Assembling Neural Crest Regulatory Circuits into a Gene Regulatory Network

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Key Words

stem cell, transcription factors, cell migration, *cis*-regulation

Abstract

The neural crest is a multipotent stem cell–like population that gives rise to a wide range of derivatives in the vertebrate embryo including elements of the craniofacial skeleton and peripheral nervous system as well as melanocytes. The neural crest forms in a series of regulatory steps that include induction and specification of the prospective neural crest territory–neural plate border, specification of bona fide neural crest progenitors, and differentiation into diverse derivatives. These individual processes during neural crest ontogeny are controlled by regulatory circuits that can be assembled into a hierarchical gene regulatory network (GRN). Here we present an overview of the GRN that orchestrates the formation of cranial neural crest cells. Formulation of this network relies on information largely inferred from gene perturbation studies performed in several vertebrate model organisms. Our representation of the cranial neural crest GRN also includes information about direct regulatory interactions obtained from the *cis*-regulatory analyses performed to date, which increases the resolution of the architectural circuitry within the network.

Contents

INTRODUCTION

The neural crest, often referred to as the "fourth germ layer" (Hall 2000), is a multipotent stem cell–like population of highly migratory cells that contribute derivatives to a wide variety of tissues and organs in the vertebrate embryo. These include but are not limited to the sensory and autonomic ganglia, adrenal and thyroid glands, smooth muscle of major blood vessels, cartilage and bone of the face, and pigmentation of the skin. As a defining feature of vertebrates, neural crest formation has been extensively studied using vertebrate model organisms ranging from lampreys and fish to frog, chick, and mouse.

Neural crest cells form over a lengthy period of time during development that starts at gastrulation and extends into late organogenesis. This process is initiated by a combination of inductive signals emanating from environing tissues, such as the underlying mesoderm or adjacent neural and non-neural ectoderm, which set up the presumptive neural crest region. As a result, the territory between neural and non-neural ectoderm, termed the neural plate border, is competent to respond to signals specifying bona fide neural crest progenitors. These cells subsequently undergo an epithelial-to-mesenchymal transition (EMT), delaminate from the neuroepithelium, and migrate along stereotypical pathways. After settling in various and sometimes distant sites in the embryo, they differentiate into a multitude of derivatives.

For more than a century, the neural crest has provided a productive paradigm for addressing essential questions regarding cell interactions that underlie induction, specification, and differentiation events during development. As such, the neural crest is the subject of an extensive literature and descriptive database that, in combination with recent genomic *cis*-regulatory and gene knockdown data, provide a critical mass of information regarding the molecular underpinnings that guide neural crest formation. Such a compelling database calls for a systematic approach to integrate diverse information into a multistep gene regulatory network (GRN) that describes the process of neural crest formation.

The accrual of molecular information relevant to neural crest induction, specification, and migration has led to the formulation of a putative vertebrate GRN that orchestrates neural crest formation (Meulemans & Bronner-Fraser 2004, Sauka-Spengler & Bronner-Fraser 2008a, Steventon et al. 2005). Because of variation between species, the main challenge has been to incorporate the pertinent data, obtained from many vertebrate developmental models, into a single, pan-vertebrate network. In addition to discrepancies in the patterns of gene

expression and differences in the deployment of paralogous genes among various vertebrates (Meulemans & Bronner-Fraser 2004), there are also remarkable differences between populations of neural crest cells originating from different axial levels within a given species. These include differences in mechanisms of delamination and developmental potential, such as the ability to generate skeletal structures (Graham et al. 2004). For example, although both cranial and trunk crest cells can generate the full repertoire of neural crest cell derivatives (McGonnell & Graham 2002), the skeletogenic potential of trunk crest cells is suppressed during normal development (Graham et al. 2004). Thus, different neural crest cell populations may well be exposed to at least a subset of unique regulatory interactions.

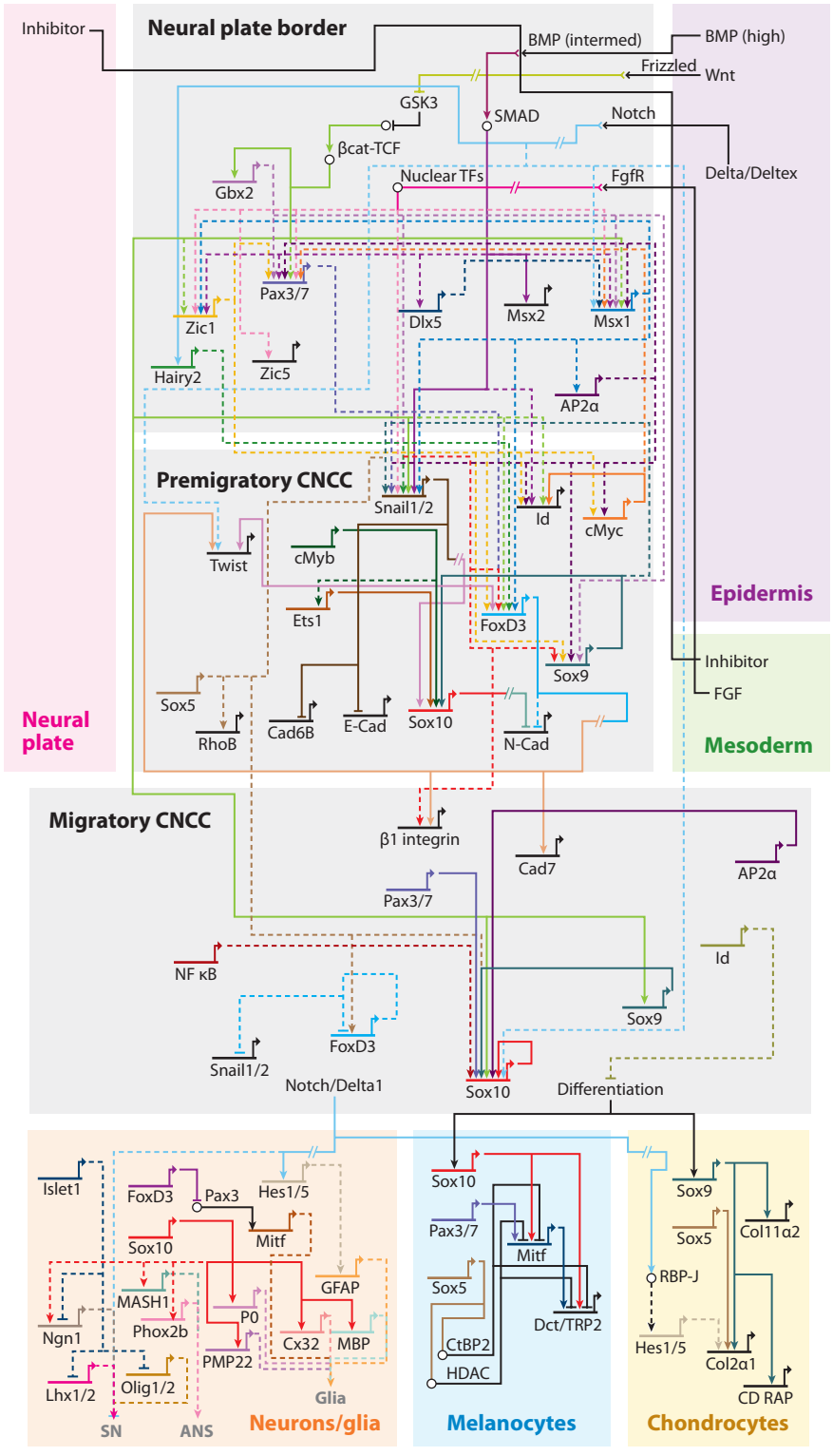
Finally, only a few *cis*-regulatory studies of neural crest genes have been reported thus far, which has made it difficult to discern direct regulatory interactions. Most known direct regulatory interactions have been elucidated in differentiating neural crest derivatives (Sauka-Spengler & Bronner-Fraser 2008a). Thus, the current formulation of the neural crest GRN is largely a consolidation of regulatory predictions. Nevertheless, many regulatory steps appear to be highly conserved even in basal vertebrate systems (Sauka-Spengler et al. 2007), which suggests that it should be possible to assemble a scaffold of regulatory interactions that may be common to all vertebrates and may function on all axial levels.

