INTRODUCTION

The neural crest (NC) is a transient embryonic cell population originating from the ectoderm, in a region lateral to the prospective neural plate. NC cells (NCCs) carry the remarkable ability to migrate in the embryo and differentiate into a vast number of derivatives. Evolutionarily, the NC played an essential role in the emergence of two of the defining features of the vertebrates: an advanced craniofacial skeleton and specialized paired sensory organs [1]. NCC are multipotent with the ability to give rise to cell types as diverse as neurons, glia, smooth muscle cells, melanocytes, chondrocytes, and odontoblasts. Because of these unique properties, the NC has
attracted the attention of developmental biologists for over a century. It is an excellent model for exploring fundamental mechanisms underlying embryonic induction, cell migration, cell fate determination, and differentiation. More recent work has also focused on the stem cell-like properties of the NC and its potential use in regenerative medicine (reviewed in [2,3]).

Several human diseases have been associated with abnormal development of the NC and its derivatives. They are collectively known as neurocristopathies. These diseases can be the result of defects in NC specification, migration, proliferation, survival, or differentiation. Because of its contribution to multiple lineages, abnormal development of the NC often results in a wide array of clinical manifestations, affecting multiple organ systems. They include conditions such as Waardenburg-Shah syndrome (aganglionic megacolon, hypopigmentation, and deafness), DiGeorge syndrome (thymic hypoplasia, craniofacial and heart defects), or Treacher-Collins syndrome (cleft palate, micrognathia, and deafness). The characterization of the regulatory inputs controlling NC formation is therefore critical to understand how these processes may be altered in neurocristopathies.

The formation of the NC is a multistep process regulated by a complex set of signaling events. The process is initiated at gastrulation by the induction of NC progenitors at the lateral edges of the neural plate, a region where neural and non-neural ectoderm meets. As the neural plate closes into a tube, NC progenitors occupy the most dorsal aspect of the neural tube. NCC eventually delaminate from the neuroepithelium and migrate along specific routes throughout the embryo (Figure 2.1). Upon reaching their final destination, NCC differentiate into specific cell types depending on their origin, history, and final position in the embryo. Here, we review the molecular players directing NC induction and specification, comparing information from four model organisms: mouse, chick, *Xenopus*, and zebrafish.

**FIGURE 2.1** NC development during the morphogenetic movements of neurulation. At the beginning of neurulation, the NC is located at the boundary between the neural plate and the non-neural ectoderm, in a region known as the neural plate border (NPB). As the neural plate folds into a tube, the NC-forming region ends up at the dorsal aspect of the neural tube. In most vertebrates, neural crest delamination and migration starts upon neural tube closure.
NC INDUCTION AT THE NPB

At the end of gastrulation, the ectoderm of the vertebrate embryo can be divided into three major domains: the non-neural ectoderm and the neural plate separated by a region known as the NPB. While the non-neural ectoderm and neural plate will develop into epidermis and central nervous system, respectively, in most species the NPB gives rise to the NC but also to another transitory cell population, the pre-placodal ectoderm (PE). The PE eventually segregates into cranial placodes that contribute to the paired sense organs (nose, ear, and lens) and to the cephalic peripheral nervous system (cranial ganglia). In anamniotes, the NPB also gives rise to primary sensory neurons (Rohon–Beard neurons) in the trunk region, and to hatching gland cells anteriorly (Figure 2.2). Topographically, the PE is positioned lateral to the NC within the head, except for the most anterior region, where the PE directly abuts the neural plate.

Besides their common origin at the NPB, NC and PE share a number of important features. Some of the same signals involved in the generation of the NC have also been implicated in the induction of the PE [4–7]. Moreover, NC and PE have a similar ability to delaminate from the epithelial structure.
from which they originate, and differentiate into a large array of cell types, including sensory neurons, glia, and supporting cells [8]. However, the NC also has a set of unique characteristics: it develops a much broader repertoire of cell types as compared to the PE, including pigment cells, cartilage, and smooth muscle cells (Figure 2.2), and it will also migrate over greater distances. In addition, unlike cranial placodes, NCC are not exclusively restricted to the head region; they arise from the entire length of the neural tube, starting from the mesencephalon.

The induction of the NC is a classical example of embryonic induction. A group of cells located at the NPB segregate from neighboring cells in response to inductive signals produced by surrounding tissues. As a consequence of these inductive interactions, a first set of transcription factors is activated that define the NPB (NPB specifier genes). In turn, these factors activate another set of genes more restricted to the NC territory, known as NC specifiers. The NC specifiers are thought to regulate the expression of downstream NC effector genes implicated in the control of NCC migration and differentiation (reviewed in [9,10]).

Timing and Tissues

The NC-forming region is located at the boundary between the neural plate and the non-neural ectoderm, and sitting atop the mesoderm. Because of their position relative to the NC, each one of these tissues has been proposed as a source of NC-inducing signal. There are some differences in the relative contribution of these tissues to NC induction among vertebrates. This is presumably related to differences in the timing of NC induction, as well as differences in the precise mechanisms of neurulation employed by each one of these organisms (Figure 2.3). For example, the sequential activation of NPB and NC specifier genes occurs in a matter of hours in Xenopus and zebrafish [13,21,22], while the same process takes much longer in birds [23]. The activation of NPB and NC specifier genes occurs at gastrulation in fish and frogs, while it is initiated at the onset of neurulation in chick and mouse embryos [11–20]. Another difference is in the timing of NCC migration. For example, unlike other species, NCC migration in the mouse is initiated prior to neural tube closure, and shortly after NC progenitors have acquired their identity [20,24].

