Received: 1 May 2017 | Revised: 26 March 2018 | Accepted: 27 March 2018

DOI: 10.1002/wdev.322

WILEY WIREs DEVELOPMENTAL BIOLOGY

ADVANCED REVIEW

Specifying neural crest cells: From chromatin to morphogens and factors in between

Crystal D. Rogers[1] | Shuyi Nie[2]

[1]Department of Biology, College of Science and Mathematics, California State University Northridge, Northridge, California

[2]School of Biological Sciences and Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia

Correspondence
Crystal D. Rogers, Department of Biology, College of Science and Mathematics, California State University Northridge, 18111 Nordhoff Street, Northridge, CA 91330-8238.
Email: crystal.rogers@csun.edu
and
Shuyi Nie, School of Biological Sciences and Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA 30332.
Email: shuyi.nie@biology.gatech.edu

Neural crest (NC) cells are a stem-like multipotent population of progenitor cells that are present in vertebrate embryos, traveling to various regions in the developing organism. Known as the "fourth germ layer", these cells originate in the ectoderm between the neural plate (NP), which will become the brain and spinal cord, and nonneural tissues that will become the skin and the sensory organs. NC cells can differentiate into more than 30 different derivatives in response to the appropriate signals including, but not limited to, craniofacial bone and cartilage, sensory nerves and ganglia, pigment cells, and connective tissue. The molecular and cellular mechanisms that control the induction and specification of NC cells include epigenetic control, multiple interactive and redundant transcriptional pathways, secreted signaling molecules, and adhesion molecules. NC cells are important not only because they transform into a wide variety of tissue types, but also because their ability to detach from their epithelial neighbors and migrate throughout developing embryos utilizes mechanisms similar to those used by metastatic cancer cells. In this review, we discuss the mechanisms required for the induction and specification of NC cells in various vertebrate species, focusing on the roles of early morphogenesis, cell adhesion, signaling from adjacent tissues, and the massive transcriptional network that controls the formation of these amazing cells.

This article is categorized under:
Nervous System Development > Vertebrates: General Principles
Gene Expression and Transcriptional Hierarchies > Regulatory Mechanisms
Gene Expression and Transcriptional Hierarchies > Gene Networks and Genomics
Signaling Pathways > Cell Fate Signaling

KEYWORDS
BMP, epigenetic, FGF, morphogen, neural crest, specification, Wnt

1 | INTRODUCTION

The complexity of the vertebrate form requires gene and protein regulation at many levels, as well as tissue interactions, cell movements and perfect timing (Martik & Bronner, 2017). Although we have yet to discover all of these controls, the development of one cell type in particular, neural crest (NC) cells, has been studied at length. Ectodermal cells respond to many instructive signals very early in development to form neural tissue (Gaur et al., 2016; Lamb et al., 1993; Rogers, Archer, Cunningham, Grammer, & Casey, 2008), epidermal/placodal tissue (Nordin & LaBonne, 2014; Schlosser, 2014), or NC tissue (Duband, Dady, & Fleury, 2015). The epigenetic and molecular specification of each of these tissue-types is followed by morphogenetic events such as neural tube closure and NC cell migration. The neural plate begins as a flat epithelial layer (Figure 1a, blue), but rises to meet in the center of the embryo and create the neural tube (Wilde, Petersen, & Niswander, 2014) (Figure 1b,d), which

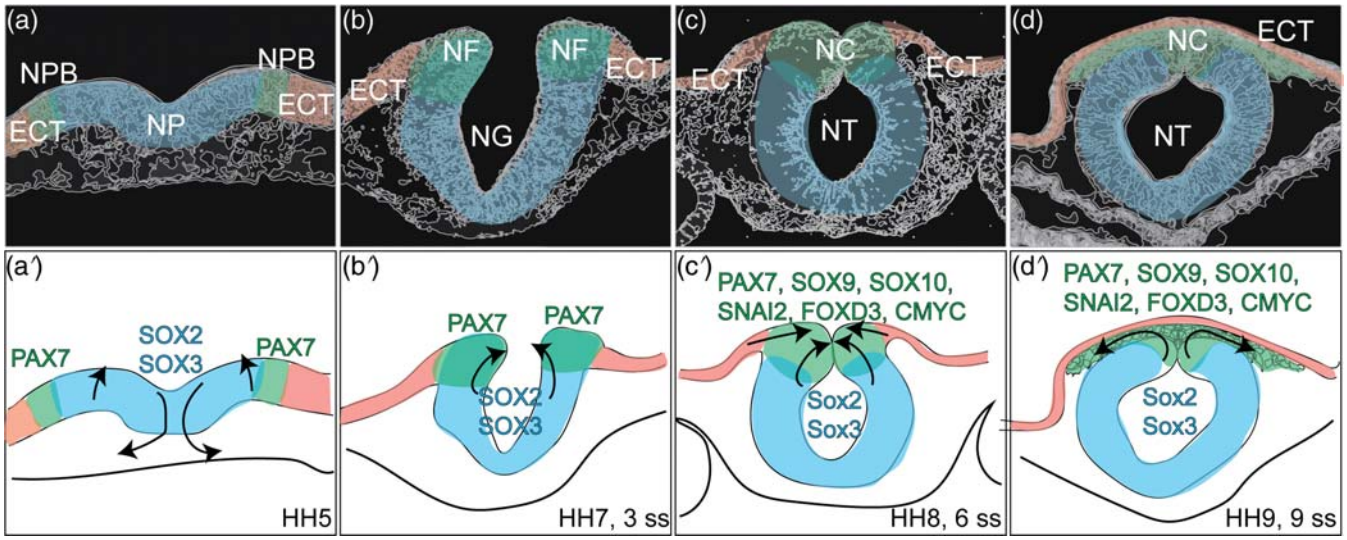

FIGURE 1 Morphogenesis and NC specification. (a, a′) In chicken embryos, the NPB (green) is specified by Hamburger Hamilton stage 5 (HH5) prior to the onset of definitive neural crest (NC) markers and expresses transcription factors such as MSX1, ZIC1, and PAX7. (b, b′) As neurulation proceeds at HH7-HH8, the neural folds rise and bend toward the midline. At this stage the neural folds are being specified as definitive NC cells. (c, c′) By HH8, definitive NC markers are expressed in the dorsal neural tube (SOX9, SOX10, SNAI2, and FOXD3), the neural tube is closed, and the ectodermal cells are converging on the midline to cover the neural tube. (d, d′) By HH9, the NC cells are beginning to undergo EMT and start detaching from the neural tube. ECT, ectoderm; NC, neural crest; NF, neural folds; NPB, neural plate border; NP, neural plate

gives rise to the central nervous system. At the same time, the nonneural ectodermal (NNE) cells meet and separate from the neural tube, covering the embryo (Figure 1d, orange), eventually differentiating into epidermis and placodes (Groves & LaBonne, 2014). In chicken embryos, NC cells, which begin as neuroepithelial cells (Figure 1c, green), undergo an epithelial to mesenchymal transition (EMT) after the neural tube closes, and leave their former neighbors, the neural and NNE, and migrate throughout the embryo creating diverse derivatives (Figure 1d, green) (Rogers, Jayasena, Nie, & Bronner, 2012). However, in different species, NC cells are specified adjacent to the neural plate (NP) and migrate while the neural tube is closing (mouse and frog) (R. T. Lee et al., 2013; Linker, Bronner-Fraser, & Mayor, 2000). The molecular and morphogenetic mechanisms that regulate the specification of each of these tissues act in concert with symphonic precision, and in this review we will highlight some of the molecular mechanisms that drive NC induction and specification.

NC cells, in their current form are thought to be a unique vertebrate trait; however, recent evidence has suggested that the NC has its origins in multiple migratory and/or pigmented cell types in closely related chordates (Abitua, Wagner, Navarrete, & Levine, 2012; Oonuma et al., 2016; Stolfi, Ryan, Meinertzhagen, & Christiaen, 2015). Analysis of both *Ciona* and Amphioxus embryos demonstrated that NC-related proteins seem to have conserved functions with vertebrate NC proteins, and that these pigmented and/or migratory cell types are controlled by conserved NC-specific transcription factors (Abitua et al., 2012; Tai et al., 2016). However, in the less-derived species, NC cells do not form the traditional derivatives such as craniofacial structures (Green, Simoes-Costa, & Bronner, 2015). Formation of the NC is mediated by a series of regulatory interactions including epigenetic changes and a tightly regulated transcriptional gene regulatory network (GRN) that is largely conserved across vertebrates (Green et al., 2015). The NP border (NPB), induced during gastrulation, includes the tissues that will give rise to the NC. However, NC cells only become morphologically recognizable as neurulation proceeds, where they manifest in an anterior to posterior fashion arising in (e.g., chicken), or adjacent to (e.g., frog), the dorsal neural tube as neural tube closure occurs. These cells are first specified in the head (cranial NC) and proceeding caudally to form cardiac and vagal NC, then trunk and finally sacral NC cells (graphical abstract).

Although premigratory NC cells are neuroepithelial as they are specified, they eventually alter the expression of their cell–cell adhesion molecules, and undergo cytoskeletal changes that result in an EMT, allowing them to delaminate from the epithelial sheet and start migrating both collectively and individually in the developing embryo (Theveneau et al., 2013). Normal formation and migration of NC cells is crucial for the development of craniofacial structures, pigment cells, and the peripheral nervous system among a multitude of derivatives. Additionally, the abilities of NC cells to migrate extensively and to differentiate into diverse cell types, are reminiscent of stem cells and metastatic cancer cells in that they utilize similar molecular pathways to self-renew (Kerosuo, Nie, Bajpai, & Bronner, 2015), migrate, invade tissues, and proliferate (Gallik et al., 2017). These unique characteristics have made NC cells an interesting and well-studied topic for many years. This review will focus on the molecular events controlling the specification of NC cells in vertebrate embryos, specifically characterizing the events in amphibians (frog) and avians (chick). Here, we give an updated view of early patterning of the NPB and the segregation of NC cells from neural ectoderm with a focus on morphogenetic events, gene regulation and the signaling involved in the process.