In this review, we attempt to integrate the most current neural crest regulatory information to generate an updated representation of the neural crest GRN. We present possible circuit connections inferred largely from loss-of-function analysis together with direct regulatory interactions, thus far documented mostly at later stages of differentiation. The goal is to build a model in which each link can be tested in several species. We also attempt to take into account separate spatial subpopulations of neural crest cells at different levels of the neural axis. As a starting point, we will focus on the regulatory state of cranial neural crest cells (**Figures 1** and **2**; **Table 1**), which are the first crest population to form and initiate migration in the vertebrate embryo. These cells contribute derivatives mainly to the facial skeleton, peripheral nervous system, and pigmentation in the head.

We present this updated neural crest GRN, created using the generic drawing software BioTapestry (**http://www.biotapestry.org/**), which employs symbolic representation of genes to describe their regulatory interactions and to integrate experimentally derived network features (**Figure 1**; Longabaugh et al. 2009). Most data in the neural crest GRN relates to cells forming at cranial levels.

INITIAL SIGNALING INPUTS INTO THE NEURAL CREST GENE REGULATORY NETWORK: BONE MORPHOGENETIC PROTEIN, WNT, FIBROBLAST GROWTH FACTOR, AND NOTCH PATHWAYS IN INDUCTION AND SPECIFICATION

The classical view suggested that neural crest cell induction occurred during the process of neurulation, as the neural folds elevated. This was thought to occur as a consequence of interactions resulting from the juxtaposition of the epidermis and the elevating neural plate (Mancilla & Mayor 1996, Selleck & Bronner-Fraser 1995). However, recent findings in frog (Monsoro-Burq et al. 2005) and chick demonstrate that neural crest induction is underway much earlier, during gastrulation (Basch et al. 2006). In chick, for instance, the transcription factor Pax7 is expressed in the neural plate border domain, where neural crest cells originate, in the mid-gastrula as early as stage HH4+. When tissue explants from this Pax7-positive domain of the gastrula were cultured in the absence of exogenous inductive signals, they were able to generate neural crest cells (Basch et al. 2006) despite the lack of added factors or other tissue interactions. Recent fate map studies show that the neural plate border region is wider and overlaps partially with the bone morphogenetic protein (BMP) 4-expressing



ABBREVIATIONS:

ANS	Autonomic nervous system
AP2α	Activating protein 2α
βcat	A subunit of the cadherin protein complex and integral component of the Wnt signaling pathway
BMP	Bone morphogenetic protein
Cad	Cadherin
CD RAP	Cartilage-derived retinoic acid-sensitive protein
cMyb	Cellular myeloblastosis oncogene
cMyc	Cellular myelocytomatosis oncogene
Col2α1	Collagen type II α1
Col11α2	Collagen type XI α2
CtBP2	C-terminal binding protein 2
Cx32	Connexin 32, gap junction β-1 protein
Dct/TRP2	Dopachrome tautomerase, tyrosine-related protein 2
Dlx5	Distal-less homeobox 5
Ets1	Erythroblastosis virus E26 oncogene homolog 1
FGF	Fibroblast growth factor
FgfR	Fibroblast growth factor receptor
FoxD3	Forkhead box D3
Gbx2	Gastrulation brain homeobox 2
GFAP	Glial fibrillary acidic protein
GSK3	Glycogen synthase kinase 3
Hairy2	Vertebrate homologue of *Drosophila* pair-rule gene hairy and downstream effector of Notch signaling
HDAC	Histone deacetylase
Hes1/5	Hairy and enhancer of split 1 or 5
Id	Inhibitor of DNA-binding/ differentiation
Lhx1/2	Lim homeobox 1 or 2
MASH1	Mammalian achate schute homolog 1
MBP	Myelin basic protein
Mitf	Microphthalmia-associated transcription factor
Msx	Muscle segment homeobox
N-Cad	Neural cadherin
NF κB	Nuclear factor κB
Ngn1	Neurogenin 1
Olig1/2	Oligodendrocyte transcription factor 1 or 2
Pax3/7	Paired box 3 or 7
Phox2b	Paired-like homeobox 2b
PMP22	Peripheral myelin protein 22
P0	Myelin protein zero
RBP-J	Recombining binding protein suppressor of hairless, a key mediator of Notch signaling
RhoB	Ras homolog gene family member B
SMAD	Sma- and Mad-related proteins, which modulate the activity of transforming growth factor β ligands
SN	Sensory neurons
Snail1/2	Vertebrate homologs of *Drosophila* snail gene
Sox	SRY (sex determining region Y)-box
TCF1	Transcription factor 1, a T-cell–specific transcription factor that mediates Wingless/Wnt signaling
TF	Transcription factor
Twist	Vertebrate homolog of *Drosophila* twist gene
Wnt	Wingless type protein
Zic	Zinc-finger protein of the cerebellum

domain during gastrula stages (Ezin et al. 2009), which is consistent with the possibility that signaling cues are already in play at this place and time.

Evidence of early specification of the neural plate border in frog and chick has been substantiated by studies in lamprey, where these events are conserved but occur at a much slower rate, which makes lamprey a suitable system for studying signaling inputs and neural plate border specifier readout with much better temporal resolution and therefore in much higher detail (Nikitina et al. 2008). Interestingly, the induction program and resulting expression of transcription factors specifying the neural plate border are shared by nonvertebrate chordates that do not possess a neural crest (Meulemans & Bronner-Fraser 2004, Sauka-Spengler & Bronner-Fraser 2008b). Thus, all evidence suggests that neural crest cell induction in vertebrate embryos occurs during gastrulation. However, the early inductive events remain unexplored in some species, such as the mouse, which highlights the importance of performing comparative analysis in numerous vertebrates.

The induction of the prospective neural crest within the neural plate border is thought to occur in response to signaling molecules emanating from adjacent tissues. The response that sets future neural crest cells apart from other border cells requires the activation of a battery of transcription factors, which imbues them with multipotency, the characteristics of proliferating cells, and the competence to respond to later neural crest–specifying signals. Identifying the signaling inputs that initiate neural crest induction has been challenging because information obtained from different vertebrate systems is sometimes contradictory. Fate map studies suggest that presumptive neural crest cells are in proximity to three different regions: presumptive epidermis, neural plate, and mesoderm. These tissues are thought to secrete signaling ligands including BMPs, Wingless-type proteins (Wnts), and fibroblast growth factors (FGFs) that have all been demonstrated as essential for the early induction, maintenance, and differentiation of neural crest cells (Knecht & Bronner-Fraser 2002). Although there are differences between neural crest populations at various levels of the neural axis, the inductive signals appear similar regardless of axial level.

Bone Morphogenetic Proteins

In frog embryos, high levels of BMP have been shown to be necessary for the acquisition of epidermal fate, whereas inhibition of BMPs is required for neural induction (LaBonne & Bronner-Fraser 1998). The neural plate border territory that lies between non-neural ectoderm (future epidermis) and neural ectoderm contains neural crest precursors, preplacodal ectoderm, dorsal neural tube, and epidermis, all of which are exposed to BMP signals. In chick explant culture experiments, juxtaposition of non-neural ectoderm and intermediate neural plate tissue, which normally forms only neural tube, can generate neural crest cells. Addition of BMP4 and BMP7, which are endogenously expressed in the non-neural ectoderm, is able to substitute for non-neural ectoderm such that neural crest cells are induced from intermediate neural plate explants (Liem et al. 1995).

Figure 1

A gene regulatory network (GRN) model (a view from all nuclei) that maps vertebrate hierarchical gene regulatory interactions during cranial neural crest cell (CNCC) development. The model is built using the BioTapestry software (Longabaugh et al. 2009). The GRN is partitioned into subnetworks that regroup regulatory interactions during induction and specification at the neural plate border, in premigratory and migrating neural crest cells, and in differentiating neural crest derivatives. Most of the linkages in the GRN model are inferred from available gene perturbation data from frog, chick, mouse, zebrafish, and lamprey. Direct regulatory interactions, based on promoter and *cis*-regulatory analysis, are indicated with solid lines. Dashed lines show potential direct regulatory interactions inferred from gene perturbation studies. Broken lines represent potential indirect interactions. Bubble nodes indicate protein-protein interactions. Transcriptional orientation was not taken into consideration because it varies among different vertebrate models.

Figure 2

A model of the GRN underlying CNCC formation that emphasizes only regulatory circuits with experimentally validated direct regulatory interactions.

