# Specification of sensory placode progenitors: signals and transcription factor networks

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**ABSTRACT** **Sensory placodes contribute to much of the sensory nervous system in the vertebrate head. They give rise to parts of the eye, ear and nose, as well as to the sensory ganglia that innervate the face, tongue, oesophagus and visceral tissues. Despite their diversity, during development placodes arise from a population of common progenitor cells, which are first specified at the border of the neural plate. The chick has been particularly instrumental in dissecting the timing of these events, and recent evidence has highlighted the close relationship of placode progenitors and precursors for neural crest cells and the central nervous system. This review focuses on the induction of placode progenitors by localised signalling events, and the transcriptional networks that lead to their specification.**

KEY WORDS: *chick, ear, eye, induction, olfactory epithelium, sensory ganglia, transcription factor*

## Introduction

The sensory placodes are unique to vertebrates and give rise to much of the peripheral nervous system in the head (for review: (Baker and Bronner-Fraser, 2001, Schlosser, 2006, Streit, 2008). They generate the lens of the eye, the olfactory epithelium that lines the nasal cavity, the entire inner ear including the cochlear-vestibular ganglion, the amniote paratympanic organ, the adeno-hypophysis and, together with neural crest cells, form the cranial sensory ganglia (Fig. 1A). Because of their vital contribution to the sense organs and their crucial role in craniofacial morphogenesis, understanding placode development has revealed much about the cellular and molecular causes of craniofacial malformations and sensory disorders. Like neural crest cells, placode precursors are induced from the neural plate border region soon after gastrulation, and occupy the cranial ectoderm surrounding the future fore-, mid- and hindbrain, but are absent from the trunk (Fig. 1C). This territory is known as the pre-placodal region (PPR) and contains sensory progenitors that are initially competent to give rise to all placodes (Saint-Jeannet and Moody, 2014, Streit, 2007). As development proceeds, placode precursors become different from each other and coalesce to form patches of thickened tissue that line the closing neural tube (Fig. 1B). While ganglia producing placodes are simple neurogenic patches from which neuroblasts delaminate, those contributing to sense organs invaginate to form cup-like structures or vesicles, which are then transformed

into more complex organs. Over time, placodes give rise to many different cell types from simple lens fibre cells to sophisticated mechanosensory hair cells in the ear, paratympanic organ and the lateral line; except for the lens and anterior pituitary all placodes produce neurons.

While placodes and their contribution to the sensory nervous system was first recognised in the late 19th century (Beard, 1886, Froriep, 1885, Knouff, 1935, van Wijhe, 1883), it is only in the last 15-20 years that the molecular mechanisms controlling their formation are beginning to be elucidated. While some of the earliest studies largely focused on amphibians, the original fate maps from the LeDouarin (Couly and Le Douarin, 1988, Couly and Le Douarin, 1985, Couly and Le Douarin, 1987) and Noden groups (d'Amico-Martel and Noden, 1980, D'Amico-Martel and Noden, 1983) set the scene for placode studies in avian embryos. Because of its amenability for experimental manipulations including temporally and spatially controlled gene knock-down or misexpression, the ease to isolate defined tissues, and its relatively slow development compared to fish and amphibians, the chick has been particularly useful to dissect placode formation over time and to unravel the molecular hierarchy involved. This review focuses on the earliest steps of placode formation, the induction of placode progenitors from the neural plate border, the transcriptional hierarchy that

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specifies placode progenitors and the similarities of this process with induction of the central nervous system.
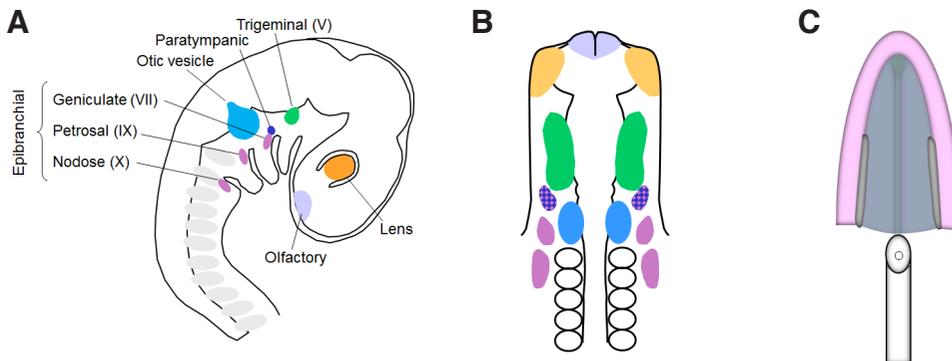
## Sensory placode derivatives

In avian embryos, morphological placodes are first visible around the time of neural tube closure (Fig. 1B) (for review, see Baker and Bronner-Fraser, 2001; Schlosser, 2006; Streit, 2008). Unlike all other placodes, the adenohypophysis and the lens do not generate any neurons. The adenohypophyseal placode develops in the anterior midline, next to the ventral forebrain, forms the anterior part of the pituitary and generates a variety of neuroendocrine cells. The lens placode develops next to the optic vesicle and differentiates into the fibre and epithelial cells of the crystalline lens. The purely neurogenic placodes – the ophthalmic and maxillomandibular trigeminal and the epibranchial placodes – produce delaminating neuroblasts that form the distal portions of the Vth, VIIth, IXth and X[th] cranial ganglia. The epibranchial placodes are located dorsal to the branchial clefts, and their neurons (VIIth, IXth and X[th] ganglia) innervate taste buds in the oral cavity to provide gustatory information, the heart and other visceral organs for viscerosensory input. Originating next to the midbrain, trigeminal placode-derived neurons (V[th] ganglion) transmit somatosensory information like temperature, touch and pain from the face. In amniotes, a recently discovered paratympanic placode lies close to the most rostral epibranchial placode (geniculate), and forms a hair cell containing pouch in the middle ear and its afferent neurons (O'Neill *et al.*, 2012). The paratympanic organ may respond to changes in air pressure. In aquatic vertebrates the lateral line system is responsible for the detection of water movement and electrical fields, and originates from two sets of placodes rostral and caudal to the ear. Finally, the olfactory and otic placodes develop next to the future olfactory bulb and the hindbrain, respectively, and after invagination, undergo morphogenesis to form more complex structures. The olfactory placode generates olfactory sensory neurons, stem cells that regenerate these neurons throughout life as well as diverse migratory neurons that leave the placode and enter the brain. The otic placode arguably forms the most complex sense organ: the auditory and vestibular parts of the inner ear, including sensory hair cells, the neurons that innervate them and many other cell types like supporting and endolymph secreting cells. Thus, sensory placodes generate an incredible diversity of structures and differentiated cell types, and it is therefore surprising that their progenitors in

the PPR initially share a common molecular signature as well as the same developmental potential and properties.

