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Highlights

The peripheral sensory nervous system in the vertebrate head: A gene regulatory perspective

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► Sequential activation of transcription factor networks subdivides the ectoderm. ► Temporal sequence of signals and transcription factors determines cell fate. ► Positive feedback loops and mutual repression stabilise cell fate.

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Review

The peripheral sensory nervous system in the vertebrate head: A gene regulatory perspective

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ABSTRACT

In the vertebrate head, crucial parts of the sense organs and sensory ganglia develop from special regions, the cranial placodes. Despite their cellular and functional diversity, they arise from a common field of multipotent progenitors and acquire distinct identity later under the influence of local signalling. Here we present the gene regulatory network that summarises our current understanding of how sensory cells are specified, how they become different from other ectodermal derivatives and how they begin to diversify to generate placodes with different identities. This analysis reveals how sequential activation of sets of transcription factors subdivides the ectoderm over time into smaller domains of progenitors for the central nervous system, neural crest, epidermis and sensory placodes. Within this hierarchy the timing of signalling and developmental history of each cell population is of critical importance to determine the ultimate outcome. A reoccurring theme is that local signals set up broad gene expression domains, which are further refined by mutual repression between different transcription factors. The Six and Eya network lies at the heart of sensory progenitor specification. In a positive feedback loop these factors perpetuate their own expression thus stabilising pre-placodal fate, while simultaneously repressing neural and neural crest specific factors. Downstream of the Six and Eya cassette, Pax genes in combination with other factors begin to impart regional identity to placode progenitors. While our review highlights the wealth of information available, it also points to the lack information on the cis-regulatory mechanisms that control placode specification and of how the repeated use of signalling input is integrated.

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Introduction

The sensory placodes give rise to most of the peripheral sensory nervous system in the vertebrate head. They form the lens of the eye, the inner ear and the olfactory epithelium and, together with neural crest cells, contribute to the cranial sensory ganglia. Initially, placodes develop as simple patches of ectoderm outside of the central nervous system, but subsequently produce a large variety of cell types ranging from simple lens fibres to sensory cells and neurons, neuroendocrine cells as well as self-renewing stem cells in the olfactory epithelium. As a defining feature of vertebrates, placodes have recently attracted much attention and the molecular pathways controlling their development are beginning to be unravelled.

Placode formation and differentiation is a long process. One of the most surprising findings is that despite their diversity, placodes arise from a common territory of multipotent precursors, the pre-placodal region (PPR), and their progenitors initially share common properties (Bailey et al., 2006; Martin and Groves, 2006; for review: Schlosser, 2006, 2010; Streit, 2007, 2008)—a hypothesis originally proposed almost 50 years ago (Jacobson, 1963a, b, c; see also Torres and Giraldez, 1998). Placode progenitors are specified from "the border", a region where neural and non-neural gene expression overlaps and where cells are initially competent to give rise to neural, neural crest and placodal derivatives, as well as epidermis (Baker et al., 1999; Basch et al., 2000; Bhattacharyya and Bronner-Fraser, 2008; Gallagher et al., 1996; Gallera and Ivanov, 1964; Groves and Bronner-Fraser, 2000; Hans et al., 2007; Köster et al., 2000; Kwon et al., 2010; Liedke, 1942, 1951; Martin and Groves, 2006; Nieuwkoop, 1958; Pieper et al., 2012; Selleck and Bronner-Fraser, 1995; Servetnick and Grainger, 1991; Storey et al., 1992; Streit et al., 1997; Waddington, 1934, 1935; Waddington and Needham, 1936). Specification of placode progenitors is controlled through a balance of inductive and repressive signals emanating from surrounding tissues: the adjacent neural plate and future epidermis and the underlying mesoderm (Ahrens and Schlosser, 2005; Brugmann et al., 2004; Litsiou et al., 2005). Subsequently, placode precursors become different from each other (Ladher et al., 2010; McCabe and Bronner-Fraser, 2009; Ohyama et al., 2007; Schlosser, 2010) and converge from an initially wide distribution within the pre-placodal region (PPR) towards focal thickenings (the placodes) (Bhattacharyya et al., 2004; Pieper et al., 2011; Streit, 2002; Xu et al., 2008). Once formed, placodes either remain as transient neurogenic patches from which neuroblasts delaminate to form the cranial ganglia or expand to deposit neuromasts along the entire body axis, as is the case for the lateral line in amphibians and fish. Alternatively, they invaginate, undergo complex morphogenetic changes and differentiate into various organ-specific cell types characteristic for the lens, otic and olfactory tissues.