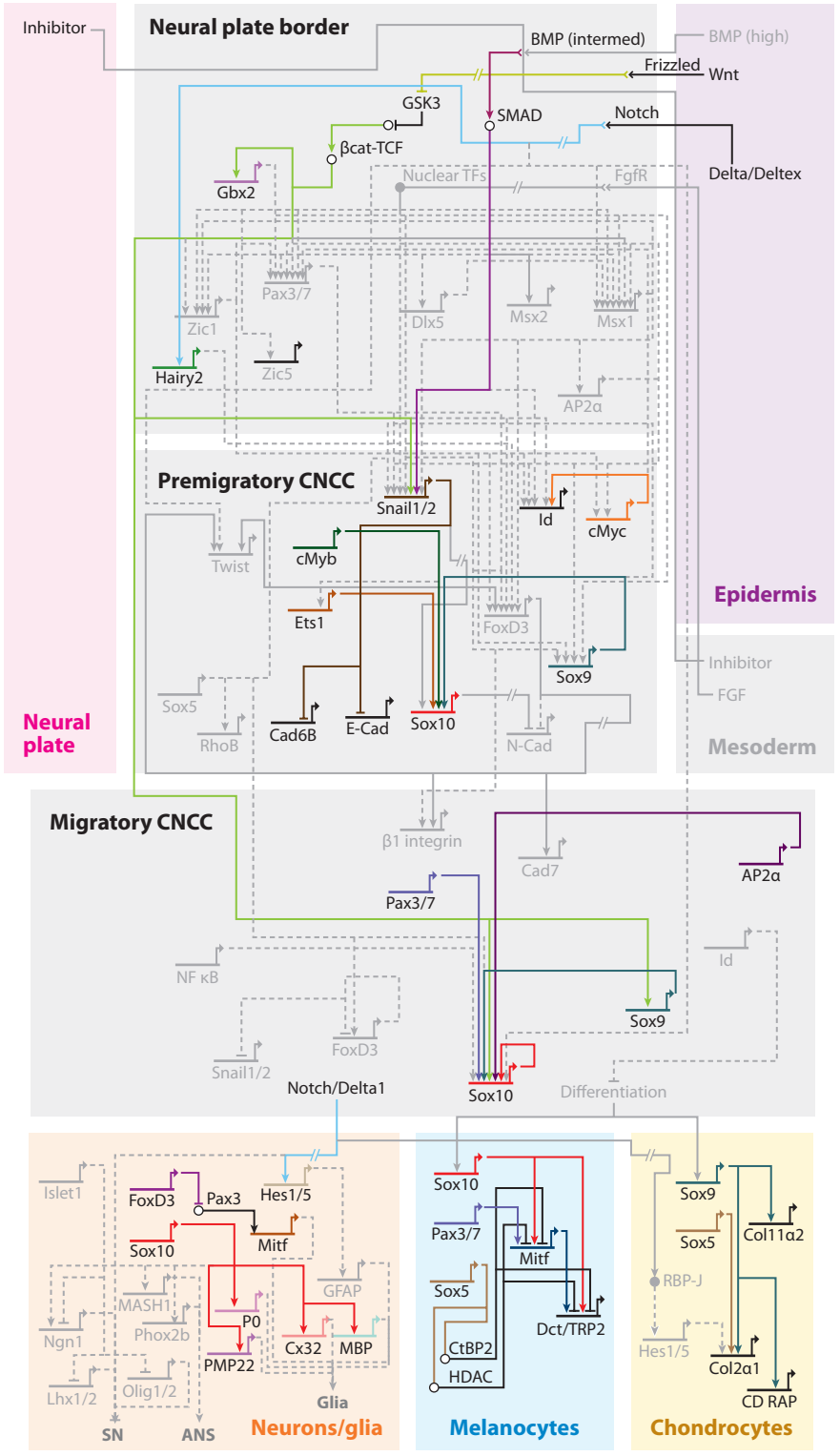


Table 1 Evidence for gene regulatory interactions in cranial neural crest cells

Source	Interaction	Target	System	Evidence
BMP	Promotes*	Msx2	Mouse	Brugger et al. (2004)
BMP	Promotes	Dlx5	*Xenopus*	Luo et al. (2001)
BMP, FGF, Wnt, Notch, Gbx2, Dlx5, AP2α, Myc	Promotes	Msx1	*Xenopus*, lamprey	Monsoro-Burq et al. (2005), Tribulo et al. (2003), Li et al. (2009), Woda et al. (2003), Nikitina et al. (2008)
BMP, Wnt, FGF, Gbx2, AP2α, Zic, Myc	Promotes	Pax3/7	*Xenopus*, lamprey	Sato et al. (2005), Hong & Saint-Jeannet (2007), Monsoro-Burq et al. (2005), Li et al. (2009), Nikitina et al. (2008)
BMP, Wnt, FGF, Msx	Promotes	Zic1	*Xenopus*, lamprey	Sato et al. (2005), Hong & Saint-Jeannet (2007), Nikitina et al. (2008)
FGF	Promotes	Zic5	*Xenopus*	Monsoro-Burq et al. (2003)
Wnt	Promotes*	Gbx2	*Xenopus*	Li et al. (2009)
Notch	Promotes*	Hairy2	*Xenopus*	Glavic et al. (2004)
Msx	Promotes	AP2α	Lamprey	Nikitina et al. (2008)
FGF, Hairy2, Zic1, Msx1, Pax3/7, Gbx2, AP2α, Sox9, Sox10, Sox5	Promotes	Snail1/2	*Xenopus*, chick	Mayor et al. (1997), Villanueva et al. (2002), Glavic et al. (2004), Sato et al. (2005), Tribulo et al. (2003), Meulemans & Bronner-Fraser (2004), Li et al. (2009), Spokony et al. (2002), Aoki et al. (2003), Honore et al. (2003), Perez-Alcala et al. (2004)
BMP, Wnt	Promotes*	Snail1/2	Mouse, *Xenopus*	Sakai et al. (2005), Vallin et al. (2001)
FoxD3	Represses	Snail1/2	Zebrafish	Lister et al. (2006)
Notch	Promotes	Twist	*Xenopus*	Coffman et al. (1993), Cornell & Eisen (2005)
Snail1/2, FoxD3	Promotes (Ind)	Twist	*Xenopus*	Aoki et al. (2003), Aybar et al. (2003), Sasai et al. (2001), Meulemans & Bronner-Fraser (2004)
cMyc	Promotes*	Id	*Xenopus*	Light et al. (2005)
BMP, Wnt, Zic, AP2α	Promotes	Id	*Xenopus*, lamprey	Kee & Bronner-Fraser (2005), Nikitina et al. (2008)
Zic, AP2α	Promotes	Myc	Lamprey	Nikitina et al. (2008)
Hairy2, Zic1, Pax3/7, Msx1, Sox10, Sox5	Promotes	FoxD3	*Xenopus*, chick	Wettstein et al. (1997), Sato et al. (2005), Tribulo et al. (2003), Honore et al. (2003), Perez-Alcala et al. (2004)
Snail1/2	Promotes (Ind)	FoxD3	*Xenopus*	Aoki et al. (2003), Aybar et al. (2003)
FoxD3	Represses	FoxD3	*Xenopus*	Pohl & Knochel (2001)
cMyb	Promotes	Ets1	Chick	P. Betancur, unpublished data
Wnt	Promotes*	Sox9	Mouse	Bagheri-Fam et al. (2006)
AP2α, Gbx2, Zic1, Sox10	Promotes	Sox9	*Xenopus*, mouse	Lee et al. (2004), Luo et al. (2003), Saint-Germain et al. (2004), Bagheri-Fam et al. (2006), Li et al. (2009), Honore et al. (2003)
Id	Represses	Sox9	*Xenopus*	Light et al. (2005)
cMyb, Ets1, Sox9, Wnt, Pax3/7, AP2α	Promotes*	Sox10	Chick, mouse, zebrafish	Betancur et al. (2010), Werner et al. (2007), Dutton et al. (2008)
Sox5, Notch, NFKappaB	Promotes	Sox10	Chick	Perez-Alcala et al. (2004), Dutton et al. (2008)
Snail1/2	Promotes (Ind)	Sox10	*Xenopus*	Aoki et al. (2003), Aybar et al. (2003)

(*Continued*)

Table 1 (*Continued*)