## The pre-placodal region: a unique territory of sensory placode progenitors

Many early avian fate maps explored the derivatives of sensory placodes (e.g. (Couly and Le Douarin, 1985, Couly and Le Douarin, 1987, D'Amico-Martel and Noden, 1983, Noden, 1992), focusing on stages just before or after placodes become morphologically distinct. More recent studies investigating the origin of placodes showed that a continuous and unique territory of sensory progenitors can first be identified at head process stages (Bhattacharyya *et al.*, 2004, Sanchez-Arrones *et al.*, 2017, Streit, 2002, Xu *et al.*, 2008): labelling experiments reveal a continuous band of ectoderm surrounding the anterior neural plate that contains precursors for all sensory placodes and has therefore been termed the pre-placodal region (PPR). An equivalent territory has also been identified by fate mapping experiments in *Xenopus* and fish (Bhat and Riley, 2011, Dutta *et al.*, 2005, Kozlowski *et al.*, 1997, Pieper *et al.*, 2011). Within the PPR, precursors for different placodes are initially intermingled with each other and with progenitors for the neural plate, neural crest and epidermis, although the degree of mixing may differ in different species (Bhat and Riley, 2011, Bhattacharyya *et al.*, 2004, Pieper *et al.*, 2011, Streit, 2002, Xu *et al.*, 2008); for review, see: Schlosser, 2006, 2010; Streit, 2007, 2008). Recent evidence suggests that they are multipotent progenitors with the potential to produce all ectodermal derivatives even at somite stages, when the neural tube is about to close (Roellig *et al.*, 2017). As development proceeds, placode progenitors segregate from other ectodermal derivatives, but it is only after neural tube closure that they converge to form distinct placodes (Bhattacharyya *et al.*, 2004, Steventon *et al.*, 2016, Streit, 2002, Xu *et al.*, 2008). Recent experiments in *Xenopus* suggest that this may at least in part be driven by neural crest cells as they migrate to form the craniofacial skeleton (Steventon *et al.*, 2016, Theveneau *et al.*, 2013).

In addition to harbouring all placode progenitors, the PPR also has a characteristic molecular signature (see also below), although only few genes have been identified that uniquely label this territory. Among them Six and Eya family members not only serve as molecular PPR markers, but are also critically important for its specification, for placode formation and the differentiation normal sense organs and cranial ganglia (for review, see: Schlosser, 2006,

**Fig. 1. Location and derivatives of placodes in the chick. (A)** *Diagram showing a side view of a chick embryo 3-4 days after laying and the position of different placodes.* **(B)** *Diagram of a 10-somite stage chick embryo. The olfactory placodes (light purple) are located anteriorly next to the future olfactory blub, the lens placode (orange) lies next to the optic vesicle and the trigeminal (green) next to the midbrain. The otic placode (blue) is found next to the hindbrain, and the three epibranchial placodes (pink) surround it more laterally. The paratympanic placode may occupy the same territory as the first epibranchial placode (geniculate; blue/pink), although there are currently no fate maps for this placode at this stage.* **(C)** *The pre-placodal region (pink) surrounds the anterior neural plate (blue/grey); neural crest cells (light grey) are absent from the most anterior neural plate border.*

2010; Streit, 2007, 2008). In vertebrates, six Six genes (*Six1-6*) and four Eya genes (*Eya1-4*) have been identified, of which *Six1, -2*, and *–4* and *Eya1* and *-2* are expressed in placode progenitors (Ahrens and Schlosser, 2005, Bessarab *et al.*, 2004, Esteve and Bovolenta, 1999, Ishihara *et al.*, 2008, Kobayashi *et al.*, 2000, Litsiou *et al.*, 2005, McLarren *et al.*, 2003, Mishima and Tomarev, 1998, Pandur and Moody, 2000). Six proteins are transcription factors that bind DNA through their homeodomain, while an N-terminal Six domain mediates the interaction with cofactors (for review, see: Kawakami, 2000; Donner, 2004; Hanson, 2001; Jemc, 2007). In a complex with other nuclear factors like Groucho or Dach, they are transcriptional repressors, while they act as transcriptional activators together with Eya proteins (Kenyon *et al.*, 2005, Kobayashi *et al.*, 2001, Li *et al.*, 2003, Ohto *et al.*, 1999, Patrick *et al.*, 2013, Pignoni *et al.*, 1997, Rayapureddi *et al.*, 2003, Tessmar *et al.*, 2002, Tootle *et al.*, 2003, Zhu *et al.*, 2002). Within the PPR, Six1 appears to play a dual role. Misexpression of Six1 alone or together with Eya2 promotes the expression of other pre-placodal genes, while simultaneously suppressing neural crest and neural plate specific factors (Brugmann *et al.*, 2004, Christophorou *et al.*, 2009) although it is currently unknown whether these genes are direct or indirect targets. In contrast, Six1 knock down in frog (Brugmann *et al.*, 2004) or misexpression of a constitutive repressor form in chick (Christophorou *et al.*, 2009) leads to loss of placode progenitors and placodal defects. In mouse, loss of Six1, Six5, Eya1 and/or Eya4 function results in defects of the olfactory epithelium, the eye and ear, as well as of the cranial ganglia. In line with these findings, mutations in the corresponding human genes are associated with various defects in sense organs including hearing loss. Thus, a large body of evidence implicates Six and Eya genes in the early specification of placode progenitors and in the maturation and differentiation of sensory placodes. Several reviews have summarised their molecular interactions and their function in development and disease in more detail (Donner and Maas, 2004, Grocott *et al.*, 2012, Hanson, 2001, Jemc and Rebay, 2007, Kawakami *et al.*, 2000, Saint-Jeannet and Moody, 2014, Schlosser, 2014, Xu, 2013).

Surprisingly, recent molecular screens for genes coregulated with *Six1/4* and *Eya2* in the chick have only identified one other transcript with PPR-specific expression, *Homer2* (Hintze *et al.*, 2017, Lleras-Forero *et al.*, 2013). *Homer2* is expressed in sensory progenitors and then becomes confined to the otic and olfactory placodes (Anwar *et al.*, 2017, Hintze *et al.*, 2017). However, unlike *Six1* it is already present at gastrulation stages in much of the non-neural ectoderm. These observations highlight Six/Eya factors as unique markers for sensory progenitor cells, and together with their functional importance identify them as key regulators of placodal fates.

