frontiers in Physiology

MINI REVIEW
published: 26 November 2020
doi: 10.3389/fphys.2020.608812

Insights Into the Early Gene Regulatory Network Controlling Neural Crest and Placode Fate Choices at the Neural Border

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The neural crest (NC) cells and cranial placodes are two ectoderm-derived innovations in vertebrates that led to the acquisition of a complex head structure required for a predatory lifestyle. They both originate from the neural border (NB), a portion of the ectoderm located between the neural plate (NP), and the lateral non-neural ectoderm. The NC gives rise to a vast array of tissues and cell types such as peripheral neurons and glial cells, melanocytes, secretory cells, and cranial skeletal and connective cells. Together with cells derived from the cranial placodes, which contribute to sensory organs in the head, the NC also forms the cranial sensory ganglia. Multiple *in vivo* studies in different model systems have uncovered the signaling pathways and genetic factors that govern the positioning, development, and differentiation of these tissues. In this literature review, we give an overview of NC and placode development, focusing on the early gene regulatory network that controls the formation of the NB during early embryonic stages, and later dictates the choice between the NC and placode progenitor fates.

Keywords: neural border, neural crest, placodes, signaling, gene-regulatory-network, ectoderm patterning, fate decision

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Specialty section:
This article was submitted to Embryonic and Developmental Physiology, a section of the journal Frontiers in Physiology

Received: *21 September 2020*
Accepted: *02 November 2020*
Published: *26 November 2020*

Citation:
Seal S and Monsoro-Burq AH (2020) Insights Into the Early Gene Regulatory Network Controlling Neural Crest and Placode Fate Choices at the Neural Border. Front. Physiol. 11:608812. doi: 10.3389/fphys.2020.608812

INTRODUCTION

The "New Head" hypothesis (Gans and Northcutt, 1983; Northcutt, 2005) suggests that the presence of a complex head is a significant evolutionary difference between vertebrates and other chordates. During evolution, the vertebrate head has appeared concomitantly with two unique tissues, which are not present (or present in rudimentary form) in earlier-derived organisms: the neural crest (NC) and the sensory placodes. These tissues are formed at the border of the neural fold on the dorsal side of the embryo: placode progenitors (PP) are present rostrally and NC precursors are located more posteriorly (**Figure 1A**). The NC cells are morphologically distinguishable at the late neurulation stage when they delaminate and migrate away from the edge of the neuroectoderm, towards the final locations where they differentiate (Shellard and Mayor, 2019; Alkobtawi and Monsoro-Burq, 2020; Thiery et al., 2020). In parallel, during neurulation, the pan-placodal ectoderm is subdivided into thickened epithelial areas defining each placode, which contribute to cranial sensory structures (Schlosser, 2008, 2010; Pieper et al., 2011; Grocott el al., 2012; Streit, 2018; Buzzi et al., 2019). Lineage tracing