Source	Interaction	Target	System	Evidence
Id	Represses	Sox10	*Xenopus*	Light et al. (2005)
Snail1/2	Represses*	Cad6B, Ecad	Chick, mouse, and human cell lines	Taneyhill et al. (2007), Cano et al. (2000)
Sox10	Represses (Ind)	Ncad	Chick, mouse	Cheung et al. (2005)
FoxD3	Represses	Ncad	Chick, mouse	Cheung et al. (2005)
RhoB	Modulates	Ncad		Groysman et al. (2008)
FoxD3	Promotes (Ind)	Cad7	Chick, mouse	Cheung et al. (2005)
FoxD3	Promotes (Ind)	β1 Integrin	Chick, mouse	Cheung et al. (2005)
Sox10	Promotes	β1integrin	Chick, mouse	Cheung et al. (2005)
Sox5	Promotes	RhoB	Chick	Perez-Alcala et al. (2004)
Notch	Promotes*	Hes1/5	HeLa cell line	Jarriault et al. (1995)
Hes1/5	Promotes	GFAP, Col2α1	Mouse	Ijuin et al. (2008)
Sox10	Promotes*	Mitf	Cell lines	Verastegui et al. (2000)
Sox5	Modulates*	Mitf	Mouse	Stolt et al. (2008)
Sox10, Mitf	Promotes*	Dct/TRP2	Cell lines	Ludwig et al. (2004)
Sox5	Modulates*	Dct/TRP2	Mouse	Stolt et al. (2008)
Sox10	Promotes *	P0, Cx32, MBP, PMP22	Mouse, cell lines	Peirano et al. (2000), Bondurand et al. (2001)
Sox10	Promotes	Phox2B, MASH1, Ngn1	Rat cell culture, zebrafish	Kim et al. (2003), Carney et al. (2006)
Sox9, Sox5	Promotes*	Col2α1	Cell lines, mouse	Lefebvre et al. (1997), Hattori et al. (2008)
Sox9	Promotes*	Col11α2, CD RAP	Cell lines, mouse	Bridgewater et al. (1998), Xie et al. (1999)
Islet1	Represses	Ngn1, Lhx1/2, Olig1/2	Mouse	Sun et al. (2008)

*Direct regulatory interaction (data available). (Ind) Possible indirect interaction.

It has been proposed that intermediate levels of BMP, obtained as a result of diffusion of secreted BMP molecules throughout the ectoderm (BMP gradient), are responsible for the induction of neural crest cells. In support of the gradient model, zebrafish BMP pathway mutants show expansion of the neural crest cell domain if BMP levels are attenuated and reduction of this domain if BMP activity is abolished. (Knecht & Bronner-Fraser 2002, Nguyen et al. 1998). Alternatively, a gradient that would create the intermediate levels of BMP required for neural crest induction may be established by antagonistic interactions with Cerberus, noggin, chordin, and follistatins, ligands secreted by the forming neural plate cells (Sauka-Spengler & Bronner-Fraser 2008a, Tribulo et al. 2003, Wilson et al. 1997). Regardless of the way a BMP gradient is established, intermediate levels of BMP alone are not sufficient to induce expression of neural crest cell markers in frog or any other vertebrate model organisms (Garcia-Castro et al. 2002, LaBonne & Bronner-Fraser 1998, Wilson et al. 1997). BMP signaling is therefore an important initial step, but additional signals are required for induction of the neural crest.

Fibroblast Growth Factors

The FGF family of growth factors represents another set of signaling cues implicated in

neural crest induction. In *Xenopus* animal cap assays, FGF2 ligand, together with attenuated BMP signaling, upregulates expression of an early neural crest cell marker, *Snail2*, whereas overexpression of a dominant negative FGF receptor blocks *Snail2* without affecting neural plate markers (Mayor et al. 1997, Villanueva et al. 2002). In frog, overexpression of FGF8, normally expressed in the paraxial mesoderm, transiently induces neural crest cells (Monsoro-Burq et al. 2003). However, exogenous FGF8 alone is not sufficient to induce the full range of neural crest markers (Noden & Trainor 2005). Furthermore, the requirement for FGF signaling may vary between species, which makes it difficult to make definitive conclusions about its universality. For example, mouse null mutant embryos lacking either FGF or fibroblast growth factor receptor (FGFR) have no obvious defects in neural crest formation (Jones & Trainor 2005). This could be explained by functional redundancy of FGF signaling factors. Similarly, in zebrafish neural crest cells develop normally in the absence of mesoderm (Jones & Trainor 2005), and mutant embryos carrying mutations in FGF signaling components show no neural crest defects.

Wnt Signaling Pathway

Wnt family members are involved in many aspects of neural crest development. Numerous family members, e.g., Wnt6, Wnt7b, Wnt3a, Wnt1, and Wnt8, are expressed in the correct tissue and at the proper time to play a role in induction (Knecht & Bronner-Fraser 2002). Wnts are present in the paraxial mesoderm in frog (Christian et al. 1991, Knecht & Bronner-Fraser 2002) and in the non-neural ectoderm adjacent to the neural folds in chick embryos (Garcia-Castro et al. 2002). Gain- and loss-of-function experiments in frog, chick, and fish have shown that the activation of the Wnt pathway is essential for neural crest cell induction and specification (Garcia-Castro et al. 2002, LaBonne & Bronner-Fraser 1998, Lewis et al. 2004). For instance, in zebrafish, an inducible Wnt inhibitor activated during early neurulation specifically interferes with neural crest cell formation without altering the formation of neurons from the central nervous system (Lewis et al. 2004). In chick, ectodermal cells express Wnt6 at the time of neural crest cell induction, and exposing neural plate explants to Wnt6 induces the formation of neural crest cells in culture (Garcia-Castro et al. 2002, Schubert et al. 2002). However, the role of Wnt signaling in induction of the neural crest during gastrulation has yet to be examined in the mouse embryo. Although Wnt1/Wnt3a double mutants exhibit defects in a wide range of neural crest derivatives (cranial skeleton, cranial and even dorsal root ganglia, and melanocytes), it is not yet clear if this results from early induction defects, as the analysis of a mutant phenotype in the neural plate border has yet to be performed (Ikeya et al. 1997). All other gene perturbation experiments used as evidence to suggest a role for Wnt signaling in mouse are confined to lineage specification and neural crest cell differentiation rather than early induction. These studies have targeted the Wnt signaling pathway components in the dorsal neural tube (Jones & Trainor 2005); this represents a relatively late time point by which bona fide neural crest progenitors reside within the dorsal aspects of the neural folds/tube. Thus, it is too late to address the role of Wnt signaling in induction events, which normally take place during gastrulation. Thus it remains unclear if Wnt signaling pathways play an inductive role at early stages.

Wnt/β-catenin in emigrating neural crest cells clearly promotes formation of sensory neurons at the expense of all other derivatives (Lee et al. 2004). Finally, due to gene duplications and the particularly large number of Wnt ligands in the mouse genome, it is possible that Wnts act redundantly during neural crest cell development in mouse. Their early inductive role may have been missed in single Wnt knockouts, but the effects of simultaneous inactivation of several Wnts have not been examined to date (Jones & Trainor 2005).

Notch Signaling Pathway

Local cell-cell signals such as Notch/Delta are also found in the vicinity of and/or on developing neural crest cells (Endo et al. 2002, Glavic et al. 2004, Williams et al. 1995). In chick, *Notch* is confined to the neural folds together with *Hairy2*, its direct downstream effector, whereas *Delta* is expressed in the presumptive epidermis (Endo et al. 2002). It has been reported that Notch-Delta signaling acts upstream of BMP4 in chick and frog embryos and can affect expression of *Snail* and other genes, termed neural crest specifiers, that define regulatory state during neural crest specification proper (Endo et al. 2002, Glavic et al. 2004). However, the function of and requirement for Notch during neural crest cell development may vary among different vertebrates. In mouse, *Delta1* null mutants have no apparent early neural crest defects even though cranial neural crest cells express several *Notch* genes (De Bellard et al. 2002, Williams et al. 1995); a different ligand may activate Notch signaling in those cells. In zebrafish, mutants in Notch pathway components appear to affect the trunk but not the cranial neural crest (Cornell & Eisen 2005), which is consistent with the possibility that this signaling pathway plays more of a role in the trunk than the cranial crest, where there may be functional redundancy with other signaling pathways.