By analyzing the expression of NC specifier genes such as Snail2, Foxd3, or Sox8/9/10 (reviewed in [25,26]), it has been possible to monitor NC induction. It is now well accepted that NC induction is a multistep process initiated during gastrulation and persisting at least until neural tube closure. Two steps can be distinguished: first, the induction of NC progenitors at the gastrula stage, followed by the maintenance of these progenitors at the neurula stage. This was demonstrated experimentally in both Xenopus and chick embryos. For example, NPB explants downregulate NC-specific genes, which indicates that further signaling events are required to elicit NC fate [27,28].

Transplantation experiments performed in the early 1990s using Axolotl embryos demonstrated the importance of the interaction between the neural plate and the non-neural ectoderm to generate the NC [29]. These findings were subsequently confirmed and expanded in Xenopus and chick embryos [30,31]. For example, explants of neural plate do not generate NCC when cultured in vitro; however, grafts of neural plate explants into the adjacent non-neural ectoderm induces expression of NC genes at their boundary. Furthermore, lineage tracing studies have demonstrated that under these experimental conditions NCC are derived from both tissues, the neural and non-neural ectoderm [30,31].
However, recent work in *Xenopus* has challenged this view. By grafting neural plate explants into ventral ectoderm, Schlosser and colleagues [32] have shown that NC-specific genes were exclusively restricted to the graft (neural plate), while PE-specific genes were induced on the non-neural side. Therefore, it has been proposed that competence to form NC is confined to the neural plate, while the non-neural ectoderm is only competent to generate PE [32], suggesting that NC induction is directly dependent on the induction of neural plate tissue. This work also implies that NC is induced in response to non-neural-derived...
signals, whereas the PE would require neural plate-derived signals. This possibility is consistent with transplantation experiments in chicken embryos showing that epidermal/neural plate interactions are sufficient to generate neural crest derivatives [30].

Early work in Uroledes demonstrated that lateral plate and paraxial mesoderm explants transplanted into the blastocoel of gastrula-stage embryos had the ability to induce ectopic NCC [33]. Since then, a number of studies have confirmed the importance of paraxial mesoderm for NC induction in *Xenopus* [34–36]. The NC specifier gene Snail2 was activated in naïve ectoderm after recombination with explants of dorsolateral marginal zone (DLMZ) isolated at the gastrula stage [34,36]. Conversely, removal of the DLMZ inhibited Snail2 expression in the embryo [35]. More recent fate map studies in *Xenopus* have attempted to identify the specific region of the mesoderm source of the NC-inducing signal. These studies indicate that the DLMZ at the gastrula stage eventually give rise to part of the intermediate mesoderm, which is positioned directly underneath the NC-forming region at the neurula stage [28]. They further provided evidence that signals from the DLMZ participate in NC induction during gastrulation, and that signals derived from the intermediate mesoderm were involved in the maintenance of the NC identity at the neurula stage [28].

In chick, recombination experiments between somitic mesoderm and neural plate explants can elicit formation of NCC [30]. However, there is also evidence that NC induction can occur in epiblast explants isolated at the gastrula stage, independent of the formation of mesoderm [16]. Zebrafish embryos, in which mesoderm formation and involution have been blocked by disruption of Nodal signaling, can still form NC progenitors, suggesting that the mesoderm and its derived signals are dispensable for NC formation in fish [37]. However, in these embryos the DLMZ adjacent to the presumptive NC territory is normally specified [38,39] and expresses Wnt8, a known NC-inducing signal [40]. Therefore, a role for mesoderm in NC induction cannot completely be ruled out based on these studies. Rather, the work suggests that the involuting mesoderm may not be required for the maintenance of NC progenitors.

In mouse, the contribution of the ectoderm and mesoderm to NC induction has not been addressed experimentally due to the difficulties in accessing the corresponding tissues in the developing embryo. In mouse mutants lacking part of the paraxial mesoderm, NC induction appears largely unaffected [41,42]. However, these mutants do not show a complete loss of mesoderm, and therefore we cannot exclude the possibility that the remaining tissues may have retained some NC-inducing activity. There is no information on the importance of the interaction between the neural plate and non-neural ectoderm in the generation of the NC in mammals.

**Signaling Factors**

At least three distinct classes of signaling molecules have been implicated in NC induction: the bone morphogenetic proteins (Bmp), the Wnt family of glycoproteins, and fibroblast growth factors (Fgf). While they all appear to have some role in NC formation, their relative importance in this process varies among species. Here, we summarize the past decade of work analyzing the involvement of these signaling pathways in NC induction.