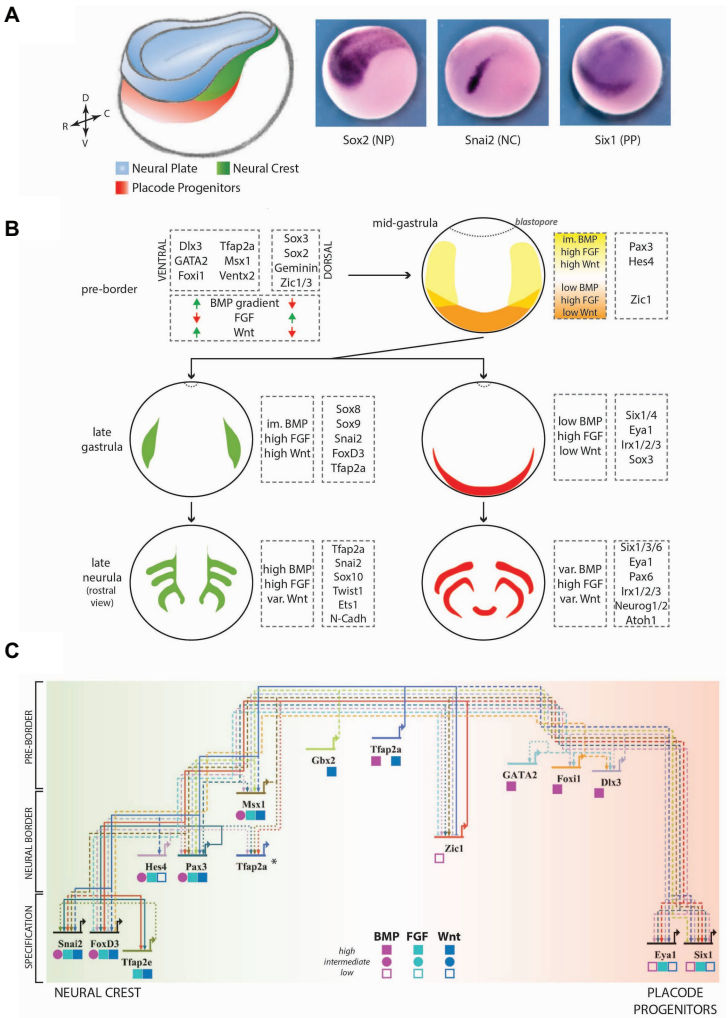

FIGURE 1 | A simplified view of the vertebrate gene regulatory network (GRN) controlling neural crest (NC) and placode induction. **(A)** Model of a *Xenopus* embryo at the mid-neurula stage, depicting the relative positions of the neural plate (NP, blue), the NC (green), and the placode progenitors (PP, red). These tissues express specific transcription factors (TFs), such as Sox2, Snai2, and Six1 respectively. DV, dorsoventral axis; RC, rostrocaudal axis. **(B)** The combined effects of signaling pathways and TFs lead to the development of different tissues in a temporally and spatially regulated manner. Here, the major genes involved at each stage have been indicated, along with the signaling levels of major secreted pathways (BMP, FGF, and WNT). Signaling pathways and genes have been selected according to their conserved functions in various vertebrate animal models and to the availability of detailed studies about their regulation and function in ectoderm patterning. At the mid-gastrula stage (pre-border stage), orange labels the anterior neural border (NB), and yellow depicts the posterior NB. At later stages, green and red depict the NC and the pre-placodal ectoderm respectively. im., intermediate; var., variable. **(C)** A synthetic view of the NB-development GRN in *Xenopus laevis*. Genes have been arranged from top to bottom according to the first stage during which their function is required. Genes positioned towards the left of the map favor the NC fate (green) while genes positioned towards the right of the map favor the PP fate (red). Gene-specific requirements of different signaling pathway activity have been depicted by shapes under the respective gene names (low, intermediate, and high). *Tfap2a has reiterated functions during the different stages, for which it interacts with different binding partners (de Croze et al., 2011; Rothstein and Simoes-Costa, 2020). Solid lines depict direct interactions, dashed lines depict epistasis interactions (either indirect or not proven to be direct) and dotted lines depict a feedback regulation. Arrows depict activation and bars depict repression. The GRN map has been constructed using the BioTapestry software (Longabaugh et al., 2005). Data from other model systems have not been included for the sake of simplicity, but the selected genes broadly display conserved functions in frog and chick. (For more detailed views of placode and NC GRNs, refer to Simoes-Costa and Bronner, 2015; Maharana and Schlosser, 2018; Prasad et al., 2019; Rogers and Nie, 2019; Thiery et al., 2020).

studies have detailed the respective contributions of the NC and the placodes (Noden, 1975; Keller, 1976; Le Douarin, 1980; D'Amico-Martel and Noden, 1983; Couly and Le Douarin, 1985, 1987; Eagleson and Harris, 1990; Garcia-Martinez and Schoenwolf, 1993; Eagleson et al., 1995; Kozlowski et al., 1997; Streit, 2002; Bhattacharya et al., 2004; Xu et al., 2008). Genetic screens conducted in multiple vertebrate species, in particular frog and chick embryos, have identified transcription factors (TFs) which uniquely demarcate NC and PP (Nieto et al., 1994; Ohto et al., 1999; LaBonne and Bronner-Fraser, 2000; Gamill and Bronner-Fraser, 2002; Plouhinec et al., 2014, 2017; Riddiford and Schlosser, 2016; Roellig et al., 2017). NC and PP originate from a common