Thus, from initial placode progenitor induction to terminal differentiation, ectodermal cells navigate a hierarchy of regulatory states with successively limited developmental potential. Emerging molecular data point to a complex gene regulatory network (GRN) that controls these events and distinguishes placode precursors from other ectodermal derivatives such as the neural plate, neural crest and epidermis. Within this network, each step in the temporal hierarchy can be identified by a specific set of transcription factors (defining the regulatory state of cells at this stage), which cross-regulate each other and which in turn are controlled by defined signalling inputs. While direct interactions and cis-regulatory modules of genes expressed in the placodes are only beginning to be elucidated, there are now sufficient gain- and loss-of-function data to begin to assemble a GRN to model the transition from multipotent placode progenitors towards differentiated placode derivatives. Such networks represent a powerful way to represent developmental processes and cell fate decisions as they allow the integration of large amounts of data into logical circuits (Betancur et al., 2010a; Davidson, 2009; Levine and Davidson, 2005; Peter and Davidson, 2011). For placode development, the main challenge is the integration of information from different animal models that differ in the timing of these events and in the experimental approaches that can be used. Even more complexity arises from the dynamic nature of the process, as illustrated by continuous changes in gene expression and the repeated use of the same signals. Here, we will first provide a brief overview of placode derivatives and their development. Then we will summarise the known molecular events that control the specification of placode progenitor cells and their patterning along the anterior–posterior axis. We will integrate this information into a gene regulatory network using BioTapestry as a tool (Longabaugh et al., 2005, 2009).

Placodes and their derivatives

During embryonic development sensory placodes are first visible as epithelial thickenings next to the developing neural tube (Fig. 1b). Two placodes are non-neurogenic: the adenohypophyseal and lens placodes. While the latter forms next to the future retina to generate the crystalline lens of the eye with lens fibre and epithelial cells, the former develops in the midline and gives rise to the anterior pituitary gland, which generates different neuroendocrine cells. The ophthalmic and maxillomandibular trigeminal placodes (profundal and trigeminal in anamniotes) and epibranchial placodes are simple neurogenic patches, from which neuroblasts delaminate to form the distal portions of the Vth, VIIth, IXth and Xth ganglia. While the trigeminal (Vth) ganglion provides somatosensory innervation from the face, the epibranchial placode-derived neurons provide viscerosensory input from the heart and other visceral organs and gustatory information from the oral cavity. In aquatic vertebrates, the pre- and post-otic lateral line placodes form a specialised sensory system for the detection of water movement and electric fields along the entire body axis generating both neurons and sensory cells. Finally, the otic and olfactory placodes form next to the hindbrain and future olfactory bulb, respectively, and undergo complex tissue reorganisation and folding after their initial invagination. The otic placode forms the auditory and vestibular part of the inner ear including sensory hair cells, the neurons that innervate them, supporting and endolymph-secreting cells, while the olfactory placode produces different cell types including olfactory sensory neurons, stem cells that regenerate them throughout life as well