**Bone Morphogenetic Protein Signaling**

According to the current model of NC induction, the diffusion of Bmp antagonists produced by the axial mesoderm, including chordin, noggin, and follistatin, creates a gradient of Bmp activity in the overlying ectoderm, such that NC forms at levels of Bmp
signaling intermediate to those required for formation of the neural plate (low Bmp signaling) and non-neural ectoderm (high Bmp signaling). This model is primarily based on work performed in frog and fish. In *Xenopus*, modulation of Bmp signaling in ectoderm explants by graded expression of noggin or expression of a dominant-negative Bmp receptor can induce epidermal, NC, and neural plate fates in a concentration-dependent manner [35,43,44]. A similar gradient model was proposed based upon genetic analysis of Bmp signaling pathway mutants in zebrafish [45,46]. More recent work suggested that this gradient could be interpreted at the level of the downstream effectors of the Bmp pathway, Smad1/5. In this study, attenuation of Smad5 expression by injection of increasing amounts of Smad5 antisense oligonucleotides resulted first in an increase and then a loss of NC progenitors [47].

Earlier work in chick suggested that Bmps expressed in the neural folds and adjacent ectoderm might function as NC inducers. Conditioned medium from Bmp4- or Bmp7-transfected cells induced NC markers in neural plate explants, mimicking the NC-inducing activity of the non-neural ectoderm [48,49]. These observations were, however, challenged by a subsequent study [50]. While recombinant Bmp4 was a potent NC inducer in a culture medium containing additives (F12-N2), Bmp4 NC-inducing activity was completely lost when using a chemically defined medium (DMEM) [50]. These observations suggest that other signals synergize with Bmp4 to induce NC in chick neural plate explants. In the chick, the timing and pattern of expression of noggin, chordin, and follistatin are not completely consistent with a role in NC induction [51–53]. However, there is evidence that Bmp attenuation in the chick epiblast might be mediated through Fgf signaling rather than Bmp antagonists [54–56]. In *Xenopus*, active Fgf signaling is also required for neutralization of the ectoderm and NC induction by Bmp antagonists [57–60], although this activity has not been directly linked to a change in Bmp signaling levels.

A requirement for Bmp antagonists in the generation of NC is not as clearly established in mouse embryos. For example, chordin/noggin double mutant mouse embryos form an excess of NC progenitors [61,62], which is opposite to what the gradient model predicts. In Bmp2 null mutant mouse embryos, NCC are induced and express several early NC markers, however these cells failed to migrate, suggesting that Bmp2 is required for the production of migratory NCC [63,64]. Mouse mutant embryos for Bmp4 or for other components of the Bmp signaling pathway die early, before NC specification [65–71]. Conditional knockouts, using the Wnt1-Cre line, to specifically delete gene function in the NC lineage have primarily demonstrated a requirement for Bmp signaling later in NC development [72–75]. However, it is important to point out that Wnt1 is first expressed in the dorsal neural tube at a time point slightly downstream of these early inductive events, and therefore the importance of Bmp signaling in NC induction in mouse embryos remains an open question.

A couple of recent reports indicate that the sole inhibition of Bmp signaling cannot account for all aspects of NC induction in *Xenopus* and chick embryos [15,76]. Both studies demonstrated that Bmp signaling may have a distinct temporal requirement during NC induction (Figure 2.4): first, inhibition of Bmp signaling (in combination with a canonical Wnt; discussed below) at the gastrula stage, followed by a period of active Bmp and Wnt signaling to maintain the NC population at the neurula stage [15,76]. This two-step model, as well as the gradient model, strongly argues for an important role of Bmp signaling in the early steps of NC induction. However, there is also strong evidence that Bmp signaling alone is not sufficient to activate expression of NC
markers [50,77–79], and therefore it must act in concert with other signals to induce the NC.

**Wnt Signaling**

A large body of evidence strongly indicates that activation of canonical Wnt signaling is critical to specify the NC in fish, frog, and chick. The canonical Wnt pathway involves binding of the extracellular Wnt ligands to frizzled proteins (receptor) and LRP/arrow (co-receptor), which in turn signals through the cytosolic adaptor protein Disheveled (Dsh), leading to inhibition of GSK-3β and subsequent stabilization of β-catenin. Wnt-dependent gene expression is activated by β-catenin together with Tcf/Lef DNA binding factors (reviewed in [80]). Interfering with any components of the canonical Wnt pathway in the ectoderm, either using dominant negative forms of Frizzled 3 (Fz3), Frizzled 7 (Fz7), and their co-receptor Lrp6, or morpholino-mediated knockdown of Fz3, Fz7, Lrp6, Kermen, Dsh and beta-catenin

**FIGURE 2.4** Spatiotemporal regulation of NPB and NC specifier genes by signaling molecules. NC induction begins at the NPB, which is mediated by several inducing signals such as Fgf and canonical Wnt from paraxial mesoderm and non-canonical Wnt from the adjacent NNE and NP, and intermediate level of Bmp activity in NPB. These signals redundantly stimulate the expression of individual NPB specifiers, including genes of the Pax, Zic, Msx, and Dlx families. Among these genes Pax3 and Zic1 in turn activate the expression of NC specifiers such as Snail1/2, Sox8/9/10, Foxd3, Twist, Myc, Id3, and Tfap2a in presumptive NC. These NC specifier genes activate NC effectors (Trp2, Col2a, c-Ret, and c-Kit), which regulate the migration and differentiation of NCC along distinct lineages. This diagram is primarily based on work performed in *Xenopus* and may not apply to all organisms. For clarity, only a subset of NPB/NC specifiers and NC effectors are shown.
are all sufficient to block NC formation in the whole embryo [81–86]. Active canonical Wnt signaling is necessary for NC induction, but it is not sufficient to induce NC in naïve ectoderm, as it also requires attenuation of Bmp signaling.