ectodermal domain, located between the dorsal neural plate (NP; future brain and spinal cord) and the ventral non-neural ectoderm (future epidermis), named the "neural border" (NB, also called "neural plate border" elsewhere; Meulemans and Bronner-Fraser, 2004; Groves and LaBonne, 2014; Pla and Monsoro-Burq, 2018; Thiery et al., 2020). At gastrula stages, *pax3/7* genes (*pax3* paralog in *Xenopus* species, *pax7* paralog in chick, and *pax3/7* ancestor gene in lamprey) mark the lateral and posterior NB, but not it's rostral most portion, while *zic1* marks the anterior NB (**Figure 1**; **Table 1**). The formation, positioning, and henceforth specification of the NB into NC and PP are regulated by the coordinated activity of multiple signaling pathways (e.g., FGF, BMP, and WNT pathways) and specific TFs (e.g., *tfap2a/b/c*, *pax3/7*, *zic1*, and *hes4*; **Figure 1B**). At neurula stages, NC and PP are marked by unique gene sets (e.g., *snai2/foxD3* and *six1/eya1* respectively, **Table 1**).

Principally, the cephalic NC and the placodes form the head sense organs and peripheral nervous system. The cranial NC forms neurons, glial cells, melanocytes, secretory cells, osteocytes, and chondrocytes (Dupin et al., 2018; Etchevers et al., 2019; Alkobtawi and Monsoro-Burq, 2020). The pan-placodal ectoderm develops into non-neurogenic placodes (e.g., adenohypophysis, lens), and neurogenic placodes (epibranchial, otic, paratympanic, trigeminal, and olfactory). In addition, aquatic anamniote vertebrates possess lateral line placodes, which generate a lateral line system comprised of mechanosensory organs in the head and the trunk (Piotrowski and Baker, 2014; Schlosser, 2014; Singh and Groves, 2016; Buzzi et al., 2019). Additionally, by a coordinated migration and morphogenesis, NC, and placode cells form the cranial sensory ganglia (D'Amico-Martel and Noden, 1983; Forni et al., 2011). In humans, defective NC development leads to neurocristopathies, which represent one-third of all developmental diseases, such as cleft palate, Waardenburg syndrome, and Hirschsprung's disease (Vega-Lopez et al., 2018). Similarly, defects in placode development lead to diseases such as BOR/BO syndrome (Kochhar et al., 2007). In order to understand the development of these tissues and uncover the molecular basis of human pathologies, functional studies have been conducted using various vertebrate animal models. In this brief literature review, we focus on the regulation of the early stages of NB development, followed by its specification into NC and PP. We particularly emphasize the common and specific pathways and the gene regulatory network (GRN) controlling the balanced emergence of both cell types around the NP.

TABLE 1 | Important references.