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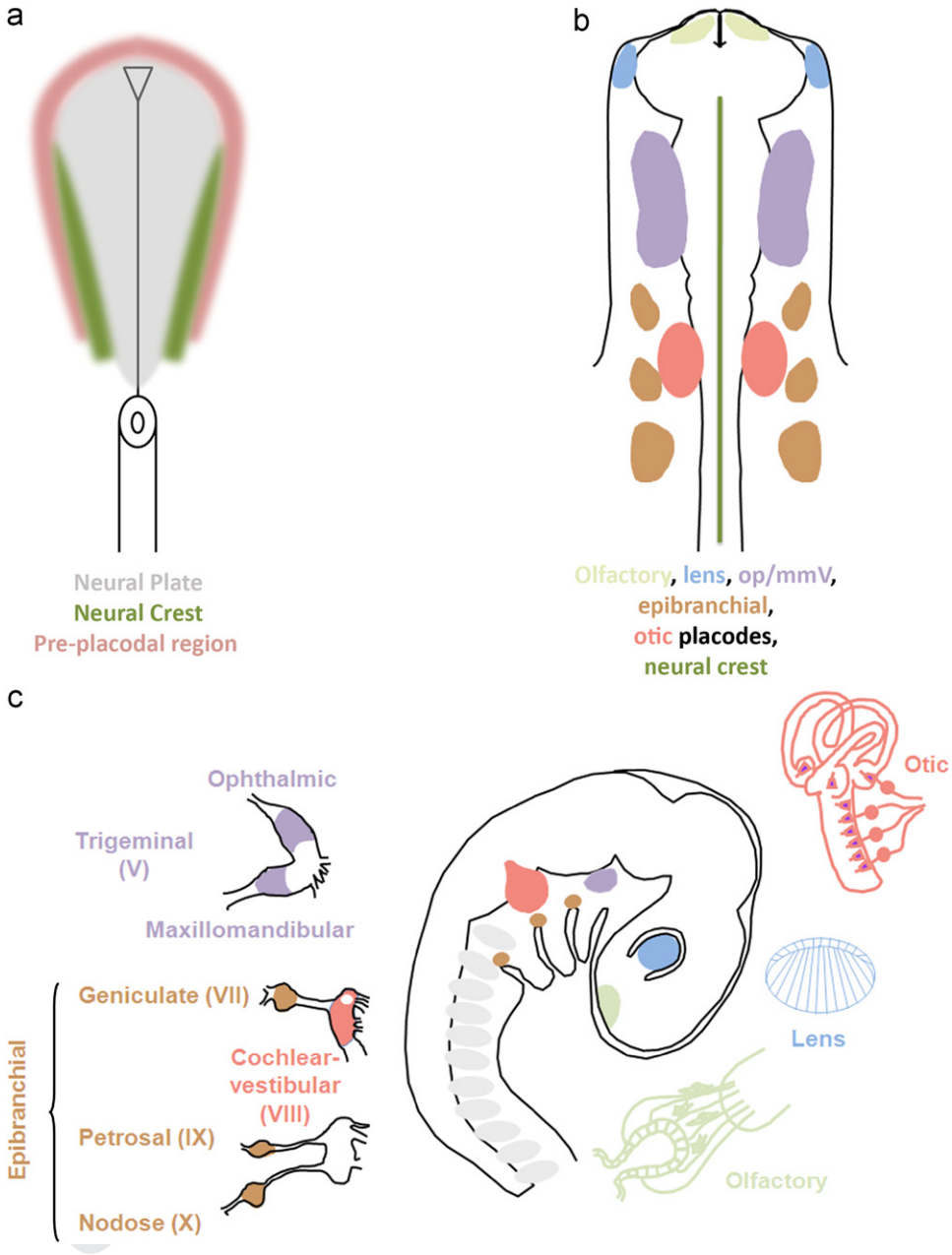

Fig. 1. Placodes and their derivatives. (A) At neural plate stages, the pre-placodal territory (pink) surrounds the anterior neural plate (grey); the neural crest territory lies more medial (green). Note: each domain is not yet clearly defined and progenitors for each cell population are intermingled. The PPR contains precursors for all sensory placodes. (B) Diagram of a 10-somite stage chick embryo; individual placodes are morphologically distinct as thickened patches of ectoderm and occupy distinct positions along the neural tube. Note: the adenohypophyseal placode is not shown and lies in the ventral midline. (C) Diagram showing placodes in a 3-day-old chick embryo and the derivatives at later stages. Left: cranial sensory ganglia; right: sense organ derivatives; modified after Webb and Noden (1993).