Finally, cells in the PPR also possess unique properties that distinguish them from other ectodermal derivatives: they are uniquely competent to respond to placode inducing signals and initially share a developmental programme. Classical experiments performed in amphibian embryos suggested that at early stages all placode progenitors are competent to become any placode (Jacobson, 1963a, Jacobson, 1963b): when the placodal ectoderm is rotated along the rostro-caudal axis at neural plate stages, such that future otic cells are now in the position of the olfactory epithelium, cells adopt a new fate according to their new position. However, when the same experiment is performed later cells retain their original identity, although inter-placodal regions can still respond to new

signals. Similar rotations were not performed in avian embryos, however in the beginning of the 21st century competence and commitment was tested in many transplantation experiments using chick-quail chimera and newly available molecular markers (Baker *et al.*, 1999, Bhattacharyya and Bronner-Fraser, 2008, Groves and Bronner-Fraser, 2000). Together these findings suggest that only the head ectoderm is competent to respond to placode inducing signals and depending on timing this property is indeed restricted to the PPR. Thus, the PPR represents a unique placode-competence field. In addition, there is strong evidence that ectodermal cells must first acquire a 'PPR state' before they can form a placode (Martin and Groves, 2006). When PPR cells from head fold stages are exposed to an otic inducer FGF2 in vitro, they rapidly turn on the ear programme (Anwar *et al.*, 2017, Martin and Groves, 2006), while early gastrula ectoderm is not responsive. However, when the same gastrula ectoderm is first transplanted into the PPR, where it initiates expression of PPR markers like *Eya2*, it can now respond to FGF2 and express ear-specific genes (Martin and Groves, 2006). This finding suggests that ectodermal cells can only respond to placode inducing signals once they have acquired PPR identity and that placode induction requires at least two consecutive steps, if not more (see also: Anwar *et al.*, 2017; Chen *et al.*, 2017).

In chick, specification assays reveal that at head fold stages, cells in the PPR are specified as placode progenitors (Bailey *et al.*, 2006). When cultured in isolation they continue to express the PPR markers *Six1* and *Eya2*. Surprisingly, all placode progenitors, even those fated to become ear and cranial ganglia, also initiate *Pax6* expression, which is normally confined to the lens, olfactory and adenohypophyseal territories, and after 3-days' culture transform into lens-like vesicles expressing *L-maf*, *FoxC1*, α- and δ-crystalline (Bailey *et al.*, 2006). Thus, all placode progenitors are initially specified as lens irrespective of their later fate. In addition, these findings suggest that placode inducing signals not only actively promote placode identity, but simultaneously must repress lens. FGFs may be key to initiate lens repression and have been implicated in the induction of most placodes. Activation of FGF signalling suppresses lens specification *in vitro* and lens formation in vivo, and is required for the formation of the olfactory, trigeminal, otic and epibranchial placodes in chick and other vertebrates (Bailey *et al.*, 2006, Canning *et al.*, 2008, Freter *et al.*, 2008, Ladher *et al.*, 2000, Maroon *et al.*, 2002, Martin and Groves, 2006, Nechiporuk *et al.*, 2007, Nechiporuk *et al.*, 2005, Nikaido *et al.*, 2007, Phillips *et al.*, 2001, Sun *et al.*, 2007, Wright and Mansour, 2003).

In summary, at head fold stages the PPR represents a contiguous band of ectoderm surrounding the future fore-, mid- and hindbrain that is characterised by unique features: it contains precursors for all sensory placode, is identified by a unique set of molecular markers (see also below), is the only region competent to respond to placode inducing signals and cells within it share the same developmental potential.

## Protecting sensory progenitors from inhibitory signals: PPR-inducing tissues and signals

In chick, ablation and transplantation experiments have identified the mesoderm underlying the PPR as the source of PPR inducing signals, although there may also be some contribution from the adjacent neural plate, like in *Xenopus*. Ablation of the future heart mesoderm at head process stages - around the onset of PPR-

specific gene expression - leads to the loss of *Eya2* and *Six4*, while removal of the prechordal mesendoderm results in loss of *Eya2* as well as the anterior PPR genes *Pax6* and *Nociceptin* (*pNoc*) (Litsiou *et al.*, 2005; Lleras-Forero *et al.*, 2013). When transplanted next to PPR-competent epiblast of the area opaca, each tissue can induce a full set of PPR markers, but also provides regional information (Hintze *et al.*, 2017, Litsiou *et al.*, 2005): future heart mesoderm generates sensory progenitors with posterior character expressing e.g. *Foxi3*, *Gbx2* and *Irx2*, while prechordal mesendoderm promotes anterior identity (*Otx2*+, *Hesx1*+, *SSTR5*+, *pNoc*+, *Six3*+). In contrast, the neural plate does not induce a full complement of PPR transcripts and therefore alone is not sufficient to impart sensory progenitor identity to non-placodal cells (Hintze *et al.*, 2017, Litsiou *et al.*, 2005).

A recent molecular screen has identified a large number of genes that are activated in response to the heart mesoderm, many of which are also enriched in the PPR (Hintze *et al.*, 2017; Lleras-Forero *et al.*, 2013). These genes may act together, in parallel and/or upstream of the Six and Eya network. To explore the molecular events during PPR induction and to identify new upstream regulators of *Six1* and *Eya2*, this study investigated the response of competent, non-placodal cells to mesoderm over time (Hintze *et al.*, 2017). Heart mesoderm or prechordal mesendoderm was grafted next to competent area opaca epiblast. The tissue exposed to mesoderm signals and the contralateral control side were then collected 3, 6 and 12 hours later, and changes in gene expression were quantified using a NanoString probe set containing more than 100 genes including known and new PPR transcripts, markers for different placodes and for neural and neural crest cells. Surprisingly, this analysis reveals that although both tissues

ultimately induce PPR with distinct regional character, they initially promote the expression of an identical set of transcription factors (see below for details), while repressing transcripts characteristic for the non-neural ectoderm and/or future epidermis (Hintze *et al.*, 2017). This is rapidly followed by the induction of a second tier of factors, prior the induction of *Six1* and *Eya2* together with some of their known regulators. It is during the second phase of PPR induction that prechordal mesendoderm and heart mesoderm induced tissues begin to diverge, and 12 hours after exposure distinct anterior and posterior identity is established (Hintze *et al.*, 2017). Thus, this new model suggests that during PPR induction cells initially adopt a common transcriptional state before they gradually diversify to generate sensory progenitors with anterior and posterior character.