In frog and fish, the source of the Wnt signal has been proposed to reside primarily in the paraxial mesoderm underlying the NC-forming region, while in chick, it is believed to originate from the non-neural ectoderm [40,50,87]. Wnt8 has been proposed as the endogenous NC-inducing signal in zebrafish, where it is expressed in a region immediately adjacent to the NC-forming region [40,88], and Wnt8 morpholino-mediated knockdown prevented expression of early NC markers [50]. In frogs, Wnt ligands have been proposed to reside in the non-neural ectoderm (Wnt7b) [89] and the paraxial mesoderm (Wnt8 [87] and Wnt3a [90]), as the spatiotemporal expression of these ligands appears to be compatible with NC induction. In Xenopus, knockdown of Wnt3a or Wnt8 prevented the expression of NPB and NC specifier genes [14,60,91]. In chick, the non-neural ectoderm expresses Wnt6 and has been proposed as the Wnt ligand involved in NC induction [50]. Consistent with this, Wnt6 overexpression enhanced NC markers expression, and Wnt6 siRNA-mediated knockdown reduced NC markers expression [92].

Mouse embryos with targeted inactivation of the β-catenin gene in the dorsal neural tube (Wnt1-Cre) have severe defects in several craniofacial skeletal elements of NC origin [93]. In these embryos, it is believed that signaling through β-catenin is required for survival and/or differentiation of cranial NCC [93]. Loss of Wnt1 and Wnt3a, both expressed in the dorsal neural tube, resulted in defects in a broad range of NC derivatives, and it has been proposed that Wnt signaling in the dorsal neural tube is primarily required for the expansion of NC progenitors rather than for NC induction [94,95]. More recently, it has been shown that sustained Wnt activity in mouse NC progenitors had little effect on the population size and instead regulated fate decisions [96]. All gene perturbation experiments in the mouse point to a role for canonical Wnt signaling in lineage specification and differentiation rather than induction (reviewed in [97]). However, an earlier role for Wnt signaling in NC induction cannot be completely ruled out, since Wnt ligands can act redundantly. Moreover, most studies thus far have targeted the Wnt signaling pathway in the dorsal neural tube using the Wnt1-Cre line, which may not interfere with early NC induction events.

A recent study has also implicated non-canonical Wnt signaling in NC induction [98]. Non-canonical Wnt ligands do not stabilize β-catenin or activate Tcf-dependent transcription; rather, they regulate changes in cell shape and motility through the activation of small Rho GTPases and Rho-associated kinases [99]. This branch of the Wnt signaling pathway has been more typically implicated in the regulation of NCC migration [100–102]. The non-canonical Wnt ligand, Wnt11R, is expressed in the neural plate immediately adjacent to the NC-forming region [103]. Wnt11R loss of function resulted in a marked reduction of Foxd3 and Sox8 expression and a partial loss of NPB genes such as Pax3 and Tfap2a [98]. The authors propose that non-canonical Wnts are acting by changing the localization and activity of the polarity kinase Par-1. Indeed, Par-1 is required for NC specification and can rescue NC-specific gene expression in embryos depleted of non-canonical Wnt ligands [98]. These observations are linking changes in cell polarity to cell fate specification, thereby raising the intriguing possibility that the cell shape changes occurring at the NPB during neural plate elevation and closure may be an integral part of the process of NC induction. This is not the first study proposing a role for non-canonical Wnt signaling in NC
induction. In chick, Wnt6 expressed in the non-neural ectoderm has been shown to mediate its NC-inducing activity independent of β-catenin [92].

**Fibroblast Growth Factor Signaling**

Historically, the first evidence for a role of Fgf signaling in NC induction came from work in *Xenopus*. In two experimental systems, dissociated cells [104] and ectoderm explants [43], basic Fgf (bFgf) in combination with attenuation of Bmp signaling was shown to induce pigment cells and Snail2 expression, respectively. More definitive evidence came from the overexpression of a dominant-negative Fgf receptor, which blocked expression of Snail2 in intact *Xenopus* embryos without affecting surrounding tissues [105]. Moreover, based on recombination experiments, it has been proposed that the non-neural ectoderm was the source of the Fgf signal [105].