as a variety of migratory neurons that leave the placode to localise in the brain. Placode derivatives have been described in great detail in other recent reviews (Baker and Bronner-Fraser, 2001; Schlosser, 2010); however, this brief summary highlights their diversity in both structure and function (Fig. 1c).

Placode progenitor distribution and their relationship with neighbouring cells

Before and during gastrulation, placode precursors are widely dispersed in the ectoderm and intermingle with future neural, neural crest and epidermal cells (Ezin et al., 2009; Fernandez-Garre et al., 2002; Garcia-Martinez et al., 1993; Hatada and Stern, 1994; Streit, unpublished) and a unique placodal territory cannot be defined. However, shortly after the neural plate is established, placode progenitors co-localise to a contiguous band of ectoderm at its border, the pre-placodal region (PPR; Fig. 1a; Bhattacharyya et al., 2004; Dutta et al., 2005; Kozlowski et al., 1997; Pieper et al., 2011; Streit, 2002; Xu et al., 2008). They continue to be interspersed with other ectodermal derivatives and segregation occurs only after neural fold formation in chick, but slightly earlier in *Xenopus*. Two recent studies in zebrafish and *Xenopus* indicate that a first lineage restriction occurs between neural/neural crest and placode/epidermal lineages due to changes in competence (Kwon et al., 2010; Pieper et al., 2012). Initially, future epidermis is competent to generate neural, neural crest and placode cells; however as development proceeds, competence for neural and neural crest is lost, while placodal competence

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persists. Conversely, a young neural plate grafted into the border region can be induced to express both neural crest and pre-placodal markers, while an older neural plate has lost competence to produce placode precursors. While these experiments argue for an early restriction of competence in the neural plate and future epidermis, they leave open the possibility that in vivo cells at the border retain plasticity to change their fate depending on local signals.

Within the PPR, precursors for different placodes are initially mixed, but segregate over time to form morphological placodes with unique identities. The degree of overlap is still under debate as is the question of whether cell movements contribute to the separation of different cells with different fates (Bhat and Riley, 2011; Bhattacharyya et al., 2004; Pieper et al., 2011; Streit, 2002; Xu et al., 2008; for review: Schlosser, 2006; Streit, 2008). On one hand, it is possible that fate map data have overestimated the extent of cell mixing for technical reasons (for discussion see Pieper et al., 2011; Schlosser, 2006); on the other hand, species-specific differences may exist that reflect distinct modes of placode formation. While little or no movement is observed in *Xenopus* (Pieper et al., 2011), in fish and chick, placode precursors appear to move extensively although it is not clear whether movement is random or directional (Bhat and Riley, 2011; Bhattacharyya et al., 2004; Streit, 2002). Ultimately, live imaging over long periods will be required to resolve these issues. At this point the question remains of whether cells within the PPR are truly multipotent and acquire different fates according to their final location, or whether cells pre-committed to specific fates segregate to their appropriate destinations. Since all placode progenitors initially share common properties (see below) and are only committed to their ultimate fate much later (Baker et al., 1999; Bhattacharyya and Bronner-Fraser, 2008; Gallagher et al., 1996; Groves and Bronner-Fraser, 2000; Henry and Grainger, 1990; Jacobson, 1963a, b, c; Waddington, 1937), it is likely that the PPR represents a territory of multipotent cells. Finally, even after placode formation cells from the surrounding ectoderm continue to be recruited into the placodal epithelium (Steventon et al., 2012; Streit, 2002; Xu et al., 2008). This observation suggests that a placode–epidermis boundary is established fairly late and its sharpening may involve cross-repressive interactions of transcription factors similar to the formation of compartment boundaries in the central nervous system (Joyner et al., 2000; Katahira et al., 2000; Kobayashi et al., 2002; Li and Joyner, 2001; Millet et al., 1999; Schwarz et al., 1999). Thus, at neurula stages placode progenitors locate to a defined territory surrounding the anterior neural plate, from which distinct placodes emerge over time.