	References	
	Xenopus	**Chick**
A. Gene		
Dlx3/5	Feledy et al., 1999; Luo et al., 2001; Pieper et al., 2012	Pera et al., 1999; McLarren et al., 2003; Khudyakov and Bronner-Fraser, 2009; Linker et al., 2009
Eya1/2	Pieper et al., 2012; Maharana and Schlosser, 2018	McLarren et al., 2003
Foxd3	Monsoro-Burq et al., 2003; Sato et al., 2005; Steventon et al., 2009; Maharana and Schlosser, 2018	Cheung et al., 2005; Khudyakov and Bronner-Fraser, 2009; Simoes-Costa et al., 2012
Foxi1/3	Matsuo-Takasaki et al., 2005; Pieper et al., 2012; Maharana and Schlosser, 2018	Khatri and Groves, 2013
Gata2/3	Pieper et al., 2012; Maharana and Schlosser, 2018	Sheng and Stern, 1999
Gbx2	Li et al., 2009; Steventon and Mayor, 2012	Steventon and Mayor, 2012
Hes4 (Hairy2b)	Nichane et al., 2008a,b; de Croze et al., 2011; Maharana and Schlosser, 2018	
Msx1	Suzuki et al., 1997; Tribulo et al., 2003; Monsoro-Burq et al., 2005	Streit and Stern, 1999; Khudyakov and Bronner-Fraser, 2009; Linker et al., 2009
Pax3/7	Monsoro-Burq et al., 2005; Sato et al., 2005; Hong and St-Jeannet, 2007; de Croze et al., 2011; Milet et al., 2013; Plouhinec et al., 2014; Maharana and Schlosser, 2018	Basch et al., 2006; Otto et al., 2006; Khudyakov and Bronner-Fraser, 2009; Linker et al., 2009; Stuhlmiller and Garcia-Castro, 2012; Vadasz et al., 2013; Simoes-Costa and Bronner, 2015
Six1	Pandur and Moody, 2000; Brugmann et al., 2004; Ahrens and Schlosser, 2005; Pieper et al., 2012; Maharana and Schlosser, 2018	McLarren et al., 2003; Christophorou et al., 2009
Snai2	Mancilla and Mayor, 1996; Monsoro-Burq et al., 2003, 2005; Steventon et al., 2009	Nieto et al., 1994; del Barrio and Nieto, 2002; Khudyakov and Bronner-Fraser, 2009
Tfap2a	Luo et al., 2002, 2003; de Croze et al., 2011; Maharana and Schlosser, 2018	Khudyakov and Bronner-Fraser, 2009; Rothstein and Simoes-Costa, 2020
Tfap2e	Hong et al., 2014	
Zic1	Mizuseki et al., 1998; Monsoro-Burq et al., 2005; Sato et al., 2005; Hong and St-Jeannet, 2007; Marchal et al., 2009; Milet et al., 2013; Plouhinec et al., 2014; Maharana and Schlosser, 2018	Khudyakov and Bronner-Fraser, 2009; Simoes-Costa and Bronner, 2015
B. Transcriptome analysis		
	Plouhinec et al., 2014; Riddiford and Schlosser, 2016; Plouhinec et al., 2017; Maharana and Schlosser, 2018	Khudyakov and Bronner-Fraser, 2009; Simoes-Costa et al., 2014; Simoes-Costa and Bronner, 2016; Hintze et al., 2017; Morrison et al., 2017; Roellig et al., 2017; Trevers et al., 2018

In this mini review article, we have gathered as many references as possible and apologize to the authors whose work could not be quoted. We add here a list of additional references for each of the genes described in the text and point to several relevant large-scale transcriptome screening. Studies using frog as a model are indicated in blue, studies using chick embryos in black; A: references describing NC and PP markers; and B: references of transcriptome analysis of NC and PP progenitors.

NEURAL CREST DEVELOPMENT, AN OVERVIEW

The neural crest is an exclusive feature of vertebrates, acquired about 500 million years ago during evolution (Sauka-Spengler et al., 2007). Since NC generates tissues typical of both ectodermal (ganglia) and mesodermal (mesenchyme, bone) origin, it has been referred to as the fourth embryonic germ layer (Hall, 2018). The NC develops from the NB positioned adjacent to the NP along the rostrocaudal axis during gastrulation and neurulation. Classically, the NC is subdivided into cranial and trunk areas, followed by further anatomical subdivisions (Alkobtawi and Monsoro-Burq, 2020). At the end of neurulation, upon neural tube closure, the NC cells start to migrate in multiple streams, delineating the main craniofacial domains and along the somites in the trunk (Theveneau and Mayor, 2012; Szabo and Mayor, 2018; Rocha et al., 2020). Upon reaching their target tissues, poorly understood genetic programs and interactions with the environment dictate NC differentiation into multiple cell types (Bronner and Le Douarin, 2012).

Before migration, NC cells follow a typical epithelial-to-mesenchymal transition (EMT), which involves the activation of specific TFs (EMT-TFs, e.g., Snail1/2, Twist1), a cadherin switch, and the fine-tuned dynamics of multiple cytoskeletal and cell-polarity proteins. This results in the loss of the polarized epithelial phenotype and acquisition of cell motility (Bahm et al., 2017; Morrison et al., 2017; Shellard and Mayor, 2019). In most species, NC migration involves "contact inhibition of locomotion" (CIL), the mechanism allowing cell dispersion *in vitro* and *in vivo*, as well as "co-attraction," a mechanism maintaining collective migration of cranial NC cells (Carmona-Fontaine et al., 2008; Wynn et al., 2013; Richardson et al., 2016; Li et al., 2019). In addition, cranial NC cells interact with placodal cells, some of which also delaminate. This helps orient the direction of migration of both cell types (Freter et al., 2013; Theveneau et al., 2013; Colombi et al., 2020). The cellular mechanisms of NC migration have been extensively reviewed elsewhere (Mayor and Theveneau, 2013; Shellard and Mayor, 2019; Alkobtawi and Monsoro-Burq, 2020; Giniunaite et al., 2020; Piacentino et al., 2020; Thiery et al., 2020).