More recent work has implicated Fgf8 as an inducer of NPB cells. Interference with Fgf8 signaling in the embryo prevented the expression of NPB genes [21,60]. Fgf8 is also capable of transiently inducing NC genes in an isolated explant assay without the supplement of Bmp antagonist; however, only a limited repertoire of genes were activated under these conditions [36]. Fgf8 is expressed in the mesoderm at gastrulation and has been proposed as the paraxial mesoderm-derived signal responsible for NC induction in *Xenopus* [36]. This proposal is based on the observation that explants of DLMZ, which normally induce NC markers in the ectoderm [34], were unable to induce NC when recombined with ectoderm explants made refractory to Fgf signaling by expression with a dominant-negative Fgf receptor (XFD). Because intact Fgf signaling is required for neutralization of the ectoderm by Bmp antagonists [57–59], an alternative interpretation would be that NC induction was blocked, not as a result of the inability of a DLMZ-derived Fgf ligand to signal in the ectoderm, but rather because the neutralization of these explants was impaired by expression of XFD [57]. Consistent with this possibility, the MAPK inhibitor, U0126, blocks neutralization by Chordin [59,60]. Consequently, it has been proposed that Fgf8 NC-inducing activity was likely to be indirect through the activation of Wnt8 in the paraxial mesoderm. While Wnt8 can rescue the expression of NC genes in Fgf8 morphants, Fgf8 is unable to rescue NC progenitors in Wnt8 or β-catenin-depleted embryos. Moreover, Fgf8 upregulates Wnt8 expression in the embryos, and Fgf8 morphants lack Wnt8 expression in the mesoderm [60].

In chicken, recent work indicates that Fgf activity is required in the ectoderm at gastrulation for NC specification. Expression of dominant-negative FGF receptor 1 at the gastrula stage prevented Snail2 and Pax7 expression in NC progenitors [56]. Interestingly, subsequent work indicated that activation of Fgf signaling in the epiblast upregulates both Bmp and Wnt signaling [106]. There is no direct evidence of Fgf signaling involvement in mouse NC induction.

A recent study in zebrafish has provided important information on the mechanism of integration of these signaling pathways in the generation of the NC [107]. In this work, the authors showed that the NPB genes Zic3 and Pax3a possess two enhancers differently regulated by Bmp, Wnt, and Fgf signaling. Zic3 and Pax3a each have an enhancer regulated by both Wnt and Fgf, as well as an enhancer regulated either by Wnt (Zic3) only or by Fgf (Pax3a) only. Changes in the relative influence/dominance of these enhancers may account for some of the differences in the importance of Wnt and Fgf signaling in NC induction in various species [107].

**Other Signaling Pathways**

Signaling through the membrane-bound protein Delta and its receptor Notch has also been implicated in NC formation in several
species. In zebrafish, Delta has been reported to be required for trunk NC formation, as Delta-deficient embryos form supernumerary Rohon–Beard sensory neurons in the trunk at the expense of NCC [108]. Interestingly, cranial NCC were unaffected in these embryos, suggesting that trunk and cranial NC are regulated by different mechanisms [108]. In *Xenopus*, constitutive activation of Notch signaling by expression of a Notch construct lacking the ligand-binding domain resulted in a dramatic expansion of neural tissues and prevented expression of epidermal and NC markers [109]. Using hormone inducible dominant-negative constructs, another study demonstrated that the timing of Notch activation was critical for NC formation. Activation of Notch at gastrulation resulted in a dramatic expansion of the NC territory without affecting surrounding tissues [110]. Notch-mediated expansion of NC progenitors is thought to occur through the activation of the transcription factor Hairy2, which in turn suppresses Bmp4 expression in the ectoderm [110]. Similarly in birds, Delta is involved in regulating Bmp4 expression levels in the ectoderm and thus is believed to be indirectly required for NC induction [110,111]. In mouse, Notch signaling has been primarily associated with NC migration, proliferation, and differentiation [112–114].

Retinoic acid (RA) signaling has been implicated in NC development as well. In *Xenopus*, RA can induce Snail2 expression in explants of anterior neural plate [115]. However, because RA functions as a posteriorizing signal in the neural tube, it was thought that Snail2 induction in these explants was secondary to the posteriorizing activity of RA [116]. Another study using *Xenopus* animal caps recombined with chick mesoderm or NP explants demonstrated that induction of Pax3 occurred independently of RA signaling, suggesting that this pathway is not directly implicated in NC induction [117]. In vitamin A-deficient quail embryos, cranial NC progenitors form properly, yet shortly after migration is initiated these cells undergo massive apoptosis [118]. Double knockout mice for the RA-degrading enzymes Cyp26a1 and Cyp26c1, which have excess RA signaling, showed normal expression of NC markers, although these embryos were affected by abnormal NC migration [119]. In the triple knockout mice for Cyp26a1/Cyp26c1, and the RA-synthesizing enzyme (retinaldehyde dehydrogenase 2; Raldh2), which presumably completely lack RA signaling, NCC were properly specified, and the NC migration defect was largely rescued in these embryos [119]. These observations suggest that endogenous levels of RA signaling in the mouse may not be essential for NC induction or migration.

Endothelin-1/Endothelin-A receptor signaling can regulate NC formation in *Xenopus*, and this pathway has been more specifically implicated in the induction and maintenance of NC progenitors [120]. The receptor (EdnrA) is detected at the NPB, and the ligand, Endothelin-1 (ET-1), is produced by the mesoderm underlying the NC territory. Interference with ET-1 signaling causes a loss of NC-specific genes and induces apoptosis indicative of a role in the maintenance of the NC progenitor pool at the neurula stage. Epistatic analysis indicated that this signaling pathway was acting downstream of the NPB gene Msx1 and upstream of the NC specifiers, Sox9 and Sox10 [120]. In mouse, ET-1 signaling is primarily required for the later phases of NC development. ET-1 is expressed in the epithelial layer of the branchial arches as well as in the mesodermal core, while the receptor is expressed in the arches mesenchyme [121,122]. Disruption of the pathway resulted in hypoplastic branchial arches due to apoptosis of the NC-derived arches mesenchyme [123].