Special properties of sensory placode progenitors

The PPR is not only defined by the location of placode progenitors, but also by special properties that distinguish it from other ectodermal cells. At neural plate and early somite stages, competence to respond to signals that induce specific placodes is restricted to the head ectoderm and for some placodes to the PPR itself (Baker et al., 1999; Bhattacharyya and Bronner-Fraser, 2008; Gallagher et al., 1996; Groves and Bronner-Fraser, 2000; Henry and Grainger, 1990; Jacobson, 1963c; Ladher et al., 2000; Martin and Groves, 2006). Recent experiments demonstrate that cells must acquire PPR properties before they can form mature placodes (Martin and Groves, 2006). When non-PPR ectoderm is exposed to the otic inducer FGF2, otic markers are not induced; however if the same ectoderm is first grafted into the PPR at head fold stages, it initiates the expression of PPR-specific genes and can now be induced to form an ear. These experiments suggest that otic induction (and possibly the induction of other placodes) occurs in at least two steps: first, cells have to acquire a PPR regulatory state before they can become inner ear.

In addition, PPR cells also share a common developmental programme: irrespective of their later fate all placode precursors are initially specified as lens (Bailey et al., 2006). When PPR explants from different rostrocaudal levels are cultured in isolation they initiate *Pax6* expression (normally confined to trigeminal, lens, olfactory and adenohypophysis precursors), followed by a set of lens-expressed transcription factors like *Sox2*, *L-Maf* and *FoxC1* (Kamachi et al., 1995; Kamachi et al., 2001; Muta et al., 2002; Yoshimoto et al., 2005). Together, these are responsible for activation of the terminal differentiation genes α- and δ-*crystallin* and execution of the lens programme. These findings imply that placode inducing signals not only impart specific placodal fates, but must also suppress the lens programme. Indeed, this appears to be the case for most placodes: activation of the FGF pathway suppresses lens specification in vitro (Bailey et al., 2006) and is required for olfactory, trigeminal, otic and epibranchial placode formation (Alvarez et al., 2003; Bailey et al., 2006; Canning et al., 2008; Freter et al., 2008; Hans et al., 2007; Ladher et al., 2000; Maroon et al., 2002; Martin and Groves, 2006; Nechiporuk et al., 2007; Nikaido et al., 2007; Phillips et al., 2001; Wright and Mansour, 2003). Thus, acquisition of PPR identity is the first step during sensory placode development: PPR cells contribute to all placodes and share common properties before they diversify.

Six and Eya family members at the core of the PPR gene network

PPR cells are identified by a unique set of transcription factors that define their regulatory state. At neural plate stages, they become molecularly distinct by expressing Six and Eya family members (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). These nuclear factors not only play an important role in conferring PPR identity (Brugmann et al., 2004; Christophorou et al., 2009), but are also crucial for many aspects of sense organ and cranial ganglion formation at later stages (Donner and Maas, 2004; Hanson, 2001; Kawakami et al., 2000; Wawersik and Maas, 2000). They are therefore considered to be key regulators of placode development. In addition, the PPR is defined by many other transcription factors that form regulatory circuits with Six and Eya genes, although none of these are PPR specific, but act as their upstream regulators or in parallel pathways (Fig. 2, Table 1).