FGF activation together with BMP and Wnt inhibition have been implicated in the induction of sensory progenitors in chick, fish and frog (Ahrens and Schlosser, 2005, Brugmann *et al.*, 2004, Litsiou *et al.*, 2005). Members of these pathways are normally expressed in both PPR-inducing mesoderm populations, while BMP and Wnt ligands are present in surrounding tissues (Fig. 2). In chick, FGF8 partially mimics the activity of the future heart mesoderm and prechordal mesendoderm: FGF8 beads induce most of the early response genes in area opaca epiblast (Hintze *et al.*, 2017, Litsiou *et al.*, 2005). In contrast, when mesoderm is grafted together with the FGF pathway inhibitor SU5402, their induction is diminished. However, when SU402 beads are added 5 hours after cells have been exposed to mesodermal signals PPR induction continues normally (Litsiou *et al.*, 2005). Thus, FGF signalling is required during PPR initiation, but is dispensable later. In contrast, BMP antagonism alone only induces very few genes, but is required
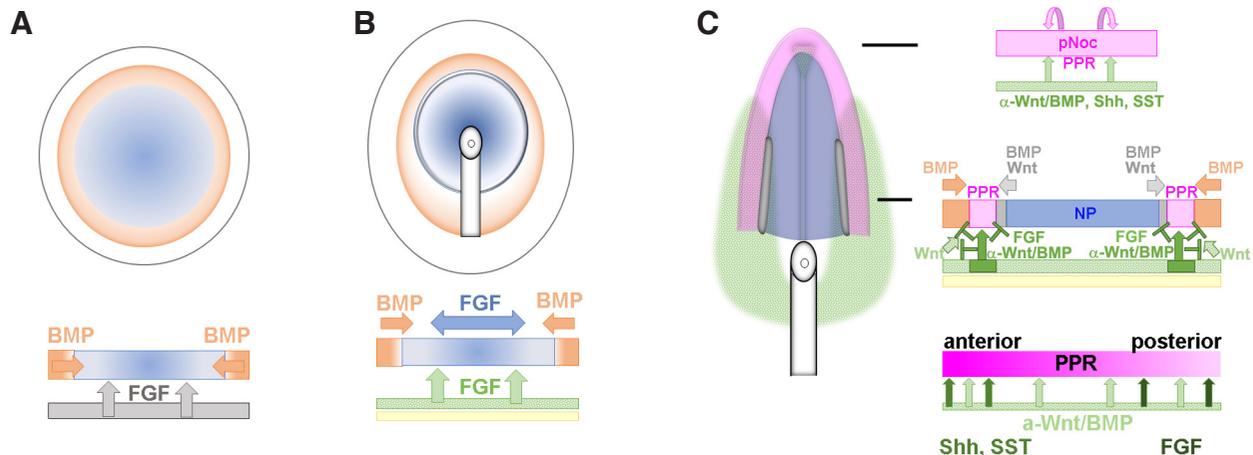


**Fig. 2. Signals involved in the neural plate border and pre-placodal region (PPR). (A)** *At pre-streak stages the epiblast is roughly subdivided into a central domain (blue) expressing pre-neural genes, and in a peripheral territory (orange) expressing non-neural factors. However, there is considerable overlap in gene expression. Pre-neural genes can be induced by the hypoblast and many are FGF dependent, while non-neural genes are under the control of BMP signalling from the extraembryonic territory.* **(B)** *During gastrulation, gene expression of pre-neural genes is maintained in the central epiblast and at late gastrula stages Sox2, a marker for the neural plate, begins to be expressed surrounding the node. Non-neural gene expression is maintained in the peripheral epiblast. FGF signalling emanates from the underlying mesoderm, as well as the future neural plate, while BMPs continue to be expressed in the lateral epiblast. At the neural plate border, pre-neural and non-neural genes continue to overlap.* **(C)** *At head process stages, the PPR becomes molecularly distinct (pink) surrounding the neural plate (blue-grey). The lateral plate mesoderm including the future heart mesoderm (green) has emerged from the primitive streak and comes to underlie much of the PPR, while the prechordal mesendoderm at the tip of the notochord underlies that anterior PPR. The two top diagrams on the right summarise the signalling events at the level of the black lines that promote anterior (top) and posterior (middle) PPR formation. Note: PPR cells are surrounded by inhibitory signals, but protected by signals from the mesoderm. Bottom diagram shows signalling along the anterior-posterior axis.*

throughout the inductive process: when BMP4 is added to grafted mesoderm many PPR transcripts are reduced including both early (e.g. *Trim24*, *N-myc*) and late factors (e.g. *Six1*, *Eya2*) (Hintze *et al.*, 2017). Conversely, when Smad6, which antagonises BMP signalling, is misexpressed in the embryo at gastrula stages PPR transcripts are expanded (Litsiou *et al.*, 2005). These findings match corresponding experiments in amphibian and fish embryos (Ahrens and Schlosser, 2005, Brugmann *et al.*, 2004, Esterberg and Fritz, 2009, Glavic *et al.*, 2004, Kwon *et al.*, 2010). Finally, Wnt antagonism does not seem to play a major role during early steps of PPR induction: in the induction assay, none of the mesoderm induced genes are regulated by Wnt manipulation (Hintze *et al.*, 2017). However, Wnt activation by misexpression of constitutively active β-catenin at gastrula stages leads to loss of PPR specific genes, while neural crest markers are expanded (Litsiou *et al.*, 2005). Conversely, Wnt inhibition using crescent results in PPR expansion at the expense of neural crest (Litsiou *et al.*, 2005). These observations suggest that while low levels of Wnt may be required for PPR specification, Wnt signalling is mainly responsible for the segregation of placode and neural crest precursors at later stages. In summary, at head fold stages sensory progenitor cells in the PPR are surrounded by inhibitory signals: BMPs and Wnts from the future neural crest and the future epidermis, as well as Wnt ligands from the lateral and posterior mesoderm (Fig. 2C). FGF in combination with Wnt and BMP antagonists from the heart mesoderm and the prechordal mesendoderm protect the PPR from these inhibitory influences and thus allow its specification next to the anterior neural plate.