Recent works have focused on premigratory NC induction and specification, starting at late gastrulation/NP stages, as denoted by the expression of early NC specifier genes (e.g., *snai2*, *foxd3*, *tfap2e*, *sox8*, and *sox9*). These earlier NC-specifiers in turn induce later NC specifiers such as *sox10*, *ets1*, and *twist1* during the second half of neurulation, when neural folds elevate and fuse dorsally (Alkobtawi and Monsoro-Burq, 2020). The NC specifiers collectively maintain their own expression by positive feedback stimulations (Lander et al., 2013).

PLACODE DEVELOPMENT, AN OVERVIEW

Placodes, the second key vertebrate innovation leading to the formation of specialized head structures, develop from the dorsal-rostral pan-placodal domain which also derives from the NB (**Figure 1A**). Post neurulation, some placodes undergo epithelial folding. Other placode cells are primed for neurogenesis and delaminate from the epithelium (Lassiter et al., 2014). However, unlike NC migration, placode migration does not seem to involve EMT: EMT markers are absent, and cells do not exhibit a mesenchymal morphology and migrate as neuronal cells through a breach in the basal lamina (Graham et al., 2007). During migration, placode cells interact with specific subpopulations of NC cells to form sensory ganglia.

The Six and Eya family of TFs are the major genes involved in early PP development. At late gastrula stages, Six1/4 and Eya1/2 are induced throughout the PP and are essential for its development (**Table 1**). These genes are also required at later stages for placode cell-proliferation and neurogenesis (Schlosser et al., 2008). Grown in isolation, PP continues expressing *six1*/*eya2*, but adopts a lens fate "by default," highlighting that additional regulators control the formation of the other placodes (Bailey et al., 2006). Although, genetic screens have identified a few genes functioning upstream/downstream of the Six/Eya complex, such as Znf462, Homer2, Hes2, Atoh1, the placode GRN remains incompletely understood (Christophorou et al., 2009; Riddiford and Schlosser, 2016; Hintze et al., 2017).

REGULATION OF NEURAL CREST AND PLACODE FATE SPECIFICATION

Neural crest and PP are specified at late gastrula and neurula stages, while the induction of the NB itself is concomitant to neural induction in dorsal ectoderm, at early gastrula stages (de Crozé et al., 2011). Both these processes are tightly regulated by the activity of signaling pathways and TFs, leading to a strict temporal developmental sequence, resulting in well-defined margins demarcating each tissue.

Secreted Signaling Pathways Broadly Pattern the Ectoderm

Levels of activity and cross-regulations between BMP, FGF, and WNT signaling pathways are particularly important for the induction of NC and PP, as they initiate spatial subdivisions of the dorsal ectoderm during gastrulation (Wilson and Hemmati-Brivanlou, 1995; Streit and Stern, 1999; Monsoro-Burq et al., 2003; Kudoh et al., 2004; Steventon et al., 2009; Stuhlmiller and Garcia-Castro, 2012; Yardley and Garcia-Castro, 2012; Schille and Schambony, 2017). Activity levels are influenced by the source of ligands and their antagonists. BMP ligands are secreted by the non-neural ectoderm and the ventral mesoderm, while the NP and the organizer produce BMP antagonists (e.g., Noggin, Chordin, Cerberus and Follistatin; Hawley et al., 1995; Wilson and Hemmati-Brivanlou, 1995; Fletcher and Harland, 2008; Patthey et al., 2008; Branney et al., 2009; Linker et al., 2009). This sets up a low-to-high gradient of BMP signaling from the dorsal midline towards the lateral zones. FGF ligands are produced by the paraxial mesoderm, while WNT ligands come from both the paraxial mesoderm and the non-neural ectoderm (Faure et al., 2002;