Despite some species-specific differences, it is generally agreed that a combination of inductive signals activates a battery of immediately downstream genes in the neural plate border that give the cells the capacity to become neural crest cells. For instance, the combination of low levels of BMP plus Wnt family members can induce expression of *Snail2* and other neural crest genes in frog explants (LaBonne & Bronner-Fraser 1998).

NEURAL PLATE BORDER SPECIFIERS

Signaling inputs into the neural plate border territory activate a battery of transcription factors whose collective expression sets presumptive neural crest cells apart from other border progenitors by conferring on them the competence to respond to neural crest–specifying signals. These genes, termed neural plate border specifiers, appear early during neurulation and include homeobox transcription factors Mxs1/2, Dlx5, Pax3/7, and Gbx2, as well as zinc finger–containing Zic proteins. Although little is known about the direct inputs that regulate their expression or about the regulatory interactions that occur among them, gain- and loss-of-function experiments suggest possible hierarchical interrelationships. Understanding their regulatory interrelationships helps expand links within the GRN, adds several testable hypotheses, and can serve as an experimental guide.

In frog, integration of inputs from the BMP, FGF, Wnt, and Notch signaling pathways activates expression of *Msx1* (Monsoro-Burq et al. 2005, Tribulo et al. 2003). *Zic1* and *Pax3* are also downstream of Wnt, BMP, and FGF signals (Sato et al. 2005), whereas FGF8 can experimentally induce *Zic5* expression but is not required to do so endogenously (Monsoro-Burq et al. 2003). Although BMP and FGF signals can regulate individual expression of *Zic1* and *Pax3*, both transcription factors need to be activated simultaneously to achieve neural crest specification. In frog embryos, high levels of either transcription factor alone (Pax3 or Zic1) promote alternative neural plate border fates (hatching gland or preplacodal progenitors, respectively) (Hong & Saint-Jeannet 2007). Furthermore, FGF8 and Wnt signals act in parallel at the neural plate border and seem to converge independently onto *Pax3* (Monsoro-Burq et al. 2005). Hairy2, a direct downstream effector of Delta/Notch input into the neural plate border territory, also participates in the regulation of neural crest specifier genes (Glavic et al. 2004). Dlx5, which is regulated by attenuated levels of BMP (Luo et al. 2001), expands the *Msx1* expression domain upon ectopic activity (Woda et al. 2003).

Because neural plate border specifiers are the first transcription factors to appear at the

border, it is not surprising that they may be directly activated by the simultaneous input of multiple signaling pathways. Although evidence for direct interactions is sparse, Brugger and colleagues show direct conversion of the intermediate levels of BMP signal onto the *Msx2* promoter (Brugger et al. 2004). Recently, Li and colleagues found that *Gbx2*, a gene essential for the anteroposterior partitioning of neural folds, is expressed in an ectodermal region that includes the future neural plate border from which crest cells will arise (Li et al. 2009). The authors demonstrated that *Gbx2* is an immediate direct downstream target of Wnt signaling. Furthermore, epistatic rescue experiments reveal that Gbx2 is positioned upstream of the earliest previously reported neural plate border specifiers, Msx1 and Pax3. These results suggest Gbx2 as a candidate for mediating the earliest Wnt inductive signaling input into the neural crest GRN.

Studying the hierarchical interrelationships between newly activated neural plate border specifiers is challenging because of the inaccessibility and/or rapidity of the induction and border specification processes in most vertebrate models. Due to their slow development, however, lamprey embryos allow unprecedented temporal resolution of neural plate border specification. This has enabled chronological ordering of the onset of gene expression among neural plate border specifiers as well as gene perturbation assays to establish their hierarchical relationships. A study by Nikitina and colleagues establishes Msx, but also the neural crest specifier AP2α, at the top of the neural plate border cascade, with many of the factors present at the border (both known border specifiers such as *Msx*, *Pax3/7*, or *Zic*, as well as early crest specifiers such as *AP2α*, *n-Myc*, or *Id*) feeding back and regulating each other's expression (Nikitina et al. 2008). It will be interesting to further investigate direct regulatory relationships at the border as well as to test similar interactions in higher vertebrates, such as chick embryo, which also have good temporal resolution of neural plate border specification.

NEURAL CREST SPECIFIER GENES

The regulatory state during neural crest specification is defined by the cumulative expression of a set of neural crest specifier genes, in the premigratory and early-migrating bona fide neural crest progenitors. Some neural crest specifiers persist in migrating and differentiating neural crest cells (such as Sox10), whereas others such as Snail2 are present only at the onset of the specification process and the EMT prior to their emigration. Some neural crest specifiers have a biphasic expression pattern in which they are present first in neural crest progenitors and again later in differentiating derivatives (e.g., Sox9). A subgroup of transcription factors such as AP2α, Snail1/2, Id, c-Myc, and Twist are expressed even before neural crest progenitors become apparent, though the timing of their onset and presence within the neural plate border varies among different vertebrates. In a basal vertebrate, the lamprey, expression of this subgroup of early-expressing neural crest specifiers begins at the early neurula stage, preceding expression of canonical neural crest markers such as *Sox10* and *FoxD3* (Nikitina & Bronner-Fraser 2009, Sauka-Spengler et al. 2007). This raises the intriguing possibility that these genes may function as a key regulatory link between the establishment of competence in the presumptive crest at the neural plate border and the specification of bona fide neural crest cells. During specification, neural plate border genes either directly or indirectly regulate neural crest specifier genes. They also receive signaling pathway inputs and undergo intricate cross-regulatory activity with other neural crest specifiers.

The regulatory control of *Snail2* exemplifies how signaling pathways and regulatory factors merge to direct the expression of a key gene involved in the EMT of neural crest cells. *Cis*-regulatory analysis shows that *Snail2* is directly regulated by intermediate levels of BMP, which are modulated by Wnt pathway input. Accordingly, the *Snail2* regulatory region contains binding motifs for Smad1, a transcription

factor that mediates BMP signaling input (Sakai et al. 2005), and Tcf/Lef1, which mediates the β-catenin-dependent Wnt signal (Vallin et al. 2001). Furthermore, in frog animal cap explants, a combination of the BMP inhibitor chordin and Wnt8 is sufficient to induce the expression of *Snail2* as well as *Id3*, a helix-loop-helix (HLH) transcriptional regulator involved in specification of the neural crest (Kee & Bronner-Fraser 2005). Overexpression of *Hairy2*, a direct downstream effector gene of Notch signaling, causes an expansion of *Snail2* expression in frog (Glavic et al. 2004) and has been proposed as a direct input into the *Snail2* regulatory region. Finally, it has been demonstrated that the neural plate border specifiers Zic1, Msx1, and Pax3/7 are independently necessary and sufficient for the expression of a group of neural crest cell specifiers including *Snail2* (Meulemans & Bronner-Fraser 2004, Sato et al. 2005, Tribulo et al. 2003). This suggests that regulatory signaling inputs activating *Snail* may be mediated by neural plate border specifiers such as Zic1, Msx1, and Pax3/7. Conversely, signaling inputs can act in parallel with upstream border specifiers to control neural crest specifier expression. For instance, in frog embryos, β-catenin-dependent canonical Wnt signals cooperate with Zic1 and Pax3/7 to activate *Snail2* expression (Sato et al. 2005).

Far less is known about the regulation of other neural crest specifiers. *Twist*, for instance, is ectopically activated upon *Snail2* and *FoxD3* misexpression in frog embryos and ectodermal explants, perhaps indirectly via Zic transcription factor (Meulemans & Bronner-Fraser 2004, Sasai et al. 2001). In contrast, expression of a constitutively activated truncated version of a Notch receptor in frog embryos downregulates *Twist* expression, simultaneously causing the neural plate to expand and the epidermis to regress. Thus, it is not clear if the loss of *Twist* expression is a result of regulatory changes caused by a shift in signaling or a secondary effect owing to neural plate expansion at the expense of the neural crest (Coffman et al. 1993, Cornell & Eisen 2005). Although it is intriguing to speculate that Zic1 mediates Notch-Twist regulation, currently no data either support or refute this possibility. Some early neural crest cell specifiers, such as Id and cMyc, appear to function within the neural crest GRN to maintain the neural crest cells in a multipotent state, mediating critical cell cycle and/or cell fate decisions by controlling the expression of genes involved in cell division and downregulating factors involved in the onset of terminal differentiation (Bellmeyer et al. 2003, Kee & Bronner-Fraser 2005, Light et al. 2005). Id is a transcriptional repressor that possesses a HLH domain for dimerization but lacks a basic domain for DNA binding. Id proteins interfere with gene expression by binding to transcriptional activators from bHLH families and preventing them from activating their direct targets. In lamprey, initial expression of *Id* at the neural plate border precedes that of *cMyc* (Nikitina & Bronner-Fraser 2009). However, in frog embryos cMyc can directly regulate *Id* expression (Light et al. 2005), which indicates that other factors, such as AP2α or Zic1, may be responsible for the initial expression of *Id* (Nikitina et al. 2008). Therefore, cMyc functions directly upstream of *Id*, via the identified *cis*-regulatory region, to maintain its expression in premigratory neural crest cells.