While these signals mediate generic PPR induction they do not account for the regional bias imparted by each mesodermal population. The anterior mesendoderm expresses high levels of *sonic hedgehog* (*Shh*). Indeed, combining prechordal mesendoderm grafts with the Shh inhibitor cyclopamine prevents the induction of anterior (*Six3*, *Otx2*), but not generic PPR factors (*Six1*, *Eya2*) (Hintze *et al.*, 2017). Thus, Shh is at least in part responsible for imparting anterior character to sensory progenitors (Fig. 2C). The mesendoderm also expresses the neuropeptide somatostatin and somatostatin-coated beads are sufficient to restore anterior marker expression (*Pax6*, *pNoc*) after mesendoderm ablation suggesting that it cooperates with Shh (Lleras-Forero *et al.*, 2013). The aPPR itself expresses *pNoc*, another neuropeptide, required for anterior identity. pNoc inhibition using pharmacological inhibitors or knock down of its receptor SSTR5 results in the loss of anterior markers like *Pax6* and *pNoc* itself (Lleras-Forero, 2013). Thus, while Shh and somatostatin participate in anterior PPR induction by the mesendoderm, pNoc seems to act in an autocrine fashion to maintain anterior PPR character once induced (Fig. 2C).

## A transcription factor hierarchy upstream of the Six-Eya network

Members of the Six and Eya families of nuclear factors lie at the heart of sensory progenitor specification. They act as a complex to activate downstream target genes (for review, see: Xu, 2013; Jemc, 2007; Kawakami, 2000; Donner, 2004; Hanson, 2001), but also promote their own expression and that of other PPR genes (Brugmann *et al.*, 2004; Christophorou *et al.*, 2009). Thus, once expressed they may maintain cells in a placode progenitor state (for review, see: Grocott *et al.*, 2012). What is the transcriptional

input that regulates the expression of *Six1* and *Eya2* in the PPR? The molecular screen described above has identified many new factors that may act in parallel, together and/or upstream of the Six and Eya network, while the time course analysis of mesoderm response genes establishes the sequence of their activation during the induction process.

In response to mesodermal signals competent epiblast rapidly upregulates of a small set of known or putative transcription factors (*Cited2*, *ERNI*, *Etv5*, *Otx2*, *Trim24*, *Znf462*, *Mynn*), while repressing others (*Dlx3/5*, *Gata2*, *Msx1*, *Tfap2a*) (Hintze *et al.*, 2017). The latter are generally considered to be non-neural or future epidermal markers (Hoffman, 2007, Knight *et al.*, 2003, McLarren *et al.*, 2003, Papalopulu and Kintner, 1993, Pera and Kessel, 1999, Pera *et al.*, 1999, Phillips *et al.*, 2006, Pieper *et al.*, 2012, Sheng and Stern, 1999, Streit and Stern, 1999, Suzuki *et al.*, 1997, Woda *et al.*, 2003, Yang *et al.*, 1998), while the former are later expressed in neural and placodal tissues and have been termed 'pre-neural' factors (Albazerchi and Stern, 2007; Bally-Cuif *et al.*, 1995; Pinho *et al.*, 2011; Rex *et al.*, 1997; Streit *et al.*, 2000; Trevers *et al.*, 2017; for review, see: Grocott *et al.*, 2012; Stern and Downs, 2012). During normal development, these genes are already expressed at pre-streak stages, with the induced transcripts being widespread in the central epiblast, and repressed genes confined to the periphery (Trevers *et al.*, 2017). However, the expression patterns of both groups overlap considerably (for review, see Grocott *et al.*, 2012). Most pre-neural genes are activated by FGF signalling, although *Otx2* also requires Wnt and BMP antagonists (Albazerchi and Stern, 2007; Hintze *et al.*, 2017; Streit *et al.*, 2000; Wilson *et al.*, 2000). In chick, these signals emanate from the hypoblast, an extraembryonic tissue equivalent to the anterior visceral endoderm in mouse, and indeed hypoblast grafts can mimic the initial step of PPR induction: when hypoblast is grafted into the area opaca it rapidly upregulates the same genes that are induced by mesoderm (Albazerchi and Stern, 2007; Hintze *et al.*, 2017; Streit *et al.*, 2000; Trevers *et al.*, 2017). In contrast, hypoblast and mesodermal signals repress non-neural transcripts, and this depends on activation of FGF signalling and BMP inhibition (Hintze *et al.*, 2017; Trevers *et al.*, 2017). For example, repression of non-neural genes by the mesoderm is overcome by supplying extra BMP4 (Hintze *et al.*, 2017). Indeed, *BMP4* and *-7* are strongly expressed in the area opaca (Chapman *et al.*, 2002, Streit *et al.*, 1998), and thus present at the right time and in the right place to promote the expression of non-neural transcripts. Thus, at pre-streak stages signals from surrounding tissues (hypoblast and extraembryonic region) begin to subdivide the epiblast into 'non-neural' and 'pre-neural' territories although there is considerable overlap between both territories.

The second tier of mesoderm-induced factors largely consist of molecules previously not associated with PPR formation, but network inference approaches identify them as putative targets of the early 'pre-neural' genes (Hintze *et al.*, 2017). Like pre-neural genes, they are already expressed before gastrulation (Trevers *et al.*, 2017). Among them the zinc-finger factor Znf462 is predicted to be a hub, where inputs from all 'pre-neural' factors converge. Indeed, knock-down of Znf462 using morpholinos reveals that this factor is required for *Foxi3*, *Gata3*, *Dlx6* and *Gbx2* (Hintze *et al.*, 2017), all of which are known to regulate *Six1* and/or *Eya2* (Esterberg and Fritz, 2009, Kwon *et al.*, 2010, Luo *et al.*, 2001, McLarren *et al.*, 2003, Pieper *et al.*, 2012, Sato *et al.*, 2010, Solomon and Fritz, 2002, Woda *et al.*, 2003). Accordingly, *Six1* and *Eya2* expression

are lost in the absence of Znf462. The Lim domain factor Pdlim4 appears to be repressed by Znf462; Pdlim4 provides positive input for *Dlx6* and is required for *Six1* and *Eya2* (Hintze *et al.*, 2017). Thus, Znf462 emerges as a new key regulator in the PPR gene network. Like the 'pre-neural' genes, many of the second-tier factors are also regulated by FGF signalling, and/or require BMP antagonism as shown by grafting experiments together with pathway agonists or antagonists similar to the experiments described above (Hintze *et al.*, 2017).