Monsoro-Burq et al., 2003; Steventon et al., 2009). Rostral to the NP, WNT antagonists limit WNT signaling (Pera and De Robertis, 2000; Wilson et al., 2001; Carmona-Fontaine et al., 2007). All these pathways are also modulated temporally as they are required at different levels at multiple stages of neural/NC/PP and epidermis specification. At the early gastrula stage, FGF signaling, along with BMP and WNT antagonists, promotes neural development while high BMP and WNT signaling lead to non-neural ectoderm development (Groves and LaBonne, 2014). Henceforth, FGF/BMP antagonists activate neural factors demarcating the dorsal ectoderm (e.g., *sox2/3*, *otx2*; Streit et al., 2000). BMP activity upregulates the expression of *tfap2a*, *foxi1*, *gata2/3*, and *dlx3/5* in the non-neural ectoderm (Nguyen et al., 1998; Luo et al., 2002; Tribulo et al., 2003; Matsuo-Takasaki et al., 2005; Esterberg and Fritz, 2009; Kwon et al., 2010; de Croze et al., 2011).

Between the neural and non-neural ectoderm, the lateral NB is characterized by high FGF, high WNT, and low to intermediate BMP activity, and uniquely marked by *pax3/7* with an overlapping expression of *tfap2a*, *msx1*, *zic1*, *gbx2*, and *hes4* (**Table 1**). In contrast, the anterior NB is subjected to high FGF/low BMP/low WNT levels (**Figure 1C**; Chang and Hemmati-Brivanlou, 1998; Piacentino and Bronner, 2018; Tambalo et al., 2020). The NB is progressively subdivided into NC, PP, dorsal neural tube, and non-neural ectoderm progenitors. Different relative levels of BMP and WNT activity control NC induction and fate maintenance (Steventon et al., 2009; Steventon and Mayor, 2012). It is not yet completely understood how the activity levels of these pathways change dynamically in time and space. One hypothesis is that morphogenesis during neurulation positions the NB close to distinct parts of the mesoderm over time: at mid/late gastrula stages, the dorsal-lateral marginal zone (immature paraxial and intermediate mesoderm precursors) is required for NC induction, while the intermediate mesoderm (pronephros progenitors) maintains NC identity at the early neurula stage. In frog and chick neurula embryos, premigratory NC progenitors exhibit increased BMP activity due to novel signaling modulators (Tribulo et al., 2003; Kwon et al., 2010; Piacentino and Bronner, 2018). Although it remains difficult to compare stages between different species, in zebrafish embryos, a low level of BMP signaling is essential for NC induction while it seems to inhibits PP formation (Nguyen et al., 1998).

Emerging functions of other signaling pathways also contribute to this complex patterning. Retinoic acid signaling contributes to NC induction and migration (Villanueva et al., 2002; Martinez-Morales et al., 2011). Notch signaling is required for *bmp4* and *snail2* expression, regulating NC induction and cell fates at the neural NB (Endo et al., 2002, 2003; Hernandez-Lagunas et al., 2011). AKT signaling is required for premigratory NC induction and maintenance (Sittewelle and Monsoro-Burq, 2018).

Transcription Factors Control Fate Decisions at the Neural Border

The integration of those multiple signals triggers the activation of specific TFs, which in turn bias NB cells towards a given fate (**Figure 1C**). Tfap2a and Gbx2, the earliest genes involved in NC induction, both activate *msx1*, *pax3*, and *hes4* (Li et al., 2009; de Croze et al., 2011). Tfap2a is required for both PP (*six1/eya1*) and NC (foxd3) fates (Luo et al., 2003; Kwon et al., 2010; Pieper et al., 2012; Maharana and Schlosser, 2018). In contrast, Gbx2 favors NC fate by inhibiting *six1* expression (Li et al., 2009). Gata2/3 and Foxi TFs (frog *foxi1a* and chick *foxi3*) promote the PP fate by directly activating *six1* expression and also upregulating *dlx3/5* expression (McLarren et al., 2003; Matsuo-Takasaki et al., 2005; Kwon et al., 2010; Sato et al., 2010; Pieper et al., 2012; Khatri et al., 2014; Hintze et al., 2017). Dlx3 (frog) and Dlx5 (chick) are necessary for PP formation through enhancer-mediated activation of *six1* (Sato et al., 2005, 2010). On the other hand, in mouse, chick, and zebrafish, Msx1 inhibits PP fate by repressing *six1* expression, thus promoting NC fate (Zhang et al., 1997; Phillips et al., 2006; Sato et al., 2010). Interestingly, a recent study in *Xenopus* suggests that Msx1 is required for *six1/eya1* expression, as Msx1 depletion slightly decreases *six1* expression, while its overexpression expands *six1/eya1* ectopically (Maharana and Schlosser, 2018). These seemingly contradictory results may be explained by distinct stage-specific requirements for each gene in different experimental settings. Accordingly, it is known that certain genes, like *tfap2a* and *msx1*, are also required for later NC developmental steps (de Croze et al., 2011; Rothstein and Simoes-Costa, 2020). Mechanistically, the Tfap2a protein dimerizes with either Tfap2c or Tfap2b, at NB and NC stage, respectively, to activate different sets of targets (Rothstein and Simoes-Costa, 2020).