Finally, Indian hedgehog (Ihh) signaling has been proposed to regulate multiple aspects of NC development in *Xenopus* [124]. Interference
with Ihh signaling using morpholino anti-sense oligonucleotides, dominant-negative constructs, and chemical inhibitors causes a loss of both NPB and NC genes, suggesting an early role in NC specification [124]. In addition, Ihh has a later function in promoting NCC migration through both autocrine or paracrine mechanisms [124]. Another member of the hedgehog family, Sonic hedgehog (Shh), has also been shown to regulate NC migration in chick [125]. In mouse, targeted deletion of the Shh receptor, Smo, in the neural crest lineage showed an important role for this signaling pathway in the patterning and growth of NC-derived facial skeletal elements [126]. There is no evidence that hedgehog signaling is involved in NC specification in amniotes.

**GENE REGULATORY NETWORK INVOLVED IN NC SPECIFICATION**

In response to signaling events mediated by Bmp, Wnt, and Fgf, distinct sets of transcription factors are sequentially activated at the lateral edge of the neural plate. A first set of genes, known as NPB specifiers, is initially broadly activated at the NPB, and their expression domain typically comprises the prospective NC tissue as well as other subdomains of the NPB. These transcription factors include several homeobox-containing proteins, Pax3/7, Mxs1/2, Dlx5, Tfap2a, and Gbx2, as well as zinc finger-containing factors of the Zic family. In turn, these factors activate a second set of genes more restricted to the NC territory, known as NC specifiers, which include, among others, genes of the Snail, Sox, and Fox family of transcription factors. These NC specifiers are thought to regulate the expression of downstream NC effector genes implicated in the control of NCC migration and differentiation. The proper expression of these three sets of genes in time and space is central to the specification of NC progenitors (Figure 2.4; reviewed in [9,127,128]). Here, we describe a subset of NPB and NC specifiers that have been identified in various species, and how they currently fit in the gene regulatory network underlying NC formation.

**NPB Specifier Genes**

In Xenopus, NPB specifiers include Msx1, Pax3, Zic1, Hairy2, Gbx2, Tfap2a, and Meis3 (Figure 2.4; for a comprehensive list of NPB specifiers, see [128–130]). They are all expressed in a region of the ectoderm that includes the prospective NC, and loss-of-function studies have demonstrated that they are required for NC formation in the embryo [13,14,21,131–135].

Among these factors, Tfap2a has been proposed as a “master regulator” of the NC regulatory cascade, as it is required for the expression of other NPB specifiers such as Msx1, Pax3, Zic1, and Hairy2 [134]. Consistent with this view, genome-wide analyses of chromatin marking patterns and transcription factors occupancy have shown that human NC enhancers are primarily occupied by TFAP2A [136], confirming that this transcription factor is a key regulator of NC fate. It is also important to mention that this regulation by Tfap2a is not unidirectional, since most NPB genes are also involved in maintaining each other’s expression or may act at multiple steps during NC development [134]. In zebrafish, Tfap2a function has been primarily associated with NC diversification [22], while the requirement of Tfap2a at the chick NPB formation has not been evaluated [23]. In mouse, Tfap2a expression begins at E7 (Figure 2.3), ahead of the activation of NC specifier genes [137], as predicted for a NPB specifier gene. Targeted deletion of Tfap2a resulted in perinatal lethality. Mutant mice showed severe craniofacial defects and failure of cranial neural tube closure, associated with increased apoptosis in the
midbrain/hindbrain region [138]. Interestingly, the expression of Pax3 was largely unaffected in these mutants [139], suggesting that Pax3 is acting upstream of Tfap2a, or that both factors are functioning in parallel pathways.

The transcription factors Pax3 and Zic1 are especially relevant to NPB formation since they are not only necessary but also sufficient to promote the formation of multiple NPB cell types. Gain-of-function experiments in the embryos have shown that Pax3 and Zic1 can promote hatching gland and PE fates [21], respectively, while their combined activity is essential to specify the NC [107]. Moreover, by manipulating the expression levels of Pax3 and Zic1 in naive ectoderm explants, it is possible to generate a pure population of NC progenitors, in the absence of other NPB cell types [21], further demonstrating the importance of the cooperation between Pax3 and Zic1 in promoting NC fate [21]. Interestingly, cells derived from ectoderm explants expressing Pax3 and Zic1 have the ability to migrate and produce a full repertoire of NC derivatives when transplanted into the embryo [140]. These observations suggest that a limited number of factors are sufficient to initiate the NC developmental program.

While Pax3 is essential for NPB formation in frogs, its paralog, Pax7, plays a critical role in NC formation in chick. Pax7 is the only factor identified so far with broad expression in the NPB at early stages that has been directly involved in NC specification in chick [16,141]. In mouse, Pax3 and Zic genes expression are initiated around E7.5 (Figure 2.3), a timing of expression that is consistent with a potential role as NPB specifiers [142–144]. In the Pax3 mouse mutant Splotch, the pool of NC progenitors fails to expand and to complete its migration, resulting in scarce cardiac NC derivatives [144,145]. Homozygous Splotch mice die in utero with persistent truncus arteriosus and pharyngeal arch patterning defects [144,146].