In vertebrates, six *Six* genes (*Six1-6*) and four *Eya* genes (*Eya1-4*) have been identified (for review: Donner and Maas, 2004; Hanson, 2001; Kawakami et al., 2000; Wawersik and Maas, 2000). Six1-6 proteins contain a Six-type DNA binding homeodomain and an N-terminal Six domain, which mediates interaction with cofactors (Kobayashi et al., 2001; Ohto et al., 1999; Pignoni et al., 1997a). Depending on the presence of such cofactors, Six1-6 proteins are transcriptional repressors or activators: together with Dach or Groucho proteins they inhibit transcription of downstream target genes, whereas when partnered with Eya proteins they act as transcriptional activators (Kenyon et al., 2005a, 2005b; Li et al., 2003; Rayapureddi et al., 2003; Tessmar et al., 2002; Tootle et al., 2003; Zhu et al., 2002). Eya1-4 proteins are unusual: they not only act as transcriptional activators, but also contain tyrosine phosphatase activity (for review: Jemc and Rebay, 2007). They are characterised by a conserved Eya domain, which harbours the phosphatase activity and is responsible for protein–protein interaction (e.g. with Six family members), and a moderately conserved Eya domain 2 surrounded by two proline/serine/threonine (P/S/T domain) stretches. The P/S/T domain is required for transactivation, while the precise function of the Eya domain 2 remains unclear.

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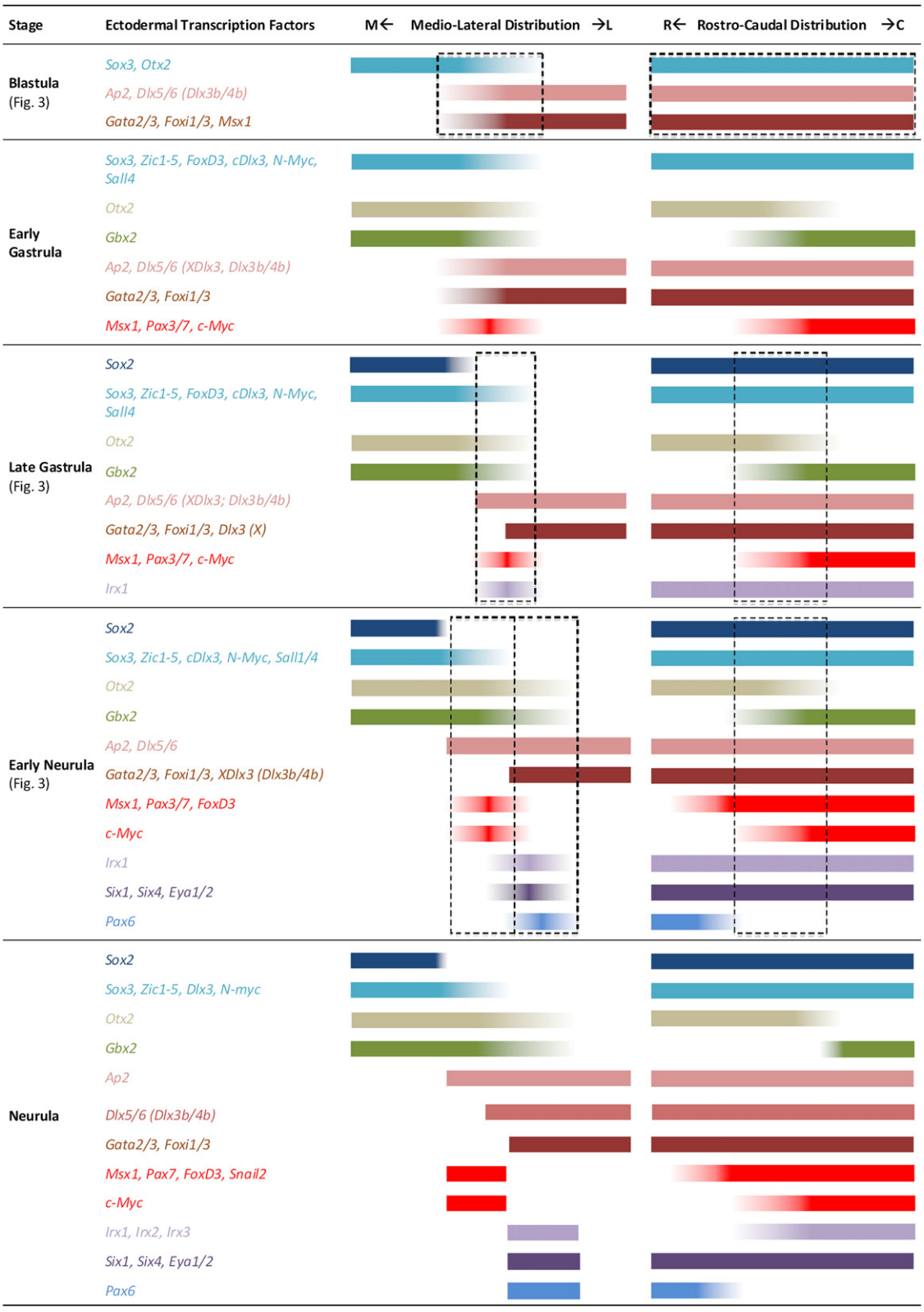