As the embryo undergoes gastrulation, both 1st and 2nd tier factors are maintained in the future neural plate and its border, labelling progenitors for the entire central and peripheral nervous system (Hintze *et al.*, 2017). However, at the neural plate border (NPB) they continue to overlap with many non-neural genes although the latter begin to segregate to form molecularly distinct subdomains. At late gastrula stages, the neural marker *Sox2* begins to be expressed close to the organiser (Papanayotou *et al.*, 2008, Rex *et al.*, 1997). While *Foxi3* and members of the *Tfap2* and *Dlx* families directly abut the *Sox2*+ domain, *Gata2/3* do not, but are expressed slightly more lateral. Thus, just prior to PPR formation and the onset of *Six/Eya* genes, the ectoderm is subdivided into four molecularly distinct domains: the central epiblast where 1st and 2nd tier factors overlap with the neural marker *Sox2*, the 'inner NPB' where these transcripts overlap with Dlx, Tfap2 and Foxi factors, the 'outer NPB' where *Gata2/3* are also present, and finally the non-neural ectoderm expressing only non-neural genes (see (Grocott *et al.*, 2012 and references therein). Around the onset of *Six1* and *Eya2* expression, individual NPB cells co-express neural, neural crest and sensory progenitor markers in different combinations suggesting that they may have the potential to develop into these lineages (see below) (Roellig *et al.*, 2017).

These spatial and temporal changes in gene expression as the embryo develops from pre-streak to late gastrulation changes, matches the sequence of events observed in the PPR induction assay. In the final step, the induction of the Six/Eya cassette by mesoderm-derived signals is accompanied by the initiation of factors known to regulate their expression in chick and frog. Among them are *Foxi3*, *Sall1*, *Gata3*, *Gbx2* and *Hey1*, as well as genes that were repressed by mesodermal signals like *Dlx6* and *Tfap2a* at earlier time points (Hintze *et al.*, 2017). Although this has not been explored in detail in chick, experiments in *Xenopus* and zebrafish suggest a complex regulatory relationship among Gata2/3, Tfap2, Dlx and Foxi1 factors (Kwon *et al.*, 2010, Pieper *et al.*, 2012). In frog, Foxi1a overexpression promotes the expression of *Dlx3*, which in turn is required for *Foxi1a*. In addition, Dlx3 and Gata2 control their own expression and that of *Dlx5*, while in fish Gata3 and Ap2 are required for *Dlx3*. Misexpression of any of these factors prevents neural plate formation, suggesting that a major function of these proteins is to restrict neural plate expansion. Experiments in chick have confirmed some of these regulatory relationships: Dlx5 misexpression not only represses neural and neural crest genes, but activates *Foxi3*, which in turn promotes *Dlx5* (Khatri *et al.*, 2014, McLarren *et al.*, 2003). Thus, once non-neural genes are expressed positive feedback loops reinforce their own expression and may make them independent from further signalling input.

Furthermore, these non-neural factors provide critical transcriptional input for the onset of *Six1* and *Eya2* at head process stages. Misexpression of Dlx5 in chick and Dlx3 in frog represses neural crest markers, while promoting PPR fate, while fish *Dlx3b*

and *-4b* mutants loose PPR gene expression *Eya2* (Esterberg and Fritz, 2009, Luo *et al.*, 2001, McLarren *et al.*, 2003, Solomon and Fritz, 2002, Woda *et al.*, 2003). In *Xenopus*, Dlx3 is required for ectodermal cells to generate a PPR in response to FGF and BMP antagonists, and has therefore been implicated as 'competence factor' (Pieper *et al.*, 2012). Indeed, evidence in chick shows that Dlx5 directly regulates *Six1*: the *Six1* enhancer element that drives its expression in the anterior PPR requires Dlx5 activity (Sato *et al.*, 2010). Together these findings suggest that Dlx factors are important activators of the Six/Eya network. Using similar experimental strategies experiments in frog and fish have shown that Tfap2, Gata and Foxi transcription factors are cell autonomously required for the expression of *Six* and *Eya* genes: in their absence ectodermal cells are not competent to respond to PPR inducing signals and cannot activate *Six/Eya* factors (Kwon *et al.*, 2010, Pieper *et al.*, 2012). Finally, Foxi3 misexpression in chick is sufficient to activate ectopic expression of the PPR markers *Six1* and *Eya2* (Khatri *et al.*, 2014). Whether this is occurs through direct binding of Foxi3 to the Six1 enhancer, or indirectly via *Dlx5* activation remains to be determined.

While the experiments described above provide evidence for activators of *Six1* and/or *Eya2*, few factors have been implicated in their repression. During PPR induction, signals from the heart mesoderm or prechordal mesendoderm repress the expression of *Msx1* (Hintze *et al.*, 2017). At pre-streak stages *Msx1* is expressed in the outer embryonic epiblast together with *Dlx5/6* and *Gata2/3* (Pera *et al.*, 1999, Sheng and Stern, 1999, Streit and Stern, 1999). During gastrulation, it is first seen in the epiblast next to the posterior primitive streak, and is then rapidly confined to a thin strip of cells along the border of the neural plate, the future neural crest, similar to *Pax7* (Basch *et al.*, 2006). Like Dlx5, Msx1 directly binds to the Six1-14 enhancer that is active in the PPR (Sato *et al.*, 2010). However, while Dlx5 promotes its activity, misexpression of Msx1, as well as Pax7 abolishes enhancer activity completely (Sato *et al.*, 2010). Thus, the expression of Six1 in sensory progenitors is tightly controlled through both positive and negative regulation. Moreover, while the Six1/Eya2 complex activates other PPR specific transcripts it also suppresses genes characteristic for alternative fates (Brugmann *et al.*, 2004, Christophorou *et al.*, 2009). Misexpression of Six1 and Eya2 results in the upregulation of PPR genes like *Six4* and *Sox11*, as well as its upstream regulators *Gata3* and *Dlx5*. In contrast, Six1/Eya2 repress neural (*Sox2*, *Sox3*) and neural crest markers (*Pax7*).

In summary, recent experiments in chick have unravelled a transcription factor hierarchy upstream of the Six1/Eya2 network. The quantification of many newly identified genes together with the analysis of PPR induction over time, highlights the sequential activation of two cohorts of pre-neural genes that label progenitors for the entire nervous system, and the repression of non-neural genes. Among the pre-neural genes, the zinc-finger transcription factor Znf426 emerges as a new hub that integrates many upstream inputs, while regulating three of the four known PPR competence factors. As PPR induction progresses, non-neural genes are now activated; they promote their own expression as well as the activation of *Six* and *Eya* family members, while repressing neural fates. Once *Six1* and *Eya2* are expressed they not only activate other sensory progenitor genes, but also their own upstream regulators, which in turn prevent spreading of neural and neural crest factors. Thus, cross-repressive interactions among transcription factors that

characterise progenitors for the neural, neural crest and placodal lineage together with positive feedback loops lead to sharpening of gene expression boundaries and ultimately segregation of different cell fates. Interestingly, individual cells at the NPB co-express neural, neural crest and placode factors suggesting that these interactions occur cell autonomously. Stochastic fluctuations in transcription factor levels may thus determine the ultimate fate of NPB cells. To unravel the mechanism single cell expression analysis combined with lineage tracing of cells with specific expression profiles will be required.