The NB marker Pax3 and the more anteriorly localized Zic1 factor are necessary and sufficient for inducing NC and PP in "naive" ectoderm (Monsoro-Burq et al., 2005; Hong and Saint-Jeannet, 2007; Milet et al., 2013; Bae et al., 2014; Plouhinec et al., 2014). *In vivo* and in ectoderm explants, fate choice is controlled by their relative levels: high Pax3 promotes a hatching gland fate (frog-specific ectoderm cell type), high Zic1 promotes PP fate, while a combination of Pax3 and Zic1 promotes NC fate. Zic1 induces PP fate in a Dlx3-dependent manner while Pax3 strongly represses *six1/eya1* expression (Maharana and Schlosser, 2018). Pax3/Zic1 together lead to the direct expression of the NC specifiers *snai1*, *snai2*, and *foxd3* (Milet et al., 2013; Plouhinec et al., 2014; Simoes-Costa et al., 2014). Consequently *in vivo*, during gastrula NB stages, the PP forms in the Zic1-positive/Pax3-negative anterior NB portion, while NC forms in the region where Pax3 and Zic1 overlap. Interestingly, there is some overlap between *pax3/7*-negative and *six1/eya1*-positive areas, thus leading to an interesting conundrum: how are cells sorted in this overlap region? In chick, a few NB cells continue expressing combinations of fate-specific markers until neurula stages and ultimately get sorted into their final domains (Roellig et al., 2017). Future studies considering the temporal and morphogenetic differences in the neurulation between different species will further address this question.

Several recent transcriptomics screens have uncovered novel regulators of NC/PP fate choice (**Table 1**). For example, in *Xenopus*, *hes4* (*hairy2b*) and *znf703*, expressed broadly at the NB, are required for NC induction. Hes4 upregulates *foxd3*, maintains NC multipotency, and, through the activity of Notch/

Delta signaling triggering Id3, promotes NC differentiation (Nagatomo and Hashimoto, 2007; Nichane et al., 2008a,b; de Croze et al., 2011). Znf703, a target of Pax3 and Zic1, is required for NC specifiers expression (Hong and St-Jeannet, 2017; Janesick et al., 2019). In chick, Axud1, a target of WNT signaling, cooperates with NB specifiers Pax7 and Msx1 for NC induction (Simoes-Costa and Bronner, 2015), while Znf462 and Pdlim4 regulate *foxi3* and *dlx5* respectively, affecting PP development (Hintze et al., 2017). These studies highlight the urgent need for functional studies weaving those numerous novel regulators into the current scaffold of the NB-GRN.