2 | MORPHOGENESIS, TISSUE INTERACTIONS, AND NC INDUCTION

2.1 | Morphogenetic movements during NC specification

The process of gastrulation allows for the creation of the three germ layers, endoderm, mesoderm, and ectoderm. The most superficial germ layer, the ectoderm, divides into neural, nonneural, and NPB cells soon after the ectoderm is specified. In many species, the formation of the NC from the unspecified ectoderm relies on the concomitant formation of the adjacent NP, and a specific transcriptome is activated in response to signaling pathways in the early embryo. In frog embryos, this early transcriptome has been described by an EctoMap, which details the spatiotemporal localization of ectoderm specification cascades (Plouhinec et al., 2017; Simoes-Costa, Tan-Cabugao, Antoshechkin, Sauka-Spengler, & Bronner, 2014). Multiple studies have detailed the direct and indirect transcriptional interactions during chicken NC specification and induction, and new details are ever emerging (Prasad, Sauka-Spengler, & LaBonne, 2012; Simoes-Costa & Bronner, 2015; Simoes-Costa, Stone, & Bronner, 2015). Due to the abundance of genes and proteins involved in NC specification, we will use mouse gene nomenclature throughout the review, highlighting specific species when necessary. As the three ectodermal derivatives are separating, neural tube and NPB cells share the expression of some transcription factors like SOX2 and PAX3/7, suggesting that their fate is not yet fixed and is ultimately determined by the instructive signals they receive (Figure 1a,a') (Roellig, Tan-Cabugao, Esaian, & Bronner, 2017). Figure 1 depicts a developing chicken embryo from mid-gastrula stage through early EMT and shows the location of different tissues as well as some of the factors that are expressed in these developing tissues during neurulation. Although morphogenesis and NC cell formation varies between organisms due to different embryonic anatomy, the regulatory networks and major factors that control specification are conserved between organisms (Green et al., 2015). The NPB, which flanks the NP bilaterally, expresses a host of transcription factors that are distinct from both the neural tube and the nonneural ectoderm. These factors, known as NPB specifier proteins, include MSX1, PAX3 (frog)/PAX7 (chick), and ZIC1 (both), and the genes that code for these proteins are expressed in the NPB in both amphibian and amniote embryos (Figure 1b,b' *Msx1* and *Zic1* would overlap with *Pax3/7* at this stage) (Basch, Bronner-Fraser, & Garcia-Castro, 2006; McMahon & Merzdorf, 2010; Monsoro-Burq, Wang, & Harland, 2005; Plouhinec et al., 2014). As development proceeds from gastrulation to neurulation and the neural folds rise to meet at the midline (Figure 1c,c'), the NPB proteins begin to activate the expression of bona fide NC transcription factors that become restricted to the dorsal neural tube, although there is still some overlap with neural tube markers such as *Sox2* and *Sox3* in both frog and chick embryos at these stages (Figure 1d,d') (Roellig et al., 2017). As the neural tube invaginates, the neural folds meet at the dorsal midline and bona fide NC markers including the *SoxE* genes, *Sox8*, *Sox9*, and *Sox10* (Aoki et al., 2003; K. M. Bell, Western, & Sinclair, 2000; Cheng, Cheung, Abu-Elmagd, Orme, & Scotting, 2000; O'Donnell, Hong, Huang, Delnicki, & Saint-Jeannet, 2006; Spokony, Aoki, Saint-Germain, Magner-Fink, & Saint-Jeannet, 2002; Wakamatsu, Nomura, Osumi, & Suzuki, 2014), as well as *Snai2* (Aybar, Nieto, & Mayor, 2003; Taneyhill, Coles, & Bronner-Fraser, 2007), *FoxD3* (Sasai, Mizuseki, & Sasai, 2001; Simoes-Costa, McKeown, Tan-Cabugao, Sauka-Spengler, & Bronner, 2012), and *cMyc* (Bellmeyer, Krase, Lindgren, & LaBonne, 2003; Kerosuo & Bronner, 2016) are expressed in the most dorsal regions (Figure 1c,c'). Subsequently, after neural tube closure, NC cells undergo an EMT, allowing them to delaminate from the neuroepithelium and to migrate throughout the embryo, starting at the level of the midbrain and then proceeding in an anteroposterior wave (Figure 1d,d'). As the NC cells leave the neural tube, they alter the expression of many adhesion molecules, but maintain the expression of most bona fide NC specifiers (Figure 1d,d').

2.2 | Tissue interactions and NC induction

The NPB is not only flanked by NP and nonneural ectoderm, but also overlays the paraxial mesoderm in cranial and trunk regions (Figures 1a and 2) (Trainor, Tan, & Tam, 1994). The proximity of these different tissues to the prospective NPB and NC-forming region allows the cells to communicate with each other via paracrine, autocrine, and direct cell–cell signaling. Each of these adjacent tissues has been proposed to act as an NC inducer after perturbation studies have deemed them necessary and/or sufficient for expression of NC markers. The original experiments in frog embryos established that interactions between the NP and nonneural ectoderm are involved in NC formation (Moury & Jacobson, 1990), and that mesoderm was sufficient to induce the expression of NC specifiers (Bonstein, Elias, & Frank, 1998; Marchant, Linker, Ruiz, Guerrero, & Mayor, 1998; Mayor, Morgan, & Sargent, 1995). Since that time, much work has been done to establish the network of intra- and extracellular factors that control NPB and NC formation from adjacent tissues (reviewed in Rogers et al., 2012; Schille, Heller, & Schambony, 2016; Shyamala, Yanduri, Girish, & Murgod, 2015), and the interaction between nonneural ectoderm as well as mesoderm and neural ectoderm is crucial for the development of presumptive NC cells.

Multiple signaling pathways have been implicated in patterning the NPB and formation of NC in different species (Table 1). In this review, we will focus on the four most well-studied pathways that function during this process in amphibian

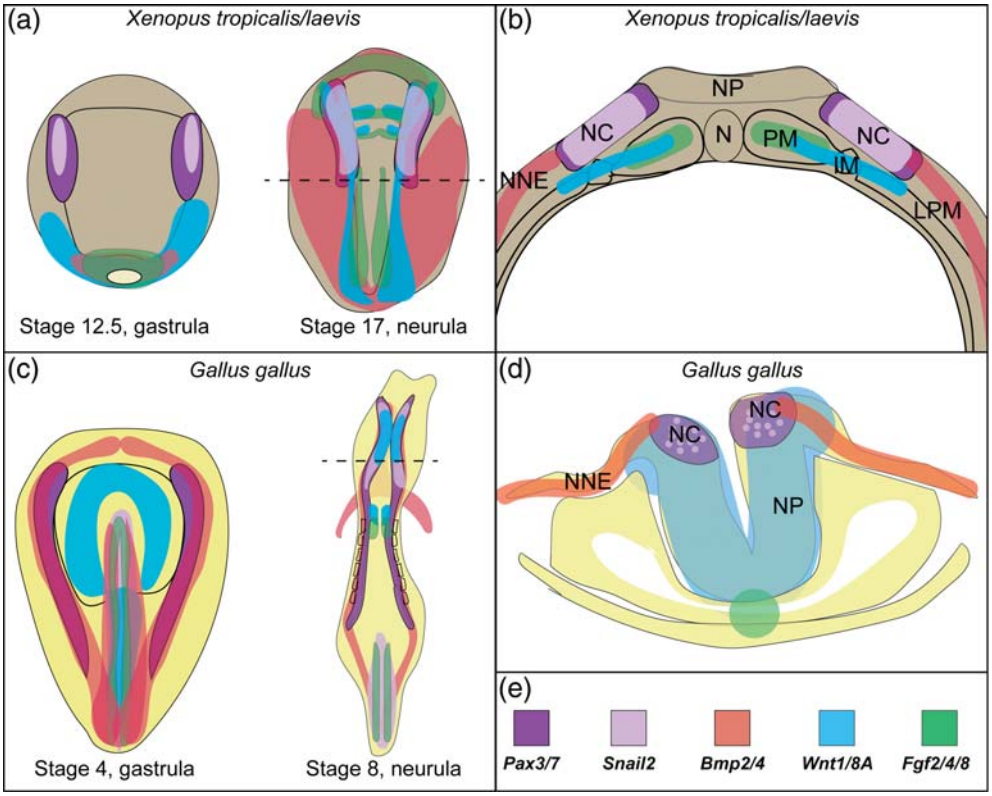

FIGURE 2 Comparative gene expression of morphogens and NC transcription factors in frog and chick. Diagrams depicting the expression of NC transcription factors *Pax3/7* and *Snai2* compared to various genes coding for morphogens that regulate their early expression (*Bmp2/4*, *Wnt1/8A*, and *Fgf2/4/8*). (a, b) In the frog embryo, *Pax3* is expressed by late gastrula stage (stage 12.5) in the presumptive NC region adjacent to the NP. Its expression is broader than *Snai2*, which is also in the presumptive NC region. At this stage, *Bmp4*, *Fgf2*, and *Wnt8* transcripts are expressed posteriorly near the blastopore. At neurula stage (stage 17), the NC cells are preparing to migrate ventrolaterally away from the midline, and the expression of both *Pax7* and *Snai2* remains adjacent to the rising neural folds. At this stage, *BMPs* remain in the nonneural ectoderm and mesoderm while *Fgf8* is expressed in the anterior region, in the brain, and *Fgf2* and *Fgf4* are in the posterior mesoderm. By stage 17, *Wnt8* is expressed in the developing neural folds. (c, d) At late gastrula stage (HH5) in the chicken embryo, *Pax7* is expressed in the NPB, but *Snai2* is limited to the primitive streak/mesoderm. *Bmp4* is expressed in the ectoderm and NPB, *Fgf8* is expressed in the mesoderm, and *Wnt8* is expressed in the NPB and the mesoderm. At neurula stage (HH8), *Pax7* is expressed in the definitive NC as well as the dorsal neural tube, and the NPB in the posterior region. *Snai2* is expressed in the definitive NC cells in the head. *Bmp4* is expressed in the neural folds and the mesoderm, and *Wnt1* is expressed in the dorsal neural folds and *Wnt8A* is expressed in the paraxial mesoderm. The differences in expression of these morphogens may explain some of the differences in NC induction between organisms. Embryos are depicted as follows: (a, c) Anterior to the top, posterior to the bottom, dorsal out. (b, d) Dorsal up, ventral down. All expression patterns (a–d) were found in Xenbase and Geisha

and avian embryos, Wingless/Int (WNT), fibroblast growth factor (FGF), and bone morphogenetic protein (BMP). NC cells are ectodermally derived, but without instructive information, the ectodermal cells do not autonomously develop into NC cells, rather, they become either neural or epidermal tissue depending on the species studied, and whether or not the tissues are dissociated (Hurtado & De Robertis, 2007; Lamb et al., 1993; Rogers, Moody, & Casey, 2009; Streit et al., 1998). Therefore, the unique location of the presumptive NPB and NC cells between multiple inducing tissues suggests that NC cells are formed concurrent or subsequent to the other ectodermal derivatives. However, evidence suggests that signals secreted from the adjacent, surrounding, and underlying tissues drive the formation of the NC from ectoderm (Figure 2). The tissues that are involved include the nonneural ectoderm as well as the mesoderm, which secrete specific members of the BMP pathway (Andree, Duprez, Vorbusch, Arnold, & Brand, 1998; Chapman, Schubert, Schoenwolf, & Lumsden, 2002; Joubin & Stern, 2001). The role of BMP signaling in NC induction has been studied in multiple species, but not all BMP-family proteins are involved in this process (Takahashi et al., 1996; Varley & Maxwell, 1996). Additionally, both the canonical and noncanonical WNT pathways have been identified as negative (Carmona-Fontaine, Acuna, Ellwanger, Niehrs, & Mayor, 2007) and positive (Ikeya, Lee, Johnson, McMahon, & Takada, 1997; Schmidt, McGonnell, Allen, Otto, & Patel, 2007; Simoes-Costa et al., 2015) regulators of NC specification, migration, and development at multiple embryonic stages. *Wnt* genes are expressed in the nonneural ectoderm and mesoderm similarly to *Bmps* at the stages of NC induction and specification, but are generally localized in the posterior side of the embryo (Figure 2a,c) (Christian, McMahon, McMahon, & Moon, 1991; Mikawa, Poh, Kelly, Ishii, & Reese, 2004), while their targets are more widespread. The FGF pathway is also a player in NC induction and specification as well as a factor that imbues general competence to ectodermal cells, allowing them to respond to the instructive signals from other tissues (LaBonne & Bronner-Fraser, 1998; Mayor, Guerrero, & Martinez, 1997). FGF