By the time premigratory and delaminating neural crest cells express transcription factors such as FoxD3, Sox9, Snail2, or Sox10, they are specified to a neural crest fate. The winged-helix transcription factor FoxD3 appears to play a role in maintaining neural crest multipotency by preventing early differentiation (Lister et al. 2006). Direct regulatory inputs responsible for FoxD3 activation and maintenance in premigratory and migrating neural crest cells have yet to be described. Similar to *Snail2* activation, evidence from studies in frog embryos suggests that a Hairy2-mediated Notch signal regulates *FoxD3* expression (Wettstein et al. 1997). In addition, the collective activity of Zic1 and Pax3/7 complemented with Wnt input induces *FoxD3* expression (Sato et al. 2005). Gain- and loss-of-function experiments in frog have also shown

that Msx1 regulates *FoxD3* expression (Tribulo et al. 2003).

The SoxE family of transcription factors, most notably *Sox9* and *Sox10*, have well-established roles in neural crest development. In frog, *Sox9* expression is dependent on the activity of AP2α (Lee et al. 2004, Luo et al. 2003, Saint-Germain et al. 2004). Moreover, in silico database searches have identified AP2α binding motifs within the early-acting *Sox9 cis*-regulatory region in mouse (Bagheri-Fam et al. 2006). In frog it has been shown that Gbx2 together with Zic1 can induce the expression of neural crest specifier genes including *Sox9* and *Snail2* while inhibiting preplacodal fate (Li et al. 2009). However, the direct regulatory inputs into *Sox9* have yet to be experimentally demonstrated.

Recently Ets1 and cMyb have been added to the neural crest GRN as new neural crest specifier genes directly regulating the onset of *Sox10* expression. Extensive characterization of the initial *Sox10*-activating *cis*-regulatory element in chick embryo (Betancur et al. 2010) reveals that the synergistic activity of Ets1, cMyb, and Sox9 directly regulates the onset of Sox10 in the cranial neural crest via an early cranial *Sox10* enhancer. The possible role of the proto-oncogene cMyb in neural crest cell development was first suggested in migrating trunk neural crest cells, where the knockdown of cMyb protein reduced *Snail2* expression (Karafiat et al. 2005). However, *cMyb* expression in chick begins much earlier, at the gastrula stage. It becomes confined to the neural folds as the neural plate begins to invaginate and later continues to be expressed in migrating crest cells (Betancur et al. 2010). Knockdown of cMyb in the cranial neural crest causes a diminution of *Sox10* expression, which confirms that this factor acts upstream of *Sox10*. *Ets1* expression is specific to the cranial crest population and first appears in neural crest progenitors in chick embryos as the neural folds are closing (Theveneau et al. 2007). Trunk neural crest cells, which normally do not express *Ets1*, arrest in the G1 phase of the cell cycle prior to separating from the neuroepithelium and synchronously enter the S phase upon delamination. Interfering with the G1/S transition prevents the delamination process from occurring (Burstyn-Cohen & Kalcheim 2002). Ectopic expression of *Ets1* in the trunk region suggests that it promotes massive migration independent of the cell cycle (Sauka-Spengler & Bronner-Fraser 2008a, Theveneau et al. 2007), which is more like migration in the cranial region. These data, together with the finding that Ets1 directly regulates *Sox10* specifically in cranial crest cells, raise the intriguing possibility that in the cranial neural crest Ets1 may have the unique function of establishing a regulatory state that activates cranial crest–specific effector genes responsible for the transition from the pre-migratory to migratory state. The differential expression of Ets1 and its regulatory relationship to other neural crest genes highlights interesting differences between neural crest populations at different levels of the neural axis.

Neural crest cell specifiers, in general, represent a node point onto which inductive inputs mediated by or acting in parallel with neural plate border specifiers converge. Those specifying transcription factors in turn control the expression of effector genes that will give neural crest cells their unique migratory and multipotent characteristics. Therefore, in the life cycle of a neural crest cell, it is critical to keep the specifier genes running as a unit in the network. For this purpose in frog, high interdependence among neural crest cell specifiers seems to exist. Gain- and loss-of-function experiments suggest that Snail2 regulates *FoxD3*, *Twist*, and *Sox10* expression, probably in an indirect fashion (Aoki et al. 2003, Aybar et al. 2003). Ectopic expression of *AP2α* in the neural plate activates the ectopic expression of *Snail2* (Spokony et al. 2002), whereas Sox10 feeds back to maintain *Snail2*, *Sox9*, and *FoxD3* expression (Honore et al. 2003). However, in mouse and zebrafish, cross-regulation among neural crest cell specifiers is less tight because knockouts of Snail1 and 2, Sox10, and AP2α have effects later, during differentiation in selective neural crest derivatives, rather than at this state

of specification (Meulemans & Bronner-Fraser 2004). Perhaps in other organisms, neural crest specifier genes have a more redundant function during specification, and then their function becomes more restricted as the neural crest advances to the differentiating state. Conversely, this discrepancy may be due to the higher rate of gene duplication and functional compensation by redundant paralogs (Lister et al. 1999, Luo et al. 2001, Yan et al. 2005). Only through characterization of *cis*-regulatory modules will we be able to understand the degree of importance of these neural crest cell specifier cross-regulatory events.

GENES REGULATING NEURAL CREST EMIGRATION AND MIGRATION

To initiate migration, premigratory neural crest cells must delaminate from the neuroepithelium. Thus, transcription factors acting on the neural crest precursor pool must not only maintain the precursors in a multipotent and proliferating state, but also activate or repress effector genes involved in their EMT. To allow cells to become less compact and acquire motility, the EMT induces changes at the cellular level that include switches in cell junctions and adhesion properties as well as major cytoskeletal rearrangements. One characteristic of the EMT process is a switch in cadherin expression that involves upregulation of type II cadherins that allow for less adhesiveness and concomitant downregulation of type I cadherins and other factors characteristic of epithelial cell types. For example, in trunk neural crest cells in the chick, forced expression of *FoxD3* downregulates N-cadherin (N-Cad, a type I cadherin) while concomitantly upregulating expression of Cad7, a type II cadherin, and β1 integrin (Cheung et al. 2005). Because FoxD3 is a repressor, the upregulation is likely to be indirect. Confirming a role for FoxD3 during delamination, misexpression of FoxD3 along the entire dorsoventral axis of the chick neural tube caused an increase in expression of neural crest cell markers, including Cad7, and promoted delamination and migration from more ventral regions of the neural tube while simultaneously repressing interneuron differentiation (Dottori et al. 2001). Normally, Cad7 is only expressed in migrating crest cells and excluded from the neural tube (Nakagawa & Takeichi 1995). Similar to FoxD3, Sox10 overexpression induces β1 integrin expression while inhibiting N-Cad expression (Cheung et al. 2005). Although it is difficult to ascribe direct gene regulatory interactions, it is clear that both FoxD3 and Sox10 affect expression of EMT effector genes, such as cadherins, whose orchestrated regulation is crucial for EMT to occur.