## Similarities in neural and sensory progenitor induction

Many of the experiments described above take advantage of the fact that the extraembryonic epiblast in chick is competent to respond to PPR inducing signals, and can differentiate into placode progenitors and mature placodes when exposed to appropriate signals e.g. a tissue graft. This induction assay rapidly recapitulates the gradual specification of ectodermal cells as sensory progenitors, and has allowed the molecular dissection of this process (Hintze *et al.*, 2017). Surprisingly, a molecular screen for potential upstream regulators of the Six/Eya network using this assay only identified a handful of PPR-restricted genes: most mesoderm-response genes

are already expressed in the pre-streak epiblast, show wide-spread expression at primitive streak stages encompassing progenitors for the central and peripheral (crest and placodes) nervous system, and later become confined to the neural plate and PPR or to the neural plate alone (Hintze *et al.*, 2017, Trevers *et al.*, 2017). These observations point to a molecular similarity between early neural and sensory progenitor cells.

Neural induction can be initiated by a graft of the avian organiser, Hensen's node, into the extraembryonic area opaca (Waddington, 1932, Waddington, 1933), leading to the formation of a *Sox2*-expressing neural plate within 7 hours (Trevers *et al.*, 2017). Analysis of transcripts upregulated 5 hours after a node graft reveals striking similarity with transcriptional profile initiated by PPR-inducing mesoderm, and to the transcriptional signature of pre-streak epiblast (Hintze *et al.*, 2017, Trevers *et al.*, 2017). Therefore, the initial steps in neural and PPR induction may be similar, if not identical, and resemble the gene expression profile in the pre-streak epiblast. Thus, cells may transit through a common transcriptional state before being committed to their respective lineage. This common state has been termed 'pre-border state', and has a transcriptional profile akin to mouse embryonic stem cell lines.

Interestingly, the transcriptional signature of sensory progenitors
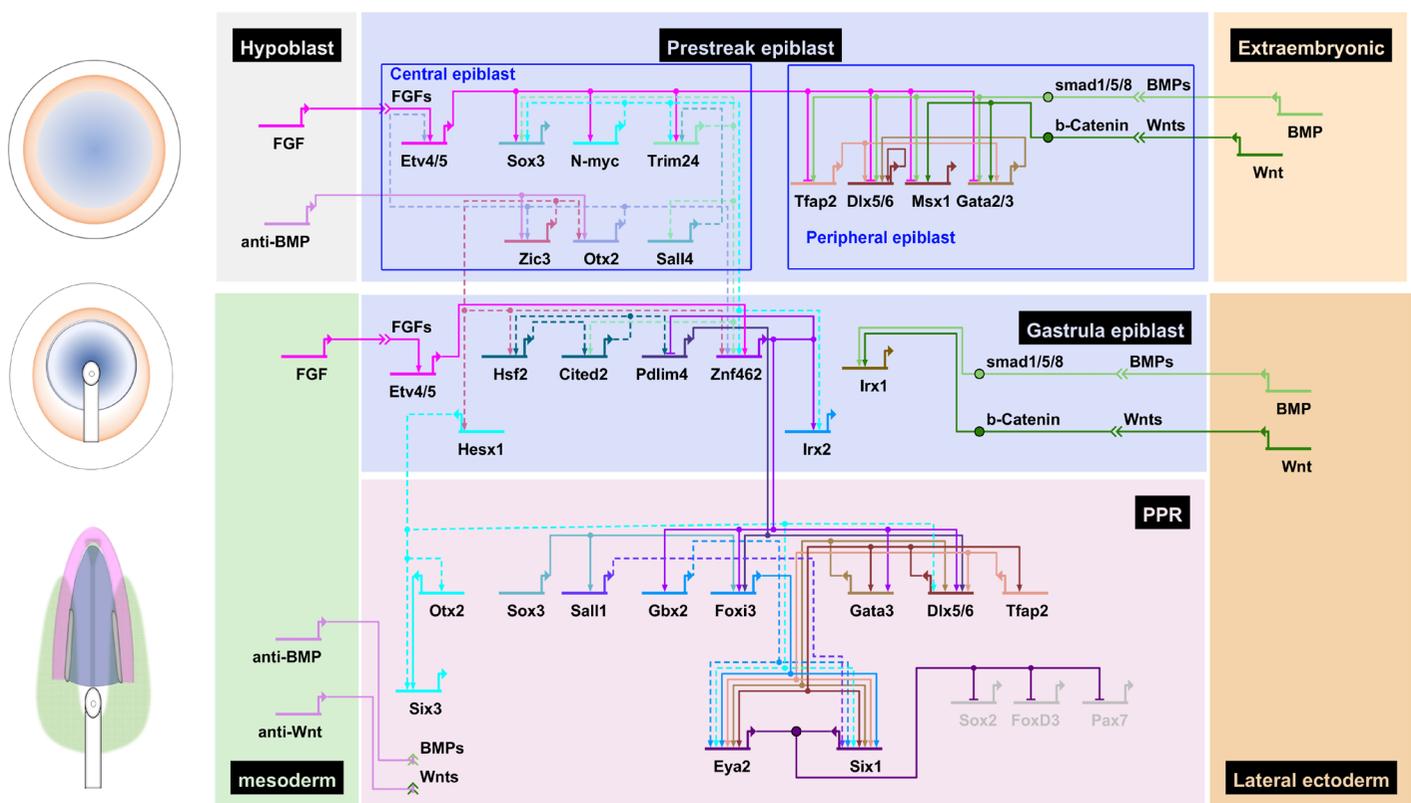


**Fig. 3. Transcription factor network upstream of pre-placodal region (PPR) specifiers Six1 and Eya2.** *Signals and transcription factors are shown from pre-streak to headfold stages; diagrams on the left show the corresponding embryonic stages. In the network, regions are colour-coded like in the embryo diagrams. Signals from the hypoblast promote the expression of pre-neural genes in the central pre-streak epiblast, while signals from the extraembryonic region promote non-neural gene expression. At gastrula stages FGFs emanate from the mesoderm and promote a second tier of factors in the central epiblast, while BMP and Wnt signalling continue to enhance non-neural gene expression. PPR formation requires BMP and Wnt antagonism, and combination of pre-neural and non-neural factors promote the expression of* Six1 *and* Eya2*, which in turn repress neural (*Sox2*) and neural crest factors (*Foxd3, Pax7*). Genes in cyan (*Hesx1, Otx2, Six3*) are confined to the anterior PPR at head fold stages, and genes in sky blue (*Irx2, Gbx2, Foxi3*) are restricted to the posterior PPR.*

in the PPR is also similar to the pre-border state: using network inference algorithms combined with community clustering to analyse the transcriptome of pre-border (pre-streak epiblast + 5 hours induced epiblast), neural plate and PPR cells from headfold stages, reveals a strong similarity between the PPR and pre-border state (Trevers *et al.*, 2017). These observations suggest that PPR cells may retain some stem cell-like character of the pre-streak epiblast, prior to their differentiation into mature placodes. This finding is reminiscent of the observation that in *Xenopus* animal pole cells from the early blastula express pluripotency markers, and that these genes are retained by neural crest cells (Buitrago-Delgado *et al.*, 2015).