TABLE 1 Recent papers detailing involvement of morphogens or their effectors in NC development

Morphogen	Specification	EMT	Migration	Differentiation	Organism	Reference
FGF		×			Chick	Martinez-Morales et al., 2011
	×				Chick	Sasai, Kutejova, & Briscoe, 2014
	×		×	×	Zebrafish	Ciarlo et al., 2017
	×				Mouse	Anderson, Schimmang, & Lewandoski, 2016
RA		×			Chick	Martinez-Morales et al., 2011
		×	×		Zebrafish	Jimenez et al., 2016
BMP	×				Frog	Shi, Severson, Yang, Wedlich, & Klymkowsky, 2011
	×				Chick	Sasai et al., 2014
	×				Mouse	Anderson et al., 2016
	×				Frog	Schille, Bayerlova, Bleckmann, & Schambony, 2016; Schille, Heller, & Schambony, 2016
			×		Chick	McLennan et al., 2017
WNT	×				Frog, chick	Garcia-Castro, Marcelle, & Bronner-Fraser, 2002
	×				Frog, chick	Sato, Sasai, & Sasai, 2005
	×				Frog	Shi et al., 2011
			×		Frog	Podleschny, Grund, Berger, Rollwitz, & Borchers, 2015
	×				Frog	Schille, Bayerlova, et al., 2016; Schille, Heller, & Schambony, 2016
			×		Frog	Maj et al., 2016
	×				Mouse*	Masek, Machon, Korinek, Taketo, & Kozmik, 2016
		×	×		Frog, chick	Rabadán et al., 2016
Notch	×		×		Frog	Vega-Lopez, et al., 2015
SHH	×				Chick	Sasai et al., 2014

*[Correction added on 15 May 2018, after first online publication: Organism was corrected from 'Frog' to 'Mouse.']

proteins are secreted from the paraxial mesoderm and they are integral in NC induction (Monsoro-Burq, Fletcher, & Harland, 2003). The roles of retinoic acid (RA), Notch and other signaling molecules have also been implicated in some stage of NC cell development (Table 1). There is not one specific signaling pathway that defines NC cells, rather, the pathways act in concert to create competent ectoderm and drive the ectodermal and/or neural cells to adopt a NC fate. Details of how these signals are established in the embryos and how downstream transcription factors are activated during NC induction are described below.

3 | SIGNALING EVENTS REGULATING NC INDUCTION

The cellular movements during gastrulation not only pattern embryonic germ layers but also set up signaling centers such as the Spemann Organizer in frog, the node in chick or mouse, or the shield in zebrafish. Each of these signaling centers secretes extracellular morphogens required for axis specification and organogenesis. Induction and development of the NC requires a specific level of signaling by the BMP, WNT, FGF, RA, and Notch/Delta pathways. In this section, we describe the roles of each of these extracellular and intercellular signaling molecules in NC induction and specification, and identify their spatio-temporal gene expression in chicken and frog embryos (Figure 2). We have detailed the general expression patterns of the genes that code for various WNT, BMP, and FGF ligands in frog and chick embryos based on published studies and the expression databases Xenbase and GEISHA. Additionally, we have compared the gene expression of the morphogens to the gene expression of the early NPB/NC specifiers *Pax3*/*Pax7* and the bona fide NC specifier *Snai2* in two stages (induction/gastrulation and specification/neurulation) in both organisms with representative section diagrams of the expression in neurula stage embryos in Figure 2 (Bang, Papalopulu, Goulding, & Kintner, 1999; Basch et al., 2006; Khudyakov & Bronner-Fraser, 2009).

As an example of the complexity involved in signaling pathways and NC development, in both frog and chick embryos, *Snai1*, *Snai2*, and *Twist1* are expressed in the developing mesoderm during gastrula stage, just prior to the induction of NPB specifiers and then re-expressed in the NC cells prior to and/or during migration (Xenbase and GEISHA) (G. W. Bell, Yatskievych, & Antin, 2004; Bowes et al., 2010; Darnell et al., 2007; James-Zorn et al., 2013; Karpinka et al., 2015). Evidence in frog embryos has shown that TWIST and SNAI2 proteins have functional redundancy and are required for mesoderm induction. Knockdown of *SNAI1* prevents *Snai1*, *Twist1* or *Snai2* gene expression and leads to a loss of mesoderm; however, only

loss of SNAI2 reduces NC cells (Zhang & Klymkowsky, 2009). Additionally, the NC phenotype is caused by the concurrent decrease in the mesodermally secreted factors, BMP4 and WNT8, which suggests that the morphogen pathways work in concert with the NC transcription factors to regulate NC specification (Shi et al., 2011). These studies identify SNAI2 as a key regulator of BMP and WNT-dependent NC specification, which complicates NC development because it suggests that feedback and feed-forward loops are involved. We attempt to dissect some of the major interactions in subsequent sections.

Recent reviews have detailed multiple signaling pathways (BMP, FGF, WNT, Notch, etc.) and how they control the development of the ectoderm and its derivatives, including the fate choice between neural, NC, epidermal and placodal cells and maintenance of those tissues (Kiecker, Bates, & Bell, 2016; Patthey & Gunhaga, 2014; Pegoraro & Monsoro-Burq, 2013; Rogers et al., 2012; Schille & Schambony, 2017; Schlosser, 2014; Stuhlmiller & Garcia-Castro, 2012a). Therefore, here we focus on the most recent experimental evidence and newly identified roles for signaling pathways in NC specification (Table 1).

3.1 | Signaling crosstalk in NC specification

3.1.1 | BMP–FGF crosstalk

The BMP and FGF pathways constantly seem to be intermingled in many developmental processes including neural induction (Cajal et al., 2014; Ishimura et al., 2000), mesoderm induction (S. Y. Lee et al., 2011; Northrop et al., 1995), and NC induction (Garnett, Square, & Medeiros, 2012; Yardley & Garcia-Castro, 2012). FGF signaling can modulate the expression of the *Bmp* transcripts, and its downstream mitogen-activated kinase (MAPK) pathway can alter the response to BMP signaling (Pera, Ikeda, Eivers, & De Robertis, 2003). In contrast, blocking BMP in frog embryos results in upregulation of *Fgf4* during the induction of neural tissues (Marchal, Luxardi, Thome, & Kodjabachian, 2009). However, the FGF family is very large, and different FGFs can either function in concert or act as antagonists of the BMP signaling pathway. It appears as though minor changes in signaling affect NC development, but the resulting NC phenotype are determined by whether the interaction is complementary or antagonistic.

In frog embryos, BMP signaling has been linked to the expression of NPB and NC specifier genes as both a positive and negative regulator (Garnett et al., 2012). Excess BMP induces nonneural ectoderm development, while too little creates NP cells (LaBonne & Bronner-Fraser, 1998). Most experiments that have tested the necessity and sufficiency for BMP/FGF/WNT signals used ectodermal progenitor cells (explants or animal caps), but a recent study in avian NP explants demonstrated that BMP4 alone was able to induce NP (mid-gastrula stage) explants to form migratory NC cells that expresses *Msx1*, *Zic1*, and *Snai2*. In contrast, NP explants treated with both BMP and FGF became placodal-like cells expressing high levels of *Six1*, *Dlx5*, and *Eya2*, suggesting the levels of BMP and FGF are tightly regulated to differentiate between NPB formation and NC formation (Shigetani, Wakamatsu, Tachibana, & Okabe, 2016). Additionally, the results suggest that even after the ectodermal derivatives have been specified, they may still be competent to respond to morphogens. Data from their interactions during early development suggest that FGF signaling functions to attenuate BMP signaling prior to NC specification, and whether the interaction leads to induction or inhibition of NC cells depends on which FGF ligand is involved as well as the embryonic stage of the organism. Experiments in mice demonstrated that in *Fgf3* null mutants, loss of FGF3 in caudal tissues resulted in expanded neuroepithelium and premature NC specification caused by increased BMP (Anderson et al., 2016). In frog embryos, ETS1, which is a direct target of MAP Kinase signaling downstream of FGF signaling, attenuates BMP signaling by binding to the *Id3* promoter with histone deacetylase (HDAC). This interaction is not required for NC specification, but affects later NC migration (C. Wang et al., 2015). However, in support of FGF-BMP teamwork, signaling from both pathways (as well as WNT) is both necessary and sufficient for the induction of ectodermal derivatives such as placodes and NC cells (Hong, Park, & Saint-Jeannet, 2008; Watanabe, Kanai, Matsukawa, & Michiue, 2015). Additionally, recent work in chick embryos showed a two-step model where FGF signaling is required in the ectoderm prior to NC induction to inhibit BMP and allow for NPB specifier expression, but BMP is subsequently required to maintain the NPB and NC population (Stuhlmiller & Garcia-Castro, 2012b). Signaling through both the BMP and FGF pathways is required for patterning of the early embryo and NC specification. Proteomic analyses are necessary to determine which specific ligands and receptors are active at the correct stages and in the appropriate spatiotemporal locations to regulate NC specification. Additionally, transcriptomic analysis after perturbation of these pathways would elucidate which gene targets they activate or repress.

3.1.2 | WNT signaling intersections

Early experiments in frog, chick, and human-induced NC cells demonstrated a requirement for WNT signaling in the induction of NPB specifier genes in vivo and in vitro (Bang et al., 1999; Leung et al., 2016). *Fgf* and *Wnt* gene expression are most pronounced in the posterior regions of both chick and frog embryos at gastrula and neurula stages (Figure 2a,d), while *Bmp* is expressed in both the posterior mesoderm (Figure 2a,c) and the ectoderm (Figure 2b,d). Interestingly, *Wnt8A* (Figure 2d), which has been implicated in NC development in both chick and frog (Hong et al., 2008), is expressed throughout the posterior mesoderm and neuroectoderm in chick embryos, and in the posterior mesoderm in frog (Ladher et al., 2000).