Snail1 and *Snail2* genes have a clear role in controlling cell adhesiveness and many other aspects of EMTs in embryonic and metastatic cells (Thiery & Sleeman 2006). Snail1 is directly responsible for the negative regulation of *E-cadherin* (*E-Cad*), a cell adhesion molecule characteristic of epithelial cells (Cano et al. 2000). Similarly, Snail2 acts directly to negatively regulate the expression of *Cad6B*, a molecule that characterizes cell-cell adhesion among dorsal neural tube cells, most of which are premigratory neural crest progenitors (Taneyhill et al. 2007). Sox5, a member of the SoxD family, is another transcription factor proposed to have a regulatory role during neural crest cell delamination. Sox5 misexpression causes an increase in the number of cranial neural crest cells generated. Sox5 upregulates *Snail2*, *FoxD3*, and *Sox10* in migrating crest cells and cell autonomously upregulates *RhoB*, a member of the Rho family of small GTPases that controls a variety of signal transduction pathways (Perez-Alcala et al. 2004). RhoB is a well-known regulator of events that change cell morphology such as actin cytoskeleton rearrangements as well as the formation of focal adhesions and stress fibers (Liu & Jessell 1998). All these cellular changes are necessary for neural crest delamination (Nobes & Hall 1995). The function of RhoB in cranial crest cells appears to be distinct from that in the trunk, where it acts as a negative modulator, downregulating N-Cad and preparing cells for delamination (Groysman et al. 2008). Again,

cis-regulatory profiling will confirm if the subcircuit initiated by Sox5 consists of direct binding to *Snail*, *FoxD3*, *Sox10*, and *RhoB* regulatory modules in delaminating cranial crest cells. Other studies have demonstrated that Sox5 can bind to *cis*-regulatory modules via known motifs, previously identified as Sox9 and Sox10 binding sites, and can modulate expression of downstream target genes by recruiting specific cofactors during neural crest cell differentiation (Hattori et al. 2008, Stolt et al. 2008). The same regulatory mechanism is likely used during cranial crest delamination. Because *Sox5* is expressed early in the premigratory neural crest, it may also be involved in the regulatory interactions that take place during neural crest specification. However, this possibility remains to be explored.

Most of the transcription factors that are involved in neural crest cell specification continue to be expressed in neural crest cells as they migrate. However, other unidentified upstream inputs, different from those that initiate expression of neural crest specifiers in the premigratory state, may be responsible for maintaining their expression during migration. Moreover, different upstream regulators may be characteristic of neural crest cells with various differentiation potentials, correlated with their future fate. For example, inactivation of Wnt signaling input sites within the *Sox9* enhancer decreased reporter expression exclusively in neural crest cells migrating into the first but not the second or third branchial arches (Bagheri-Fam et al. 2006). *Cis*-regulatory analysis in mouse has shown that during neural crest migration, *Sox10* is directly regulated by Pax3, AP2α, and Sox9 but also receives Wnt signaling input (Werner et al. 2007). Analysis in zebrafish confirms that a Wnt signal feeds directly to the *Sox10* regulatory element during migration but also strongly suggests SoxE, nuclear factor κB (NF κB), and Notch signals as potential direct Sox10 regulatory inputs (Dutton et al. 2008). These studies also demonstrated that there is no direct regulatory interaction between FoxD3 and Sox10 despite the presence of FoxD3 binding motifs in *Sox10 cis*-regulatory regions. However, FoxD3 has been reported as a negative regulator of *Sox10* (Pohl & Knochel 2001, Sasai et al. 2001). It is plausible that the negative feedback of a FoxD3 repressor on the *Sox10* regulatory module may have been missed because assays employed to identify direct regulators are more targeted to isolation of positive regulatory influences (activators). Conversely, FoxD3 may function as a regulator of Sox10 activity via still unidentified enhancers. Alternatively, the loss of *Sox10* expression after FoxD3 inactivation may suggest that their functional interactions are not direct and perhaps involve an intermediary inhibitor.

Prior to and during neural crest migration, cells acquire signaling receptors that allow them to interact with their environment and help guide them along specific pathways. In the cranial region such molecules include Neuropilin-1/2, Roundabout homologs Robo-1/2, and Ephrin receptors (Sauka-Spengler & Bronner-Fraser 2008a). However, the transcription factors that regulate expression of these signaling molecules remain elusive. Similarly, not much information is available regarding the upstream regulators of genes that are involved in cell cycle decisions prior to cranial neural crest cell delamination; only a few regulatory interactions that prevent cells from undergoing apoptosis have been described. In Sox9 null mice, neural crest cells undergo massive apoptosis (Cheung et al. 2005). Similarly, zebrafish neural crest cells lacking Sox9 within the branchial arches show a predominant cell death phenotype (Yan et al. 2005). Gain- and loss-of-function experiments in frog suggest a direct regulatory connection between Sox9 and another antiapoptotic factor, Snail1 (Aoki et al. 2003).

In summary, the combined regulatory function of neural crest specifier genes and their downstream effectors endows neural crest cells with the characteristics that render them mesenchymal, proliferative, and motile. However, out of the many neural crest downstream effector genes, the direct regulatory inputs and links to upstream neural crest specifiers are known for only a few, which makes it difficult to assign

their precise positions within the neural crest GRN.

THE TRANSITION FROM MIGRATION TO DIFFERENTIATION

How neural crest cells lose their migratory and multipotent characteristics as they prepare to differentiate remains an open question. It is logical to postulate that a separate set of gene batteries is deployed in each neural crest lineage. *Cis*-regulatory analysis combined with functional and binding affinity assays have revealed several subcircuits of direct gene regulatory interactions for each lineage. After neural crest cells have migrated and reached their final destinations, typically expression of most early neural crest cell specifiers, including Snail/Snail2, FoxD3, Id, and AP2α, is downregulated, although the direct regulatory interactions triggering or mediating this downregulation are elusive (Meulemans & Bronner-Fraser 2004). Nevertheless, some evidence suggests that FoxD3 participates in repression of *Snail1b* (previously *Snail2*) in zebrafish. Its absence causes prolonged expression of *Snail1b* when it would normally be turned off (Lister et al. 2006). Exogenous expression of FoxD3 in frog causes repression of endogenous *FoxD3*, indicating that FoxD3 can directly downregulate its own expression in a negative autoregulatory loop (Pohl & Knochel 2001). Downregulation of FoxD3 in migrating cells prior to differentiation does not take place in all neural crest–derived lineages. Although absent from melanoblasts, *FoxD3* expression persists in neural/glial precursors, where it prevents Pax3 from binding to the promoter of microphthalmia-associated transcription factor (Mitf) and thus prevents sensory precursors from assuming a pigment cell fate (Thomas & Erickson 2009). These data demonstrate the importance of negative regulation in cell fate acquisition in cell types with multiple developmental potentials such as the neural crest. It will be essential to study the differential upstream inputs that confine FoxD3 or other repressive circuits that could act as regulatory switches between different lineages.

Notable exceptions are SoxE transcription factor family members Sox9 and Sox10, which persist in specific subpopulations of neural crest cell derivatives and appear to be master regulators of terminal differentiation in the majority of neural crest derivatives (Kelsh 2006, Sauka-Spengler & Bronner-Fraser 2008b). The necessity of different SoxE genes for specification of distinct neural crest sublineages has recently been demonstrated in zebrafish (Arduini et al. 2009). Sox9 and Sox10 are maintained in cartilage and neuron/glial/melanocyte lineages, respectively, such that Sox10 persists in melanoblasts and elements of the peripheral nervous system, whereas Sox9 is characteristic of neural crest–derived chondrocytes. Experiments in frog suggest that the HLH transcriptional repressor Id prevents premature neural crest cell differentiation during neural crest migration. Constitutive expression of Id family members in migrating neural crest cells populating the pharyngeal arches, most of which would normally adopt a cartilage fate, extends *Sox10* expression, which is normally downregulated in this population when the cells enter the arches (Light et al. 2005). Furthermore, overexpression of Id3 in Sox10-expressing melanoblasts or Sox9-expressing neural crest–derived cartilage cells inhibits SoxE expression, which affects melanocyte and chondrocyte differentiation (Light et al. 2005). Thus, downregulation of Id is necessary for the initial steps of neural crest cell differentiation to occur. It is plausible that endogenous downregulation of Id indirectly releases inhibitors that feed into the neural crest specifier module. Maintaining expression of *Sox9* and *Sox10* until the time of differentiation, however, may be independent of the Id regulatory cascade. Strong evidence indicates that Id helps establish the time window during which cells respond to differentiating signals (Light et al. 2005). At the proper time, it may release activator genes involved in differentiation and maintenance of *Sox9* and *Sox10* expression in their respective differentiated lineages.