Because NPB specifiers are the first group of genes activated in response to extracellular signals, it is expected that their cis-regulatory sequences contain response elements for the signaling pathways implicated in NC induction (Figure 2.4). It is only recently that these potential direct interactions have been analyzed at the molecular level. In zebrafish, the two NPB genes Zic3 and Pax3a each possess two intronic enhancers, which are differently regulated by Bmp, Wnt, and Fgf signaling [107]. This study provides the first evidence that Pax and Zic gene families are directly activated by NC-inducing signals. Another NPB specifier, Gbx2, which is essential for the positioning of the neural folds and required for the expression of NC specifiers, is an immediate direct target of canonical Wnt signaling in Xenopus [132]. Moreover, rescue experiments have shown that Gbx2 act upstream of Pax3 and Msx1 [132], thereby positioning Gbx2 as one of the earliest factors mediating the Wnt NC-inductive signal. In Xenopus and zebrafish, Msx1 expression at the NPB is activated by attenuation of Bmp signaling [147]. Consistent with these observations, in mouse a Bmp-responsive enhancer has been identified upstream of the Msx2 gene, which appears to respond to intermediate levels of Bmp signal [148]. Msx1/2 double knockout mice have delayed NCC migration in the pre- and post-otic regions and elevated number of apoptotic cells in the NC populations contributing to the cranial ganglia and the first pharyngeal arch [149].

NC Specifier Genes

NPB specifier genes, which are broadly expressed at the NPB, are thought to activate a subset of genes with a more restricted expression in the NC lineage, known as NC specifier genes. Several families of transcription factors have been identified as NC specifiers, including
c-Myc, Id3, Snail1/2, Foxd3, Sox8/9/10, and Twist (Figure 2.4; for a comprehensive list of NC specifiers, see [128–130]). Typically, these genes are activated in NC progenitors before they initiate their migration, and their expression is either maintained or downregulated as NCC migrate in the periphery and differentiate along specific lineages. Their expression pattern in NC progenitors is well conserved across species, but there are also some variations in the precise timing of their expression.

For example, the SoxE family of transcription factors, which include Sox8, Sox9, and Sox10, has well-established roles in NC development (reviewed in [150–153]). While they all show expression in NC progenitors, they also show differences in their onset and sequence of expression in various organisms. In Xenopus, Sox8 is the first SoxE family member detected in the prospective NC at the late gastrula stage [154], immediately followed by Sox9 [155], while Sox10 is activated slightly later at the early neurula stage [156,157]. In zebrafish, Sox8 is not expressed in the NC; it is Sox9b that is first detected in NC progenitors [158], with a later onset of expression for Sox10 [159]. In contrast to other species, Sox10 is not maintained in migrating NCC in zebrafish. In chick and mouse embryos, the expression of Sox9 and Sox10 is initiated before Sox8 expression [160,161], and, at least in chick, Sox9 is the first SoxE gene expressed in NC progenitors [162,163].

Morpholino-mediated knockdown of Sox8, Sox9, or Sox10 in Xenopus results in the loss of expression of several NC specifiers, such as Snail2, Foxd3, and Twist [154,155,157]. However, because Snail2, Sox9, Foxd3, and Twist precede Sox10 expression in NC progenitors, Sox10 is more likely to be involved in maintaining the expression of these genes [156]. In zebrafish, Sox10 mutations do not prevent NC specification; instead, NCC fail to migrate and undergo apoptosis [159]. Similarly, Sox10 knockout mice exhibited massive NCC death in the trunk prior to or shortly after delamination [164]. In chick and mouse, it has also been shown that Sox9 functions in NC progenitors formation, delamination, and in the development of specific NC derivatives [163,165].

Regarding the upstream regulators of SoxE genes, very little information is currently available. In Xenopus, Sox9 expression is activated by Tfp2a [166], and in mouse, putative Tfp2a binding motifs have been identified within the Sox9 cis-regulatory region [167], suggesting that Tfp2a may directly regulate Sox9 expression. There is also evidence that Sox8 and Sox9 may directly regulate Sox10 expression in Xenopus [154]. A more recent study, using a combination of chromatin Immunoprecipitation (ChIP) and reporter assay, has demonstrated that Sox9, Ets1, and cMyb directly bind and activate a Sox10 enhancer in the chicken cranial NC [168].

The winged-helix transcription factor Foxd3 is expressed in the premigratory NC and is an important regulator of NC development in all vertebrates. In mouse embryo, Foxd3 is required for maintenance of multipotent NC progenitors. A NC-specific deletion of a floxed allele of Foxd3 resulted in a broad loss or severe reduction of multiple NC derivatives [24]. Foxd3 is also likely to control NC survival because these mutants have an increase in NC apoptosis. In zebrafish, knockdown of Foxd3 indicated that it is required for the differentiation of a subset of NC lineages but did not appear to be involved in NC induction or migration [169]. Zebrafish Foxd3sym1 homozygous mutants start with normal numbers of premigratory NCC [170]. Foxd3sym1 mutants have increased NCC apoptosis in a region of the hindbrain corresponding to third NC stream, indicating a role for Foxd3 in the survival of at least a subpopulation of NCC [170]. In chicken embryos, misexpression of Foxd3 within the dorsal neural tube causes an expansion of the NC domain [17,171]. Similarly, in Xenopus Foxd3 overexpression in
the embryo or in explants induced a broad array of NC-specific genes [172]. Expression of a dominant-negative Foxd3 construct inhibited NC differentiation in the embryo, a phenotype that can be rescued by Snail2 expression [172].