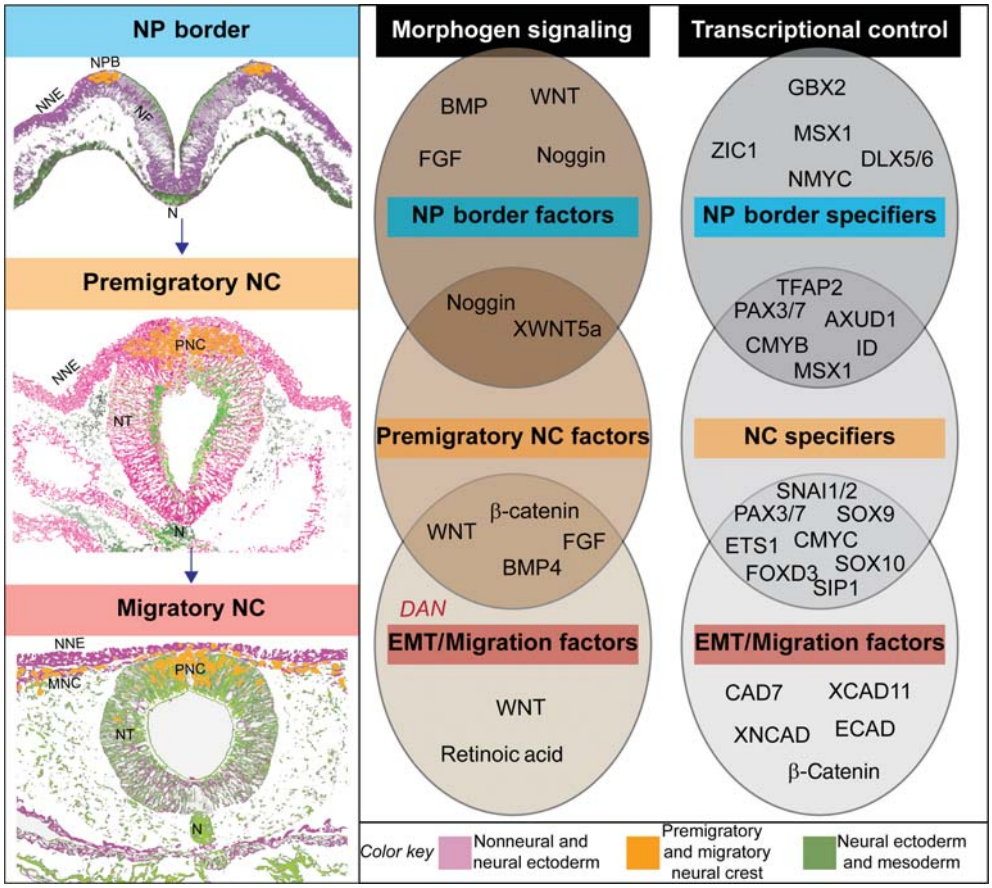

FIGURE 3 Developmental stages and molecules involved in NC specification and migration. Prior to differentiation, NC cells go through three stages (left column), neural plate border (NPB), premigratory NC (PNC), and migratory NC (MNC). The nonneural ectoderm (NNE) develops lateral to the NPB cells during neurulation as the neural folds (NF) rise to form the neural tube (NT). At these early stages, signaling from WNTs, FGFs, BMPs, and BMP antagonists (Noggin, Chordin) (middle column) drives the specification of the NPB specifiers (right column). As the NT closes, NC cells are specified in the dorsal NT, and are marked by NC specifier transcription factors. Along with signaling from WNTs, BMPs, RA, and FGFs, the NC specifiers down regulate adhesive cadherins, upregulate migratory cadherins and the cells leave the neural tube. The neural and nonneural ectoderm is pink and green, premigratory and migratory NC are orange

WNT plays both antagonistic and promoting roles in NC development. Work in *Xenopus* and mouse embryos demonstrated that the WNT antagonist, DKK1 inhibits the formation of NC cells in the anterior neural fold, and that loss of DKK1 in those tissues caused ectopic anterior NC formation (Carmona-Fontaine et al., 2007). Due to the varying expression patterns and wide range of family members, the role of WNTs has been understudied in NC development. Until recently, there was little known about the link between signaling by the WNT ligand and its intracellular transcriptional messengers and their role in NC induction and specification. Both canonical and noncanonical WNT signaling play a role in the formation of NC cells, and the specification of the NPB is dependent on both. A recent study in chick embryos identified the *Axud1* gene as a direct WNT1/β-Catenin target that not only interacts with two NPB master regulators (MSX1 and PAX7), but functions to activate the expression of *Foxd3*, an NC specifier (Figures 3 and 6) (Simoes-Costa et al., 2015). In mouse, NPB specification is modulated by the canonical WNT effector Grainyhead-Like 3 (GRHL3), which is also required for neural tube closure (Kimura-Yoshida, Mochida, Ellwanger, Niehrs, & Matsuo, 2015). The separation of neural ectoderm relies on the presence of WNT inhibitors such as DKK1, while the surface ectoderm utilizes GRHL3, and the specification of NPB and NC cells requires balance of both tissues (Kimura-Yoshida et al., 2015). Recent examples of noncanonical WNT signaling in *Xenopus* embryos demonstrated that ROR2 activates β-Catenin-independent signaling, and it is required for the formation of the NPB. *Xenopus* embryos mutant for ROR2 fail to restrict BMP signaling, which leads to a loss of NC cells (Schille, Bayerlova, et al., 2016), and PAR-1 expression is able to rescue the loss of NC due to a noncanonical WNT knockdown (Ossipova & Sokol, 2011). In addition to its traditional roles, WNT signaling also coordinates with BMP signaling to induce SOX9 phosphorylation and SUMOylation, which is required for its function as an NC specifier (J. A. Liu et al., 2013).

3.2 | Other signaling pathways

3.2.1 | Retinoic acid

RA, a morphogen derived from Vitamin A (retinol), functions in many aspects of development. RA is a unique morphogen in that it diffuses through cell membranes and is able to affect changes in gene expression directly (Duester, 2008) whereas the other molecules that we highlight function with a more traditional ligand-receptor signal transduction mechanisms. First, RA is required for the induction and patterning of mesodermal and NP tissues (Sive, Draper, Harland, & Weintraub, 1990; Villanueva, Glavic, Ruiz, & Mayor, 2002), which have been established as necessary elements to allow NC formation. RA has long been recognized as a modulator of NC migration, and when it accumulates in migratory NC, it functions as a teratogen and

causes defects in craniofacial morphogenesis (Table 1) (Jimenez et al., 2016; Laue et al., 2011; Martinez-Morales et al., 2011; Uribe, Hong, & Bronner, 2017; Watanabe, Goulding, & Pratt, 1988; Watanabe & Pratt, 1991). The early role of RA in patterning regulates NC specification and development, possibly due to the importance of RA in mesoderm specification. However, the RA pathway also intersects with other signaling pathways like BMP, FGF, and EGF to control development. It functions subsequent to BMP signaling to pattern anteroposterior mesoderm (Naylor et al., 2016) and endodermal cell types (Davenport, Diekmann, Budde, Detering, & Naujok, 2016). RA and FGF signaling pathways also function together to pattern the posterior portion of embryos and control NC migration (Martinez-Morales et al., 2011). For example, knockdown of the RA receptor-ϒ causes defects in NC progenitor cells in zebrafish embryos in addition to aberrant paraxial mesoderm development (Wai et al., 2015). There are contrasting studies with regards to RA in NC development. Both increases and decreases in RA appear to negatively affect NC cell survival and migration, suggesting that its levels are tightly regulated to allow for normal embryonic development (Chawla, Schley, Williams, & Bohnsack, 2016).

3.2.2 | Hedgehog family proteins

There is very little known about the role of Hedgehog family proteins (Sonic and Indian) in NPB and NC cell specification, although there is more known about their role in NC migration (Powell et al., 2015; Testaz et al., 2001; Tolosa, Fernandez-Zapico, Battiato, & Rovasio, 2016). However, two recent studies have begun to dissect the function of Hedgehog ligands and effectors in these early developmental processes. Indian Hedgehog, which is expressed in the mesoderm, is required for early and late NC cell specification as well as NC migration in *Xenopus* embryos (Aguero, Fernandez, Lopez, Tribulo, & Aybar, 2012). Additionally, loss of the receptor Dispatched-1, which is required for the secretion of lipid-modified Hedgehog proteins, inhibits cranial NC specification and results in craniofacial defects (Schwend & Ahlgren, 2009; Schwend, Loucks, & Ahlgren, 2010). More work needs to be done to determine the functions of Hedgehog and their downstream targets in NPB and NC specification.

3.2.3 | Cell adhesion, morphogenesis, and specification

Cell adhesion proteins have long been studied for their role in the process of NC EMT (reviewed in Barriga & Mayor, 2015; Strobl-Mazzulla & Bronner, 2012a; Taneyhill & Schiffmacher, 2017); however, recent analyses have determined that these proteins may also have additional roles in NC specification and induction. Recent evidence has determined that NC specifiers and EMT-inducing factors like SNAI2 have alternate early roles in cell fate specification. SNAI2 functions early to repress the expression of *P-Cadherin* in the chicken embryo allowing the mesendoderm to form, which later signals to the overlying ectoderm to form NC cells (Acloque, Ocana, Abad, Stern, & Nieto, 2017). Neural tube formation and NC cell specification are tightly linked. Adhesion molecules function to maintain the separation between the two tissues to allow for normal development. In mouse embryos, transcription factors like Grainyhead-Like-2 (GRHL2) directly preserve the nonneural ectoderm by maintaining the expression of E-Cadherin (ECAD) and preventing aberrant NC specification, and if they are lost, the NNE exhibits NC-like migratory phenotypes creating developmental defects (Pyrgaki, Liu, & Niswander, 2011; Ray & Niswander, 2016). Additionally, Cadherin-11 (Cad11) has been implicated as both a negative and positive regulator of cranial NC specification. Loss of function experiments in frog embryos using translation-blocking morpholino oligomers demonstrated that CAD11 actually increased NC cell proliferation in a canonincal-WNT dependent manner, suggesting that it must be present to control normal NC development (Koehler et al., 2013); however, the levels of CAD11 must be tightly balanced because loss of function experiments using dominant negative CAD11 constructs led to a β-catenin-dependent loss of NC cells (Borchers, David, & Wedlich, 2001). HDACs function to control the regulatory circuits that maintain the dynamic expression of cadherin proteins, and loss of HDAC function led to aberrant expression of Cadherin-6B (CAD6B) and the inhibition of NC specification in chick embryos (Murko et al., 2013). Additionally, the atypical cadherin WNT-PCP component Van Gogh Like 1 (VANGL1) is required in frog for the specification of a small subset, but not all, of NC genes to be expressed (Deichmann et al., 2015). Even with a small sample of instances where adhesion molecules were directly related to NC specification events, it is clear that multiple overlapping and parallel pathways control the process of NC specification (Box 1).