DISCUSSION

Research in multiple model systems has highlighted essential elements of the GRN governing NB induction and NC/PP fate choice (a frog-specific simplified NB-GRN is shown in **Figure 1C**). Importantly, the functions of the key regulators are largely conserved across species (**Table 1**). However major questions remain unanswered. Genetic and transcriptome screens show that the NB-GRN is largely incomplete. Moreover, while complex epistasis relationships begin to be established, most direct regulations await a functional validation. Furthermore, complex feed-back and feed-forward mechanisms between signaling pathways and NB specifiers remain incompletely understood (Litsiou et al., 2005; Garnett et al., 2012). BMP signaling activates Tfap2a, Foxi1, and Gata3, which then regulate each other (McLarren et al., 2003; Ahrens and Schlosser, 2005; Litsiou et al., 2005; Kwon et al., 2010; Pieper et al., 2012; Khatri et al., 2014). Gata2 upregulates both BMP and WNT ligands (Sykes et al., 1998). The NB specifiers Pax3, Zic1, Msx1, Hes4, and Tfap2a regulate each other in a feed-forward loop and require additional WNT signaling (Monsoro-Burq et al., 2005; Sato et al., 2005; Maczkowiak et al., 2010; de Croze et al., 2011; Simoes-Costa and Bronner, 2015). Frog PP specifiers *six1/eya1* affect NB and NC specifiers expression (*pax3*, *foxd3*) as well as NB inducers (*tfap2a*, *msx1*, *dlx3*, *gata2*, *foxi1*; Maharana and Schlosser, 2018). As a whole, these complex cross-talk and feedback regulations stabilize fate choices.

Another debated question is how multipotency, a key characteristic of NC and placodes, is controlled during NB development (Baggiolini et al., 2015). Whether high (NC) or more limited (placodes), the diversity of NC/placode derivatives surpasses other cells' potential at a similar stage and promotes the formation of the New Head. While the molecular basis of placode multipotency remains unexplored, a first model has proposed that NC progenitors retained blastula-type multipotency (Buitrago-Delgado et al., 2015). However, this model is debated since single-cell transcriptomes have shown that the multipotency gene signature proposed by Buitrago-Delgado et al. was not specific to multipotent cells (Briggs et al., 2018). Rather, functional analysis of the vertebrate-specific genetic innovations Nanog/Oct4 (and their orthologs Ventx/Pou5) before or after gastrulation rather suggests that NC progenitors *de novo* activate pluripotency regulators after NB induction (Scerbo and Monsoro-Burq, 2020). This reinitiates multipotency and promotes the ectomesenchyme fate. From an evolutionary perspective, the cranial NB/NC-GRN requires Ventx/Nanog, Pou5/Oct4 and later NC specifier Ets1 to promote jawed structures formation in gnathostomes (Simoes-Costa and Bronner, 2016; Martik et al., 2019; Soldatov et al., 2019; Scerbo and Monsoro-Burq, 2020). Later on, NC specifiers' downregulation leads to the loss of pluripotency and the initiation of cell differentiation (Dottori et al., 2001; Sasai et al., 2001; Teng et al., 2008; Betancur et al., 2010; Mundell and Labosky, 2011; Dupin et al., 2018).

Despite their limitations, all these studies shed light on the two alternative models proposed for NB development. The "binary competence" model proposes that early in development, the competence to develop either NC or placodes is restricted to the NB and the non-neural ectoderm, respectively (Schlosser, 2008; Pieper et al., 2011, 2012). The "NB" model proposes, that early on, the multipotent NB generates both NC and PP, the relative positions of which are determined at later stages by distinct specifiers. Recent experiments suggest a combination of both models *in vivo*: at blastula to late-gastrula stages, the multipotent NB shows co-expression of markers of either fate and no spatial segregation of fate-biased cells (NB model), but as development proceeds, the capability to form either NC or PP would restrict to subzones of the border (binary competence; Roellig et al., 2017; Briggs et al., 2018; Maharana and Schlosser, 2018). When single-cell transcriptomics studies will explore these early stages with increased resolution in the near future, it will be interesting to re-evaluate how cell lineage choices are controlled at the NB. Altogether, the recent functional analyses of early ectoderm patterning have shed important novel information, increasing knowledge of the GRN acting to promote NC and/or PP for the benefit of future studies of human pathologies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

The authors gratefully acknowledge financial support provided by the European Union's Horizon 2020 Research and Innovation Program under Marie Skłodowska-Curie (grant agreement No 860635, ITN NEUcrest); by Université Paris Saclay; by Centre National de la Recherche Scientifique (CNRS); by Agence Nationale pour la Recherche (ANR-15-CE13-0012-01-CRESTNETMETABO); and by Institut Universitaire de France.

ACKNOWLEDGMENTS

We are grateful to Dr. M. Alkobtawi for proofreading the manuscript and to all members of the AM-B team for their support.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.