Another possibility is that the inhibitory activity of Id maintains *Sox10* and perhaps *Sox9* expression at low levels. It has been suggested that low concentrations of Sox10 sustain the multipotency of neural crest cells and at higher levels inhibit neuronal differentiation and promote glia and melanoblast formation (Kim et al. 2003, Paratore et al. 2001). Resolving regulatory interactions to the detail that would allow unraveling of these complex events remains a challenge. The battery of genes involved in maintaining neural crest cells may change such that new regulatory interactions emerge, some of which may involve redeployment of transcription factors involved in early neural crest cell specification to perform a later function in cell differentiation. For example, the way that Sox9 and Sox10 acquire new, instructive roles in directing the fate of certain neural crest derivatives may involve acquisition of new cofactors.

DIFFERENTIATION OF THE CRANIAL NEURAL CREST

Neural crest cells give rise to a wide variety of derivatives ranging from melanocytes, glia, and neurons to skeletal components of the head. In general, the type of derivative depends upon the axial level from which the neural crest cells originate and the time of their emigration from the neuroepithelium. For example, midbrain and rhombomere (r) 1 and r2 neural crest cells contribute to the neurons and glia of the trigeminal ganglion as well as to the skeleton of the upper and lower jaw. Neural crest cells from r4 give rise to neurons of the proximal facial ganglion and the hyoid bone. Neurons of the proximal and jugular ganglia and skeletal components of the postpharyngeal arches are derived from postotic neural crest streams r6 and r7 (Graham et al. 2004, Lumsden et al. 1991, Schilling & Kimmel 1994). The vagal neural crest forms the enteric nervous system as well as cardiac and aortic arch components. The trunk neural crest forms sensory and autonomic ganglia and the adrenal medulla.

The time of migration also influences the types of derivatives that neural crest cells form. Early migrating cranial neural crest cells populate the pharyngeal arches to generate bone, cartilage, and connective tissue (skeletal structures), whereas the later wave stays close to the central nervous system and generates the neurons and glia of the cranial ganglia (Graham et al. 2004). Melanocytes are derived from neural crest cells from all axial levels. In mouse, a subpopulation of neural crest cells within a dorsomedial domain of the neural tube at the midbrain-hindbrain junction migrates exclusively into the developing dermis and expresses melanocyte lineage markers (Trainor 2005).

Of all the cranial neural crest derivatives, melanocytes and chondrocytes are the two lineages in which the most *cis*-regulatory work has been performed, and this research allows predictions regarding regulatory subcircuits. In melanocytes, Sox10, in synergy with Pax3, directly regulates *Mitf* by binding to a proximal region of its promoter (Bondurand et al. 2001, Verastegui et al. 2000). Then, in collaboration with Mitf, Sox10 directly regulates expression of an enzyme necessary for melanin synthesis, dopachrome tautomerase (*Dct/TRP2*; Ludwig et al. 2004).

Sox5 also plays a direct modulatory role in melanocyte differentiation. Sox5 and members of the SoxD family of transcription factors are characterized by their lack of a transactivation domain (Lefebvre et al. 1998). It has been speculated that they regulate transcription by recruiting other coactivators or corepressors to regulatory regions. On one hand, in melanocytes, Sox5 binds to the *Mitf* and *Dct/TRP2* promoter regions through Sox10-identified binding elements. It recruits the corepressors, C-terminal binding protein 2 (CtBP2) and histone deacetylase (HDAC), to compete with Sox10 for binding of these regulatory regions and therefore modulates Sox10-inducing activity (Stolt et al. 2008). During chondrocyte development, on the other hand, Sox9 directly regulates expression of important cartilage markers such as Collagen type II α1 (Col2α1) (Lefebvre et al. 1997), Col11α2 (Bridgewater et al. 1998), and Cartilage-derived retinoic acid–sensitive protein (CD RAP)

(Xie et al. 1999) by binding to sites in identified enhancer regions. Interestingly, Sox5 null mice have skeletal defects and particularly craniofacial defects (Smits et al. 2001). This suggests another role for Sox5 in chondrocyte development. Consistent with this possibility, Sox5 was recently found to cooperate with Sox9 and other cofactors in chondrocytes to regulate expression of Col2α1 by binding to Sox9 target sites (Hattori et al. 2008). These inputs at the effector level of the neural crest GRN are a few notable examples of how precise gene regulatory subcircuits can guide a neural crest subpopulation to differentiate into specific derivatives.

Little is known about direct regulatory interactions in the specification and differentiation of cranial neural crest cells into glia and neurons. Most knowledge about direct regulatory interactions in neurogenic neural crest derivatives comes from experiments performed in trunk neural crest cells. These studies show that differentiation into neural crest–derived neurons and glia requires redeployment of factors utilized earlier during neural crest induction and specification. As an example, Notch and Delta proteins are expressed in neural crest cells that populate the presumptive trigeminal ganglion region, where they undergo gliogenesis and neurogenesis. The Notch signaling pathway promotes gliogenic differentiation while inhibiting neuronal differentiation (Nakamura et al. 2000, Ohtsuka et al. 1999). Furthermore, different mediators of Notch signaling appear to control, in part, the cell fate decision between gliogenic and skeletogenic differentiation. Whereas the Deltex-mediated Notch pathway controls gliogenesis, simultaneous activation of the key mediator of Notch signaling, recombining binding protein suppressor of hairless protein (coded by the *RBPJ* gene), and the Deltex-dependent Notch pathways leads to chondrogenic specification (Ijuin et al. 2008), which is mediated by the previously characterized Notch downstream effectors Hes1 and Hes5 (Jarriault et al. 1995). The downstream readouts used to differentiate the gliogenic and chondrogenic lineages were glial fibrillary acidic protein (GFAP) and Col2α1, respectively. Thus, the above-mentioned studies place these specific markers as potential effector genes that act directly downstream of Notch signaling inputs mediated by Hes1 and Hes5 (Ijuin et al. 2008).

In addition to its role in melanocyte differentiation, Sox10 also controls specification of glial and neuronal fates in neural crest derivative specification. Sox10 appears to further participate in the differentiation of glia, as its expression within this lineage persists into terminal differentiation stages (Kelsh 2006). During glial differentiation, Sox10 directly regulates the expression of protein zero (P0) (Peirano et al. 2000), myelin basic protein (MBP), peripheral myelin protein 22 (PMP22) and the gap junction protein connexin 32 (Cx32), thus affecting all major components of the myelination process (Bondurand et al. 2001). Finally, evidence concerning the direct regulatory role of Sox10 during differentiation of neural crest–derived neurons comes from studies of sensory and autonomic lineages in the trunk. In mouse neural crest cell cultures, Sox10 regulates the expression of mouse achaete-scute homolog 1 (MASH1) and the paired homeodomain (Phox2b), transcription factors that are essential for autonomic neurogenesis (Kim et al. 2003). Sensory neurons derived from the dorsal root ganglia transiently express Sox10, which has been shown to regulate the expression of proneural gene *Neurogenin1* in zebrafish (Carney et al. 2006). Similar interactions involving direct Sox10 regulatory inputs and expression of the sensory neuronal marker Neurogenin1 may take place during cranial neurogenesis.

Finally, it is important to stress the role of negative regulation during the steps of terminal differentiation into neural crest derivatives. A recent study by Sun and colleagues (2008) shows that LIM-homeodomain factor Islet1 specifically regulates subprograms within different sensory neuron lineages. At the end of the neurogenic phase of development, Islet1 is specifically required to repress/terminate the expression of genes such as *Neurogenin1* or

NeuroD family members. Interestingly, Islet1 is also required to repress several transcription factors not normally expressed in the sensory ganglia but found in the spinal cord and hindbrain, such as LIM-homeobox genes *Lhx1* and *Lhx2* and oligodendrocyte markers *Olig1* and *Olig2*. This suggests that Islet1 inhibition also serves as a control switch that keeps cells within the sensory lineage (Sun et al. 2008).

CONCLUSION AND FUTURE PROSPECTS

In this review, we present an overview of the GRN orchestrating the formation of neural crest cells, with a focus on the cranial level (**Figures 1** and **2**). Formulation of this network relies on information largely inferred from studies of the molecular mechanisms underlying neural crest formation in several vertebrate model organisms. It also includes all known *cis*-regulatory information obtained to date, which provides evidence for direct regulatory interactions and architectural circuitry between the molecular factors involved. The neural crest GRN presented here can be used as a guide for future experiments to test if predicted direct regulatory connections hold true for different vertebrate model organisms. The future promise of high throughput *cis*-regulatory and transcriptional profiling of neural crest cells at each regulatory step will provide further information that can be assembled into a high-resolution GRN.

DISCLOSURE STATEMENT

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