4 | THE PUTATIVE NC GRN

Studies from multiple organisms (frog, chick, zebrafish, mouse and lamprey) have provided evidence for the existence of a putative pan-vertebrate NC GRN that describes the hierarchical interactions between signaling molecules and transcription factors during NC formation (Figure 6) (reviewed in Betancur, Bronner-Fraser, & Sauka-Spengler, 2010a; Martik & Bronner, 2017; Meulemans & Bronner-Fraser, 2005; Simoes-Costa & Bronner, 2015). Inductive signals (BMP, FGF, WNT, Notch, and their antagonists) from the NP, nonneural ectoderm, and underlying mesoderm interact to establish a broad region at the border of the NP by activating a battery of transcription factors (NPB specifiers). The cells in this region are capable of giving rise to NC and dorsal neural tube derivatives. These NPB specifiers, through positive and negative regulatory interactions, further

BOX 1

INDUCED PLURIPOTENT STEM-DERIVED NC CELLS

The successful creation of induced pluripotent stem cells (iPSCs) from adult cells arose in 2006, when the Yamanaka lab published the factors required to reverse the process of differentiation (Takahashi & Yamanaka, 2006). Since that time, researchers have created specific protocols to differentiate these induced stem cells into multiple derivatives. The novelty, benefit, and relevance of human-iPSCs, and their ability to become multiple cell types including NC cells, has brought NC science out of the embryo model and into the realms of human therapeutics. Recently, the focus of multiple labs has been the creation of in vitro models of NC cells using iPS technology (Figure 4). The iPSC-NC cells can be used to study development, but also may be a useful tool for therapeutic treatment of neurocristopathies such as aganglionosis of the enteric nervous system, or cardiac defects. To create iPSC-NC cells that can be directly compared to NC cells in vivo (Figure 4a), multiple protocols have been identified. Primarily, non-NC cells (fibroblasts, keratinocytes, etc.) are isolated in vitro (Figure 4b) (V. K. Bajpai et al., 2017). To revert terminally differentiated cells like fibroblasts to a stem-like fate, cells are generally transfected or treated with exogenous Yamanaka Factors (OCT3/4, SOX2, KLF4, and CMYC) (Figure 4b). Next, those induced stem-like cells are treated with multiple factors including WNT/WNT inhibitors, BMP, FGF, and IGF and other factors that can force them to adopt an NC fate (Avery & Dalton, 2016; Menendez et al., 2013). Recent work demonstrated that the concentration of these factors is crucial. Use of a chemical FGF inhibitor SU5402 in iPSCs caused NC iPSCs to differentiate prematurely (Jaroonwitchawan, Muangchan, & Noisa, 2016). Similarly, some labs have created in vitro NC populations using direct differentiation of embryonic stem (ES) cells by treating ES cells with morphogens such as WNTs, BMPs and FGFs (Leung et al., 2016). To date, multiple labs have identified methods to differentiate human fibroblasts and ES cells into induced NC cells. The differentiation of these cells is marked and controlled by transcriptional regulators that are activated to induce NC cells in vivo such as Yes-Associated Scaffold Protein 65 (YAP) proteins (Hindley et al., 2016) and NC GRN-related transcription factors such as TFAP2A (Rada-Iglesias et al., 2012), PAX7, SNAI2, and FOXD3 (Kerosuo et al., 2015). These cells can differentiate into derivatives of NC cells including cartilage, melanocytes, neurons, and smooth muscle, cells. Induced NC cells can be used as another tool for NC research, but in addition, they are a step toward personalized stem-cell therapy treatments and cures for incurable diseases caused by abnormal development or differentiation of NC cells.

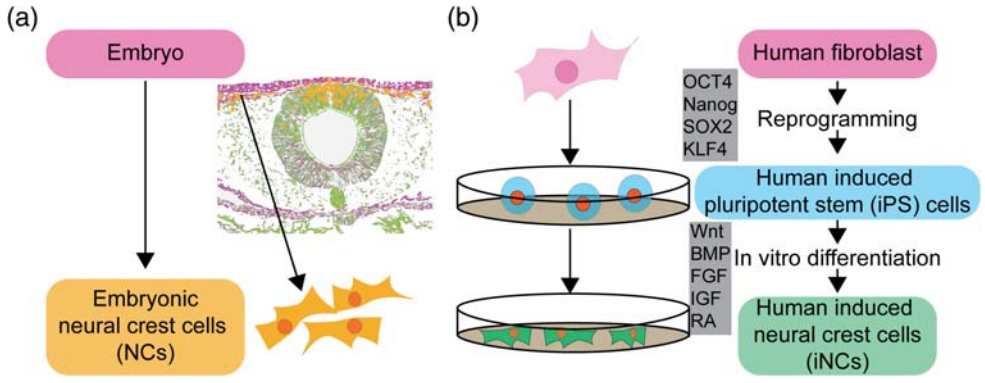

FIGURE 4 Embryonic NC cells vs. human induced NC cells (HiNCs). Schematic comparing (a) the development and isolation of embryonic NC cells versus (b) the technique used to create human induced NC cells from differentiated fibroblasts. To create human induced pluripotent NC cells, fibroblasts must first be de-differentiated by reprogramming them using the Yamanaka stem cell factors, Oct4, Sox2, Nanog, and Klf4. Then by using the appropriate media (various), those stem cells can be differentiated into NC cells in vitro

refine the NPB domain between NP and nonneural ectoderm. Then, the combinatorial expression of NPB specifiers activates another set of transcription factors (NC specifiers), which in turn regulate downstream effectors important for production of bona fide NC cells. This hierarchical activation of transcription factors endows premigratory NC cells in the dorsal neural tube with the ability to undergo EMT and become migratory. This section of the review will concentrate on the formation of premigratory NC in the cranial region.

4.1 | NPB specifiers

As described in the previous section, the initiation of NC development occurs early during gastrulation, concurrent with or subsequent to neural induction. The interactions of inductive signals activates the expression of a set of genes coding for

transcription factors, including *Tfap2*, *Zic1*, *Hairy2*, *Msx1/2*, *Dlx5/6*, *Pax3/7*, *Gbx2*, and *Foxi1/2* (de Croze, Maczkowiak, & Monsoro-Burq, 2011; Glavic, Silva, Aybar, Bastidas, & Mayor, 2004; Li, Kuriyama, Moreno, & Mayor, 2009; Matsuo-Takasaki, Matsumura, & Sasai, 2005; McLarren, Litsiou, & Streit, 2003; Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005; Nichane, Ren, Souopgui, & Bellefroid, 2008; Sato et al., 2005; Woda, Pastagia, Mercola, & Artinger, 2003). These transcription factors confer competence onto the NPB to form NC cells, but do not restrict the fate of these cells. In addition to NC cells, the NPB region can also give rise to neural tube roof plate cells, dorsal interneurons, sensory neurons like Rohon-Beard cells, and preplacode ectodermal precursors depending on the combinations of varying levels of NPB specifiers that can be asymmetrically expressed (Aruga, Tohmonda, Homma, & Mikoshiba, 2002; Bang et al., 1999; Goulding, Chalepakis, Deutsch, Erselius, & Gruss, 1991; Hong & Saint-Jeannet, 2007; Litsiou, Hanson, & Streit, 2005; Y. Liu, Helms, & Johnson, 2004; Mansouri & Gruss, 1998; McLarren et al., 2003; Tremblay, Pituello, & Gruss, 1996; Woda et al., 2003). These transcription factors also cross-regulate each other to stabilize their expression at the NPB, and their expression is often retained (e.g., PAX7 and MSX1 in the chick) in the progenitors through later stages of development. Below, we summarize recent findings about important NPB specifiers that are critical for NC specifier expression. Recent evidence from *Xenopus* embryos has introduced NKX6.3 as an additional NPB specifier protein that is sufficient to induce the majority of the traditional NPB and NC specifier genes (Figure 6) (Zhang et al., 2014). However, most NPB factors can individually activate only a subset of NC specifier genes, and only in combination can they carry out the entire NC specification program (de Croze et al., 2011; Feledy et al., 1999; Glavic et al., 2004; Hong & Saint-Jeannet, 2007; Li et al., 2009; Luo, Matsuo-Takasaki, Lim, & Sargent, 2001; Monsoro-Burq et al., 2005; Sato et al., 2005; Tribulo, Aybar, Nguyen, Mullins, & Mayor, 2003).

Transcription factor AP2-alpha (TFAP2) is an early marker of the NPB in *Xenopus* and in the basal vertebrate *Petromyzon marinus* (lamprey) (de Croze et al., 2011; Meulemans & Bronner-Fraser, 2002; Meulemans & Bronner-Fraser, 2005; Nikitina, Sauka-Spengler, & Bronner-Fraser, 2008). TFAP2 has a unique dual role during NC development (i.e., chromatin modification discussed later and NC differentiation): TFAP2 functions first during NPB development and then during NC specification (de Croze et al., 2011; Khudyakov & Bronner-Fraser, 2009; Nikitina et al., 2008; W. D. Wang, Melville, Montero-Balaguer, Hatzopoulos, & Knapik, 2011). Recent studies in frog have placed its early function upstream of *Pax3* and *Zic1* in NPB induction (Hong, Devotta, Lee, Park, & Saint-Jeannet, 2014). In *Xenopus*, *Tfap2* is directly activated by PRDM1A (BLIMP1), which is activated by Notch signaling in the NPB (Hernandez-Lagunas, Powell, Law, Grant, & Artinger, 2011; Powell, Hernandez-Lagunas, LaMonica, & Artinger, 2013). When establishing the NPB, TFAP2 activates many other NPB specifiers (Figure 6, *Hairy2*, *Msx1*, *Pax3/7*, *Zic1*, *Foxi*), and its expression is stabilized by positive feedback from MSX1 (Bhat, Kwon, & Riley, 2013; de Croze et al., 2011; Nikitina et al., 2008). Later, during NC specification, TFAP2 activates NC specifiers (*Snai1Snai1/2*, *Sox9*, *Sox10*), and this function is independent of its earlier NPB function (de Croze et al., 2011; Schorle, Meier, Buchert, Jaenisch, & Mitchell, 1996; Van Otterloo et al., 2012; W. D. Wang, Melville, et al., 2011). As one of the earliest NPB specifiers in *Xenopus*, *Tfap2* is an immediate target of WNT/β-catenin signaling, although direct binding of TCF/LEF elements on its promoter have not been verified. In zebrafish, TFAP2 may only function as an NC specifier as its expression is limited to prospective NC cells from late gastrula stages (Knight et al., 2003). However, TFAP2 has been identified as a facilitator of NC cell evolution based on its control of *SoxE* expression in lamprey embryos (Van Otterloo et al., 2012).

Gastrulation brain homeobox 2 (*Gbx2)* is another gene that is turned on at the NPB (de Croze et al., 2011; Li et al., 2009; von Bubnoff, Schmidt, & Kimelman, 1996). Similar to *Tfap2*, *Xenopus Gbx2* is a direct target of WNT signaling. The *Gbx2* promoter harbors TCF/LEF elements, and these elements are occupied by β-catenin during NPB specification (Li et al., 2009). In addition to activating the expression of *Pax3*, *Msx1*, and *Snai2*, GBX2 activates *Foxd3* robustly and it appears to be necessary for suppressing border cells from adopting a preplacodal fate (Li et al., 2009; Monsoro-Burq et al., 2005). Recent evidence from mouse embryos has suggested a putative feed-forward loop where PAX3 and ZIC1 activate the expression of *Gbx2* (Figure 6) (Bae et al., 2014). In frog embryos, TFAP2 cooperates with ZIC to complement the NC-specification function of GBX2, and TFAP2 may function to suppress a neural fate, since depletion of TFAP2 expands *Sox2* expression (de Croze et al., 2011). Thus, GBX2 and TFAP2 in combination with ZIC1 appear to be sufficient to induce a majority of the NPB genes and initiate the NC program.