The cooperative activity of Zic1 and Pax3, in combination with inputs from Wnt signaling, has been proposed to regulate the NC expression of Foxd3 in *Xenopus* [13]. A more recent study has identified two Foxd3 enhancers (NC1 and NC2) that drive differential expression in the chick cranial (NC1) and trunk (NC2) NC. Mutational analysis, in vivo ChIP, and morpholino knockdowns demonstrated that the transcription factors Pax7 and Msx1/2 cooperate with the NC specifier gene Ets1 to bind to the cranial NC1 regulatory element. At the trunk level, Pax7 and Msx1/2 function together with the NPB specifier gene Zic1, which directly binds to the NC2 enhancer [161]. This work provides strong evidence directly linking Foxd3 expression in the NC territory to the activation of the NPB specifiers Pax3/7, Zic1, and Msx1/2.

The two members of the Snail family of zinc finger transcription factors, Snail1 and Snail2, have a critical role in NC development. Functional studies in chick and *Xenopus* demonstrate that overexpression of these genes resulted in an expansion of the NC territory, while their inhibition prevented NC formation and migration [173–175]. In *Xenopus*, Snail1 is expressed in the prospective NC slightly earlier than Snail2, and epistatic analysis indicated that Snail1 was an upstream regulator of Snail2 during NC development [175,176]. Indirect evidence suggests that Snail2 expression might be regulated by Zic1 and Pax3 in *Xenopus*, while Msx1 may carry that function in zebrafish [13,147]. Snail2 null mouse are viable and have normal NC formation, migration, and differentiation [177]. Homozygous null mutation in Snail1 is embryonic lethal because of its early role in gastrulation [178]. An NC-specific deletion of the Snail1 gene demonstrated that Snail1 was not essential for NC formation and delamination [179]. The double knockout of both Snail1 and Snail2 demonstrated that this gene family is not required for the generation, delamination, or migration of the NCC in mouse embryos [179], indicating that the Snail gene family does not have an evolutionary conserved function during NC formation.

The protooncogene c-Myc and its downstream effector Id3 have a fairly broad expression domain at the NPB in *Xenopus*. It includes both the prospective NC and PE [180–182]. Because c-Myc and Id3 expression is initiated prior to most NC specifiers, such as Snail2 and Sox9, it has been proposed that c-Myc and Id3 may function as a bridge between NPB and NC specifier genes. Consistent with this possibility, knockdown of Id3 results in the downregulation of early NC specifier genes Snail2, Sox10, Foxd3, and Twist [181,182]. Forced expression of Id3 in *Xenopus* NC prevented the development of most NC derivatives, presumably by maintaining NCC in a progenitor state beyond their normal course and thereby preventing their timely differentiation [181].

**CONCLUSIONS AND PERSPECTIVES**

In this chapter, we have summarized the major signaling pathways involved in NC induction and have presented a sample of the molecular players—NPB and NC specifiers—directing NC specification, comparing information from different model organisms.

Studies in fish, frog, and chicken point to some differences in the source and nature of the signaling molecules involved in the induction of NC progenitors in vertebrates. This presumably reflects differences in the timing of neural and NC induction, as well as differences in the precise mechanisms of neurulation employed by each one of these organisms.
NC induction in mammals is not as well understood as in other species, due to the difficulties of manipulating these processes early in the embryo. The development of new tools to specifically remove gene function in the presumptive NC territory, at an earlier stage than what can be achieved using the Wnt1-Cre line, will be critical to evaluate the level conservation of these mechanisms and pathways in the induction of mammalian NCC. In that respect, Pax3-Cre-mediated deletions have not yet provided information different from what has been learned using Wnt1-Cre.

NC specification is guided by the careful orchestration of regulatory circuits that can be assembled in a gene regulatory network. This regulatory cascade is temporally and spatially regulated by factors (NPB and NC specifiers), often acting in an iterative manner, which adds to the complexity of the network. While a great deal has been learned in the last few years on the expression and the role of these factors individually, the challenge is now to better understand how they interact with one another. Perturbation experiments in several model organisms have been extremely useful to delineate the interactions between the components of this network. However, so far only a small number of these interactions have been validated to discriminate direct from indirect regulations (reviewed in [128,183]). This is an essential step in establishing the functional linkage between these factors and defining their position within the NC gene regulatory network. The validation of these interactions through the identification of cis-regulatory sequences using ChIP and ChIP-Sequence is the focus of extensive work in many laboratories around the world. This effort will provide invaluable information to assemble this gene regulatory network. The comprehensive characterization of the regulatory inputs controlling NC formation is essential to understand how these processes may be altered in pathological situations.

Acknowledgments

We thank Dr. Jane McCutcheon for comments on the manuscript. We would like to apologize to colleagues whose work is not cited here, due to space limitations. Work in J-P S-J’s lab is supported by a grant from the National Institutes of Health (ROI-DE014212).

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