The above experiments indicate a surprising similarity in the initial response to neural and PPR inducing signals. If the induced transcriptional state is indeed identical it should be possible to initiate induction with any tissue, and then complete it with another, which then determines the final outcome. A graft of Hensen's node takes 7 hours to induce *Sox2+* neural tissue. However, when the extraembryonic epiblast is first primed by head mesoderm for 3 hours, the node only takes 4 hours to induce *Sox2* (Trevers *et al.*, 2017). Likewise, 3 hours exposure to a node primes the epiblast to respond more rapidly to mesoderm grafts and generate sensory progenitor specific gene expression (Trevers *et al.*, 2017). Furthermore, the hypoblast also induces the pre-border state and can substitute for node or mesoderm within the first 3 hours of the induction process (Trevers *et al.*, 2017). Together, these findings indicate that the epiblast responds to neural and PPR inducing tissues in the same manner by initiating a unique transcriptional response (pre-border). In normal development, this transcriptional profile resembles that of the pre-streak epiblast, which in turn may be induced by the underlying hypoblast. Thus, initiation of neural and sensory progenitor induction initially follows a similar programme and cell fates diverge later.

## Models for cell fate segregation at the neural plate border

Experiments in *Xenopus* have previously suggested a 'dual competence model' to explain the segregation of neural, neural crest, placodal and epidermal lineages (Pieper *et al.*, 2012) for review: (Schlosser, 2008). Based on a series of transplantation experiments, the model proposes that changes in competence divide the ectoderm into two territories, one competent to give rise to neural and neural crest cells, versus another competent to generate sensory placodes and epidermis. When the neural plate from neurula stage embryos is transplanted into the crest or placode territory, it can be induced to generate neural crest cells, but not placodes. When the same experiment is performed with future epidermis, the epidermis can generate placodes, but no neural crest cells. This led to the conclusion that restriction of competence initiates segregation of ectodermal lineages. This model differs from the neural plate border model (NPB model) in chick (for review: Grocott *et al.*, 2012), which proposes that cell fates are gradually restricted over time, and that NBP cells retain the ability to generate neural, neural crest, placode and epidermal cells for some time and respond to local cues that drive them towards different fates.

The dual competence model implies that cells should lose competence to respond to neural and PPR-inducing signals at different times. However, in chick this is not the case: competence of the extraembryonic epiblast to generate neural tissue in response to an organiser graft and to produce PPR in response to head mesoderm is lost at exactly the same stage (HH4+) (Litsiou, 2004, Litsiou *et al.*, 2005, Storey *et al.*, 1992). Furthermore, recent evidence indicates that both neural and PPR induction share common features with induced cells going through the same transcriptional state before they diversify (Trevers *et al.*, 2017). These findings are compatible with the NPB model, but difficult to reconcile with the dual competence model.

In chick, the epiblast is gradually subdivided into domains with different gene expression profiles (see above). This begins before gastrulation where two broad territories can be distinguished, which do however, overlap considerably. As development proceeds new genes become expressed under the influence of local signals and gene expression boundaries gradually sharpen, but continue to overlap at the NPB. Sharpening of these boundaries is mediated by cross-repressive transcription factor interactions, and by positive feedback loops in which 'like' promotes 'like'. Indeed, the same changes in expression patterns are observed in *Xenopus* at blastula stages (Pieper *et al.*, 2012), and experiments using gain- and loss-of-function of transcription factors suggest similar regulatory relationships. It is therefore likely that the mechanisms that segregate cell fates in the ectoderm in avian and amphibian embryos are very similar, or identical.

Recent evidence in chick reveals that NPB cells are indeed a special cell population where neural, neural crest and placode markers not only overlap, but also are co-expressed in the same cell (Roellig *et al.*, 2017). Using antibodies for Sox2, Pax7 and Six1, proteins generally considered to be specific for neural, neural crest and placode progenitor cells, this study reveals that NBP cells are heterogeneous expressing different combinations and levels of these factors. This heterogeneity is apparent at gastrula stages and persists in the neural folds and in the non-neural ectoderm until at least the 10-somite. Lineage tracing Sox2+ cells reveals that they continue to generate neural crest cells even at neural fold stages, and this is consistent with previous single cell labelling studies in chick and mouse (Baggiolini *et al.*, 2015, Bronner-Fraser and Fraser, 1988, McKinney *et al.*, 2013). Using misexpression and knockdowns this study shows that different levels of Sox2 and Pax7 drive cells towards neural and neural crest lineages, respectively. These findings are incompatible with the dual competence model, and the most parsimonious explanation is that cells in the NBP have the potential to generate multiple fates, and that this property is maintained long after neural plate formation. Thus, NBP cells appear to be unique multipotent progenitors for the entire nervous system and future studies involving single cell analysis combined with lineage tracing will be required to unravel the molecular mechanisms that control their commitment to a single lineage.

## Concluding remarks

Placode progenitors give rise to much of the peripheral nervous system in the vertebrate head, and arise together with neural crest cells in the ectoderm surrounding the neural plate. Recent studies in chick have called existing models for cell fate segregation in the ectoderm into question, which suggest that the neural plate border is subdivided into distinct domains of different progenitors. In contrast, it turns out that neural plate border cells are heterogeneous with individual cells expressing different combinations

and levels of transcription factors previously thought to be neural, crest and placode 'specifiers'. This heterogeneity and cell mixing persists until the time of neural tube closure suggesting neural plate border cells are open for different fates much longer than previously thought. The discovery of an unexpected similarity between neural, neural crest and placode induction agrees with these findings, and the identification of many new potential players and their assembly into a new predictive network, provide a new framework to explore lineage segregation at the neural plate border with a new perspective.

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