Msh homeobox *(Msx*) expression and activity requires both WNT activity and graded BMP signaling. *cis*-Regulatory analysis demonstrated a BMP response element in the *Msx2* promoter (Brugger et al., 2004; Tribulo et al., 2003). Although direct binding sites for WNT effectors are not yet identified in the promoters for either *Msx* gene, MSX1 requires WNT activity and PAX3 to activate the expression of downstream transcription factors including *Snai2*, *Foxd3*, and *Twist1* (Monsoro-Burq et al., 2005). In fact, in chicken, MSX1 physically interacts with the WNT effector, AXUD1, to promote the transcription of *Foxd3* (Simoes-Costa et al., 2015). Supporting its role as an early NC inducer, *Msx1* is also expressed in the NPB in tunicates, which may be analogous to early NPB and NC cells (Stolfi et al., 2015). It is also required for suppressing the expression of

the NP marker, *Sox2*, supporting the possibility that MSX1 acts downstream of TFAP2 (de Croze et al., 2011; Monsoro-Burq et al., 2005).

Paired box (PAX3/7) and ZIC1 can synergistically activate multiple key NC specifiers such as *Snai1/2*, *Foxd3*, *Twist*, *Gbx2*, *Ets1*, and *Sox8/9* to trigger the NC developmental program, as well as activating regulators required to maintain signaling pathways for NC induction in frog embryos (Bae et al., 2014; Milet, Maczkowiak, Roche, & Monsoro-Burq, 2013; Monsoro-Burq et al., 2005; Plouhinec et al., 2014). At the NPB, *Pax3* and *Zic1* levels are controlled by BMP, WNT, and FGF signals, which collectively regulate the activity of *Pax3* and *Zic* enhancers (Garnett et al., 2012; Sanchez-Ferras, Bernas, Laberge-Perrault, & Pilon, 2014). Due to this combinatorial effect, the expression of *Pax3* and *Zic1* is not uniform at the NPB, and higher PAX3 levels favor a hatching gland fate while higher ZIC1 levels lead to preplacodal fate (Hong & Saint-Jeannet, 2007).

In addition to the well-known NPB specifiers listed above, new factors are emerging as regulators of these specifiers. CDX proteins, which regulate axial elongation in urochordates, have recently been shown to directly control the expression of the NPB and NC specifiers *Pax3*, *Msx1*, and *FoxD3* (Sanchez-Ferras et al., 2016). With the advent of new systems biology analytics, we imagine that the list of NP and NC regulators including transcription factors, signaling factors and epigenetic modifiers, will expand with time.

4.2 | NC specifiers

A second cohort of transcription factor genes: *Snai2*, *SoxE* (*Sox8*, *9*, *10*), *TFAP2*, *Twist*, *cMyc*, *Id*, *Foxd3*, *Ets1*, and *cMyb* are induced by the concerted action of NPB specifiers in frog embryos (Figure 6). The factors are required to generate NC cells that are competent to respond to migratory signals as well as to repress the neural fate. As with the NPB factors, these proteins also regulate the expression of each other. A subset of NC specifiers (e.g.,CMYC and ID3) and their regulatory circuits act to maintain the multipotency and suppress premature differentiation of NC cells (Light, Vernon, Lasorella, Iavarone, & LaBonne, 2005). At the same time, another subset of specifiers (FOXD3, SNAI1/2, SOX10, etc.) maintain their expression during NC delamination and migration, functioning to activate EMT and cell migration program by altering the expression of adhesion molecules.

ETS1 is a direct regulator of *FoxD3* and *Sox10* expression in chicken embryos (Betancur, Bronner-Fraser, & Sauka-Spengler, 2010b; Simoes-Costa et al., 2012). It is exclusively expressed in cranial NC cells, and its expression pattern is regulated by a combinatory input from TFAP2, PAX7, MSX1/2, SOX9, as well as positive feedback from FOXD3 and ETS1 itself (Barembaum & Bronner, 2013). ETS1 not only influences the activation of cranial-specific effector genes and regulates cranial-specific delamination, but also acts with other cranial NC proteins such as SOX8 and TFAP2, to drive the differentiation of NC cells along the skeletal lineage (Simoes-Costa & Bronner, 2016; Theveneau, Duband, & Altabef, 2007).

Both CMYC and ID3 play conserved roles in maintaining NC progenitors in an undifferentiated state as well as controlling the size of this progenitor pool (Bellmeyer et al., 2003; Kee & Bronner-Fraser, 2005; Kerosuo & Bronner, 2016; Light et al., 2005; Wei et al., 2007). *Id3*, a direct target of the proto-oncogene CMYC, controls the cell cycle to mediate the decision between proliferation and apoptosis, and help bias cells toward an NC lineage and away from NP fate (Kee & Bronner-Fraser, 2005; Light et al., 2005). In zebrafish, ID3 also acts downstream of the BMP5-MAPK pathway to regulate NC proliferation (Shih et al., 2017). Besides activating the NPB specifiers *Msx1* and *Pax3/7*, chicken CMYC also interacts with MIZ1 to regulate the survival and cell cycle progression of NC cells (Kerosuo & Bronner, 2016). In addition, the P53-mediated programmed cell death pathway also cross talks with NC regulators (e.g., PAX3, SNAI2 and ETS1) and regulates the balance between NC progenitor cell maintenance and EMT in both chick and mouse embryos (Rinon et al., 2011; X. D. Wang, Morgan, & Loeken, 2011).

FOXD3, SNAI1/2, SOX9, and SOX10 coordinate the establishment of a major NC cell trait, the ability to undergo EMT (Cheung et al., 2005). Specifically, these transcription factors change the cell adhesiveness by repressing different cell adhesion molecules. FOXD3, which is directly transcriptionally regulated by PAX7, MSX1/2, ZIC1, and ETS1, downregulates expression of the scaffolding gene, *Tspan18*, which is required for the maintenance of CAD6B expression in chick embryos (Fairchild, Conway, Schiffmacher, Taneyhill, & Gammill, 2014; Fairchild & Gammill, 2013; Simoes-Costa et al., 2012). In addition, FOXD3 overexpression leads to the down regulation of N-cadherin (NCAD) in chick embryos (Cheung et al., 2005). SNAI2, a direct WNT target (Vallin et al., 2001), binds to the E-box element of the *Cad6B* promoter and represses *Cad6B* expression (Coles, Taneyhill, & Bronner-Fraser, 2007; Taneyhill et al., 2007). In yet another regulatory loop, the *Snai2* regulatory region is directly targeted by the cytoplasmic tail of CAD6B in cooperation with β-catenin (Schiffmacher, Xie, & Taneyhill, 2016). SNAI2 also functions with partner proteins such as LMO4, a LIM adaptor protein, to act as a cofactor during EMT in neuroblastoma and in chicken NC cells (Ferronha et al., 2013; Ochoa, Salvador, & LaBonne, 2012). After being phosphorylated downstream of BMP and WNT signaling, SOX9 also cooperates with SNAI2 and maintains *Snai2* expression during EMT (J. A. Liu et al., 2013; Sakai, Suzuki, Osumi, & Wakamatsu, 2006; Yan et al., 2005). *Sox10*, which is directly regulated by SOX9, ETS1, and CMYB, can also repress *Ncad* expression in chick embryos (Betancur et al., 2010b; Cheung

et al., 2005). In *Xenopus* and zebrafish, *Twist* is expressed in premigratory and migrating cranial NC cells and the protein has been shown to physically interact with SNAI1/2 and regulate EMT (Lander et al., 2013) by repressing *Ecad* to promote cell dispersion and migration (Barriga, Maxwell, Reyes, & Mayor, 2013). In chick, where *Twist* is not expressed in NC cells at this stage, Smad interacting protein-1 (SIP1) seems to play a similar role in repressing *Ecad* and promoting the separation of delaminated NC cells (Rogers, Saxena, & Bronner, 2013).

4.3 | Added complexity to the GRN

The NC GRN in its current state is comprised of a hierarchical and looping network of transcription factors. The expression of some transcription factors and signals are reiterated or continued during various stages of NC development and most are not unique to NC. Rather, it is their unique combination at appropriate developmental stages that helps define the NC. In addition, other cellular processes, such as chromatin remodeling, posttranscriptional (microRNAs, lnRNAs, RNA binding proteins) and posttranslational modifications, and other signaling pathways (apart from WNT, Notch, BMP and FGF) act to modulate this network of transcription factors to define the "transcriptional" state of the NC along its path to becoming a migratory cell (Bonano et al., 2008; Gammill & Bronner-Fraser, 2002; Hu et al., 2012; Hu, Strobl-Mazzulla, & Bronner, 2014). Thus, the future NC GRN model will have to reflect this added complexity.

Another challenge is to generate a pan-vertebrate NC GRN and to reflect interactions at various axial levels as well as interspecies differences. We have updated the GRN to indicate species where direct interactions are shown (Figure 6), however, there are still many unresolved or indirect connections in the network. The current version of the NC network describes formation of the cranial NC, where most of the studies are performed (Betancur et al., 2010a). Recent studies have identified transcriptome differences between multiple axial levels of NC cells (Lumb et al., 2017; Trinh et al., 2017). Moreover, much of the data for the putative GRN comes from gain and loss of function studies from *Xenopus*, lamprey and chick. Collating all the data into one GRN is proving to be difficult. Another key issue is deciphering the temporal relationship between NPB specifiers. Due to inaccessibility/redundancy (mouse/zebrafish) and/or rapid induction and developmental differences of these cells (chick, frog, zebrafish) this issue remains unresolved. By focusing on *cis*-regulatory analysis of various key promoters, some of these issues can be resolved and each direct input to the GRN can be tested across multiple species.

The same signaling cues that control NC specification and formation in different vertebrate embryos and pattern the neural tube at different anteroposterior axial levels are also likely to instruct distinct subpopulations of NC cells to take on cranial, vagal, cardiac, or trunk fates. Strikingly, recent evidence in mouse embryos identified axial-level gene expression differences even within the cranial NC cells. Comparative analysis of the RNA-sequencing data from the migratory cranial crest streams between rhombomeres 1–2 and rhombomere 4 showed that there are approximately 120 transcripts that are expressed differently between the two closely related streams (Lumb et al., 2017). There are some differences in expression of the NC specifiers that control the cranial versus trunk fates (RNA-sequencing information showing genes upregulated in cranial NC in chick embryos can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75125). Recent evidence in chicken embryos demonstrated the ability to reprogram the trunk NC into a cranial identity by misexpressing the cranial-specific transcription factors SOX8, TFAP2B, and ETS1 in premigratory trunk NC cells, suggesting that the specific expression of each of the NC specifiers is tightly regulated for a purpose, to create different cell populations using the same/similar proteins.

