses *Snai1/2*. Similarly, pre-placodal tissue express *Six1/4* and *Eya1/2*, which repress *Pax7*. This promotes the spatial segregation of these domains. **C.** *Sox10* integrates many neural crest specification genes and utilizes a positive feedback loop to maintain neural crest identity during migration.

Table 1.
Definitions of GRN Elements.

This table describes the terminology employed to describe developmental GRNs.

Network: A gene regulatory network defines the transcriptional relationships within a cell type across developmental time. Networks progress by sequential activation of modules.
Regulatory State: A regulatory state is a group of nodes that are active in a cell at a particular time point.
Module: Modules describe the complete transcriptional identity of a cell at discrete developmental stages during embryogenesis. Modules contain multiple sub-circuits that describe distinct active processes.
Node: A single factor (transcription factor, signaling effector, noncoding RNA, or epigenetic remodeler) that receives input from other nodes or environmental signaling and acts to activate or repress another node within the network.
Regulatory link: A regulatory link represents an interaction (activating or repressing) between nodes and can be direct (with evidence of cis-regulatory binding) or indirect (acting through other genes).
Sub-Circuit: A sub-circuit consists of a combination of interlinked nodes that are responsible to execute a specific developmental function.