4.4 | Chromatin remodeling

Epigenetic regulators facilitate the expression of NC specifiers at the correct time in development. New techniques such as the Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) are currently being used to identify active versus inactive enhancers of NPB and NC specifier genes (Figure 5, Box 2). ATAC-seq uses a mutated hyperactive transposase (TN5) to cut exposed DNA and simultaneously ligate the DNA fragments to adapters for PCR and sequencing, thus it can efficiently identify accessible chromatin regions, for example, active enhancers. In recent years, we have gained knowledge of how epigenetic modification regulates NC specification. For example, *Sox10* expression is regulated by both DNA methylation and histone methylation. DNMT3B binds to and methylates the promoter of *Sox10* to regulate the duration of NC production (Hu, Strobl-Mazzulla, Simoes-Costa, et al., 2014). Conversely, histone demethylase KDM4A (JMJD2A), removes the H3K9me3 repressive mark at the promoters of *Sox10* as well as several other NC specifiers (e.g., *Snai2*, *FoxD3*, *Sox8*) during NC specification (Matsukawa, Miwata, Asashima, & Michiue, 2015; Strobl-Mazzulla et al., 2010). At the same time, NSD3, a lysine methyltransferase, adds H3K36me2 active marks at the *Sox10* promoter, leading to its active transcription (Jacques-Fricke & Gammill, 2014). Additionally, the activity of SNAI2 is facilitated by chromatin modifications. SNAI2 represses *Cad6B* expression during EMT, which is mediated by the recruitment of HDAC repressive complex to the *Cad6B* promoter (Strobl-Mazzulla & Bronner, 2012b). SNAI2 also directly interacts with PRC 2 to repress *Ecad* expression (Tien et al., 2015).

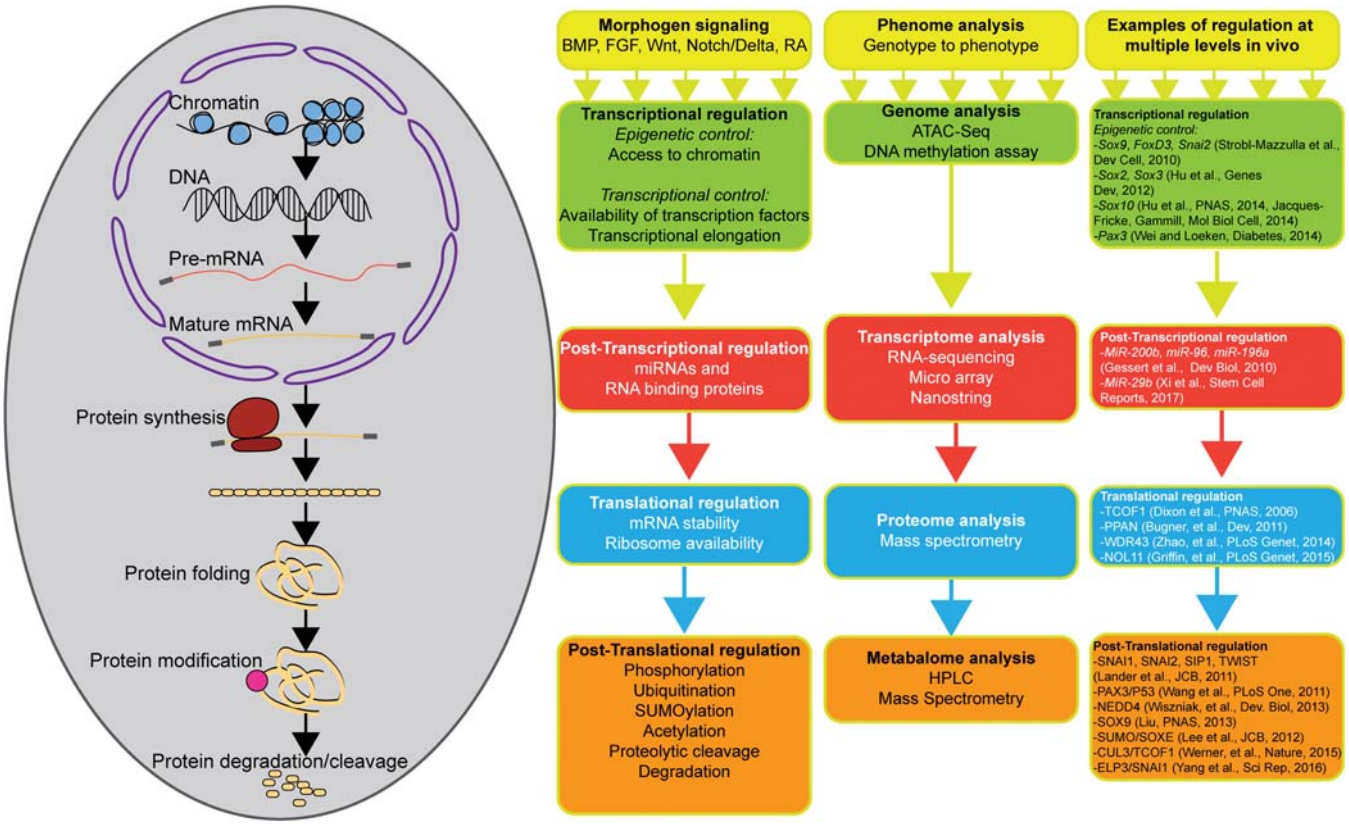

FIGURE 5 Recent technological advances have allowed more in depth study of the NC regulatory levels. Diagram shows the different intracellular control levels that regulate how and when NC cells form and the assays that can be used to perform systems analysis of NC formation. (Left) Depiction of a cell with multiple levels of genetic and proteomic regulation and where in the cell these processes take place (cytoplasm vs. nucleus). Specific types of cellular regulation are identified at three different levels: The general levels of regulation starting from extracellular morphogens that activate intercellular cascades followed by regulation of DNA transcription, mRNA availability, stability and translation, and protein stability, modification and degradation. Column 2 shows the types of analyses used to identify the changes at the previous levels. Finally, specific examples of genes, mRNAs, microRNAs and proteins that are regulated at these levels during NC development with references

The chromatin architecture influences the expression of NC genes. In *Xenopus* embryos and human induced NC cells, chromatin-remodeling complexes containing CHD7 and PBAF directly regulate the enhancer regions of *Sox9* and *Twist* thus promoting NC specification (R. Bajpai et al., 2010). Also in *Xenopus*, expression of *Hmga2* (which can bend the DNA double helix) is activated downstream of MSX1, and promotes the expression of multiple NC specifier genes including *Twist*, *Snai2*, *Sox9*, and *Sox10* (Macri et al., 2016). In addition to modifying the accessibility of transcriptional machinery, transcriptional elongation is also important for NC specification. In frog, CDK9 and Cyclin T1 of the positive transcription elongation factor complex (P-TEFb) are required for the expression of NC specifiers, *cMyc* and *Sox10* (Hatch et al., 2016).

4.5 | Posttranslational modification

The stability and activity of NC transcription factors is regulated by posttranslational modifications, such as phosphorylation, sumoylation and ubiquitination (Figure 5, Box 2). Such modifications target transcription factors to various subcellular compartments and alter their function, and as a result, affect the transcriptional readout of the cell in response to extrinsic signals. As described above, phosphorylation of SOX9 by PKA (cAMP-dependent protein kinase A) enhances SOX9 function, including its interaction with SNAI2, and treatment with a cAMP/PKA inhibitor prevents SOX9 induced EMT in quail embryos. The transcriptional activation of the *Snai2* promoter is also enhanced by PKA signaling (J. A. Liu et al., 2013; Sakai et al., 2006). Additional modification alters SOXE protein function; the small ubiquitin-like protein, SUMO, modifies SOXE factors (SOX9/SOX10) and PAX7 (Luan et al., 2013) in frog NC cells and promotes the maintenance of the NC progenitor pool as well as direct the specification of the otic placode instead of NC cells (P. C. Lee et al., 2012; Taylor & Labonne, 2005). Sumoylation of SOXE proteins inhibits NC specification by modifying their recruitment of cofactors and favors the corepressor GRG4 over the coactivator, CREB-binding protein/p300 (P. C. Lee et al., 2012). SNAI1, a highly labile protein that is expressed in *Xenopus* NC cells, but not chicken, is targeted for ubiquitination by GSK3β in the absence of active WNT signaling (Zhou et al., 2004). Similarly, SNAI2, TWIST, and SIP1 are modified by the E3 ubiquitin ligase PPA during EMT

BOX 2

MULTI-LEVEL REGULATION AND SYSTEMS APPROACHES TO NC BIOLOGY

Multi-level regulation of NC specification

New technology has allowed NC biologists to delve deeper into the intracellular environment to characterize the molecular processes that regulate the induction and formation of NC cells. There are multiple levels at which NC regulatory networks control the access to, expression of and stability of NC-related factors. Within the cell, there are four categories of regulation (Figure 5). The bulk of studies that have begun to analyze nontraditional methods of gene and protein regulation with regards to NC specification and development have been published in the past 10 years. This recent interest is most likely due to the invention of technologies that make the study of changes in chromatin (e.g., bisulfite sequencing, ATAC-seq), whole transcriptomes (e.g., RNA-seq, ChIP-seq), and identification of proteins (e.g., Mass-Spec) more accessible to basic research labs. ***Transcriptional and epigenetic regulation*** consists of both epigenetic modifications to the genomic DNA and chromatin as well as direct gene-level transcription events. Access to the enhancers and promoters of NC genes is tightly regulated by a host of epigenetic modifiers in vivo and in vitro during the formation of NC cells (Rada-Iglesias et al., 2012). Multiple NC-related genes in chicken such as, *Sox10* (Hu, Strobl-Mazzulla, Simoes-Costa, Sanchez-Vasquez, & Bronner, 2014; Jacques-Fricke & Gammill, 2014), *Sox9*, *Foxd3*, and *Snai2* (Strobl-Mazzulla, Sauka-Spengler, & Bronner-Fraser, 2010), *Sox2* and *Sox3* (Hu, Strobl-Mazzulla, Sauka-Spengler, & Bronner, 2012), and *Pax3* in mouse (Wei & Loeken, 2014), have been identified as targets of transcriptional control. At the chromatin level, recent studies in frog have shown that the Polycomb Repressive Complex (PRC) interacts with SNAI1/2 proteins to control the levels of histone H3K27 trimethylation on target promoters, which allows NC-promoting genes to be expressed (Tien et al., 2015). Additionally, DNA methyltransferase 3B (DNMT3B) methylates the *SOX10* promoter region to control the duration of NC production, while DNMT3A binds to the promoter regions of *Sox2* and *Sox3* and represses them allowing for the formation of NC cells (Hu et al., 2012; Hu, Strobl-Mazzulla, Simoes-Costa, et al., 2014). Transcriptional control can range from availability of transcription factors and their partners to the presence of transcriptional elongation factors such as CDK9 and CYCLINT1 of the P-TEFβ complex, which are also required for NC specification (Hatch et al., 2016). Additional transcriptional control can arise from combinatorial transcription regulators like YAP, which is required for *Pax3* expression in frog (Gee, Milgram, Kramer, Conlon, & Moody, 2011). ***Posttranscriptional control***: Although a number of microRNAs have been implicated in the processes of NC EMT (Banerjee, Dutta, & Pal, 2016) and derivative differentiation (Ding et al., 2016), less is known about their role in NC specification. However, in vitro studies in ES cells identified that *miR-29b* inhibited the NC fate and drove cells to the neural tube epithelium fate (Xi et al., 2017) while in vivo studies in frog embryos highlighted the importance of the RNA binding proteins FMR1 and FXR1 and the tight regulation of multiple miRNAs in the development of eye and craniofacial cartilage (Gessert, Bugner, Tecza, Pinker, & Kuhl, 2010). Ultimately, miRNAs, their functions and regulation remain understudied in embryonic development. ***Translational and posttranslational control***: Evidence exists that there are controls at the pre- and posttranslational levels for both activators and inhibitors of NC crest formation. At the translational level, proteins like mouse TCOF1 (Dixon et al., 2006), *Xenopus* Peter Pan (PPAN) (Bugner, Tecza, Gessert, & Kuhl, 2011), zebrafish WDR43 (Zhao et al., 2014), and *Xenopus* NOL11 (Griffin, Sondalle, Del Viso, Baserga, & Khokha, 2015) are required for ribosome biogenesis, and knockdown of the aforementioned proteins leads to craniofacial defects resulting from abnormal NC development. Due to the need for dynamic expression, some of the major NC specifiers are posttranslationally modified during development to regulate the stability of the proteins. For example, the *Xenopus* E3 ubiquitin ligase CUL3 in complex with its vertebrate-specific substrate adaptor KBTBD8 is implicated in Treacher Collin's syndrome due to its importance in NC specification (Werner et al., 2015). Even major NC transcription factors are posttranslationally modified. In chick embryos, the SUMOylation of PAX7 (Luan, Liu, Stuhlmiller, Marquez, & Garcia-Castro, 2013) and SOX9 (J. A. Liu et al., 2013) is required for NC specification and delamination, respectively. In *Xenopus*, the stability of multiple NC transcription factors including, SNAI1/2, Zeb2/SIP1 and TWIST is controlled by the E3 ubiquitin ligase, partner of paired (PPA) as well as ELP3 (Lander, Nordin, & LaBonne, 2011; Yang, Li, Zeng, Li, & Mao, 2016). Multiple components of the ubiquitin ligase pathway including mouse NEDD4 and *Xenopus* CUL3 (Werner et al., 2015; Wiszniak et al., 2013), are implicated in regulating NC cell development. Overall, the importance of regulating cells as complex as the NC is highlighted by the myriad of ways the genes and proteins that orchestrate their development is controlled. As new technologies arise, we are sure to discover additional factors involved at each of the regulatory levels.

A systems view of NC development

Molecular and cellular biology are no longer limited to candidate approach-based science due to the rapid advances in systems-level technologies, which has opened up a world of possibilities. With the advent of the “omics” and data

science, we are now able to study entire organismal changes at every level from pretranscriptional regulation to post translational (Figure 5). New studies of methylomics, genomics, transcriptomics, and proteomics have brought big data to the NC field. Additionally, with the inclusion of big data comes a need for bioinfomatic expertise. The identification of novel targets of NC-specific transcription factors using techniques like chromatin-immunoprecipitation followed by sequencing (ChIP-Seq) (Bildsoe et al., 2016; Tien et al., 2015) and RNA-sequencing (RNA-seq) after gene perturbation (Lumb, Buckberry, Secker, Lawrence, & Schwarz, 2017; Simoes-Costa et al., 2014) in addition to screens to identify open enhancers (Minoux et al., 2017) has increased the resolution of the NC GRN.

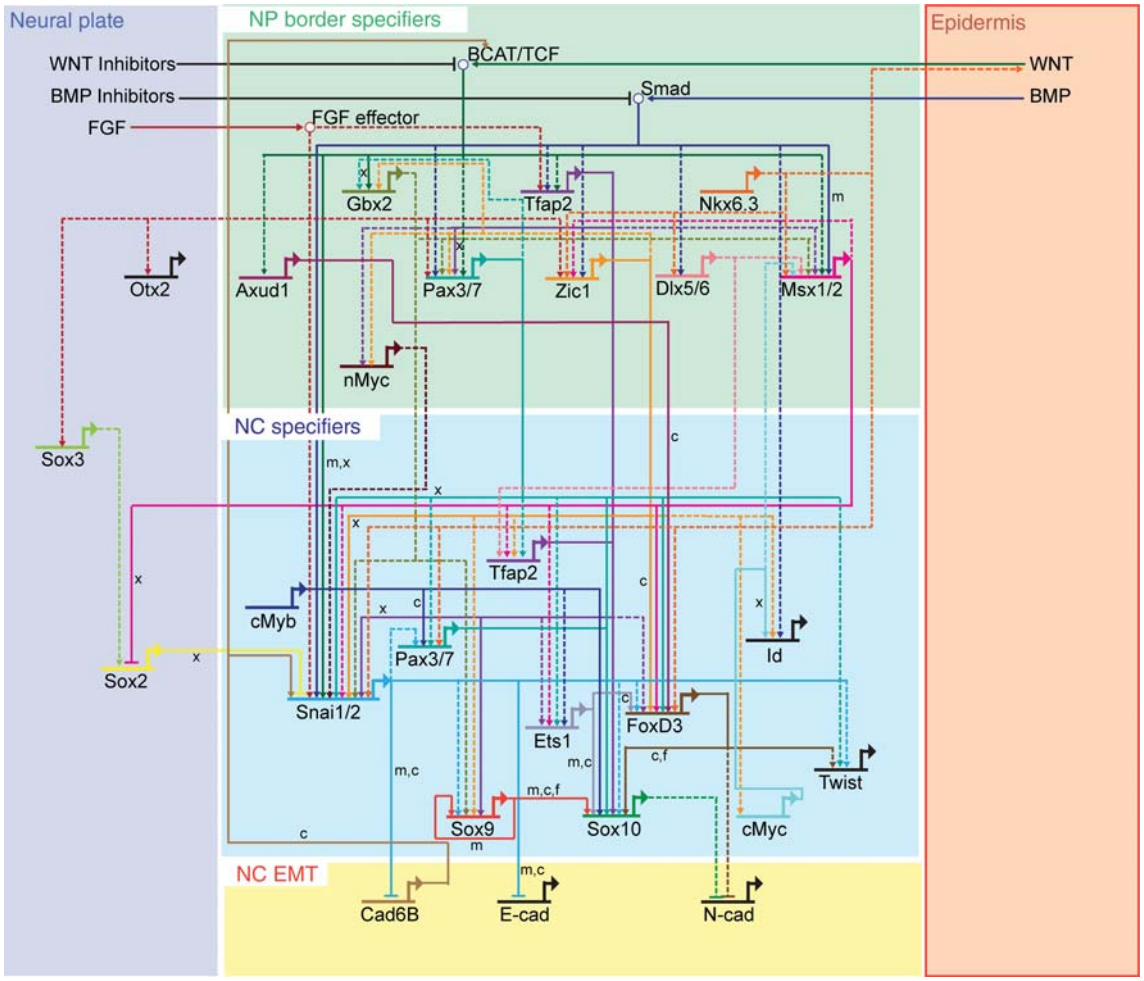

FIGURE 6 The NC gene regulatory network (GRN) is constantly evolving. This GRN was compiled from gene perturbation studies from multiple species including chick, frog, mouse and zebrafish. The GRN displays the regulatory interactions between signaling factors (top level) and their intracellular effectors and downstream transcriptional targets. The NP (left) and epidermis (right) are depicted on either side of the NC as they would be in a developing embryo. NPB specification (green), premigratory NC specification (blue), and NC EMT factors (yellow) are shown. Pax3/7, Tfap2 are both represented twice because they have been identified as both border specifiers and NC specifiers. Solid lines indicate direct regulatory interactions based on promoter and cis-regulatory analysis. Verified animal models are marked next to each direct interaction (c, chick; m, mouse; x, frog, f, fish). Signaling factors are represented as proteins in all capitals while GRN transcription factors are lower case and capitalized, but not italicized, to demonstrate that they represent both functional proteins and gene targets. Dashed lines represent functional interactions without evidence of direct interaction so far. Bubble nodes: Protein–protein interactions

(Lander et al., 2011). In addition, the ubiquitin ligase, NEDD4, promotes NC cell survival in mice by maintaining the expression of NC specifiers (Wiszniak et al., 2013).

5 | CONCLUSIONS

The induction and specification of NC cells requires many parallel and cross-talking pathways. Although the morphogenetic processes of vertebrate embryonic development are highly varied between organisms, the NC cells arise from the NPB cells, and similar signaling factors and GRN-players are implicated in the NC cell fate. NPB specifiers like MSX,

ZIC, and PAX3/7 are conserved throughout the vertebrates, and even play a role in the development of possible NC precursors in tunicates (Figure 1) (Green et al., 2015). Comparative analysis of the gene expression patterns of morphogens such as *Bmps*, *Wnts*, and *Fgfs* with both NPB specifiers (*Pax3/7*) and definitive NC specifiers (*Snai2*) shows that both *Bmps* and *Wnts* are expressed in similar domains and cells that will eventually give rise to or signal directly to NC cells, while *Fgfs* may play a less direct role in NC induction, but are clearly important (Figures 2 and 3). BMP signaling is required early in development to create a competent ectoderm that can respond to signaling from other pathways including WNT, FGF, RA, and Notch/Delta. There is also crosstalk between the signaling pathways, which controls the expression of NC specifiers and their targets (Figures 3 and 6). Once the embryos have begun the cellular movements of gastrulation, which aligns tissues that send signals to the NPB, the NC GRN is initiated, and the NC specifiers not only interact with and activate each other, they also progress NC development by altering the expression of cell adhesion molecules and allowing the process of EMT to commence. Recent research demonstrated that epigenetic control and access to chromatin is an important regulatory step in controlling the timing of these events, which differ between organisms. By late gastrula stage in frog embryos for example, NC cells have already been specified and express NC specifier genes such as *Snai2*, and *SoxE* genes, but in chick embryos, the NPB is only just forming in mid-gastrulation (Figure 2). With the advent of new technologies like ATAC-Seq (Figure 5), the study of NC cell development has transcended candidate-based approaches and we have entered the era of systems-level approaches to questions about NC formation. In addition, with the ability to reprogram adult cells into induced pluripotent NC cells (Figure 4), researchers are now able to perform NC research outside of the embryo, and conceive of using these cells for personalized therapeutics. Although the future of NC biology lies in systems approaches, there will always be a need to follow the candidates and refine the networks that control the development of this important vertebrate innovation.

ACKNOWLEDGMENTS

We apologize to investigators whose relevant work has not been cited because of space constraints. We would like to thank Lisa Sorrells from CSUN for her contributions to the graphical abstract.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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How to cite this article: Rogers CD, Nie S. Specifying neural crest cells: From chromatin to morphogens and factors in between. *WIREs Dev Biol*. 2018;e322. https://doi.org/10.1002/wdev.322