Fig. 2. Distinct regulatory states as ectodermal cells progress towards pre-placodal progenitors. The medio-lateral and rostro-caudal distributions of different ectodermal transcription factors are represented schematically, from pre-gastrula to neurula stages. TFs are organised and colour-coded according to their expression domains across multiple species. Hatched boxes (black) indicate the regulatory states described in the network depicted in Figs. 3 and 5 (see brackets on the left). See main text for full narrative description including references for gene expression data. Note: we use *Ap2* as a generic symbol for the Ap2 transcription factor family. Dlx gene nomenclature and expression across species is complex (see text); in addition to dynamic changes over time, differences are also observed along the anterior–posterior axis at neurula stages at least in chick (see, e.g. Streit, 2002). The diagrams represent approximations.

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Table 1
Interactions during PPR and placode specification. The table summarises all interactions described in the text and the appropriate references. Experimentally verified direct interactions are indicated by

	Source	Interaction	Target	System	Evidence
From blastula to nerula stages	FGF	Promotes	Sox3	Chick	Streit et al. (2000), Wilson et al. (2001), Albazerchi and Stern (2007)
	Dlx5 (Dlx3b/4b), Dlx3, Six1/Eya2	Represses	Sox3	Chick, *Xenopus*	McLarren et al. (2003), Woda et al. (2003); Pieper et al. (2012)
	BMP, FGF, Wnt	Promotes	Zic1-5	*Xenopus*	Monsoro-Burq et al. (2003), Sato et al. (2005), Hong and Saint-Jeannet (2007)
	Pax3	Represses	Zic1-5	*Xenopus*	Hong and Saint-Jeannet (2007)
	BMP, Wnt	Promotes	Pax7	Chick	Litsiou et al. (2005), Patthey et al. (2008)
	FGF, Six1/Eya2	Represses	Pax7	Chick	Christophorou et al. (2009), Stuhlmiller and García-Castro (2012)
	Wnt	Promotes	Pax3	*Xenopus*	Bang et al. (1997), Hong and Saint-Jeannet (2007)
	BMP, Foxi1, Wnt	Promotes	Msx1	Chick, *Xenopus*	Suzuki et al. (1997), Wilson et al. (2001), Matsuo-Takasaki et al. (2005), Hong and Saint-Jeannet (2007)
	Dlx5 (Dlx3b/4b), FGF	Represses	Msx1	Chick	Stuhlmiller and García-Castro (2012) McLarren et al. (2003)
	Ap2, BMP, Dlx5 (Dlx3b/4b), Foxi1, Gata2/3	Promotes	Dlx5 (Dlx3b/4b)	Chick, *Xenopus*	Luo et al. (2001), McLarren et al. (2003), Matsuo-Takasaki et al.(2005), Kwon et al. (2010), Pieper et al. (2012)
	Six1/Eya2	Represses	Dlx5 (Dlx3b/4b)	Chick, *Xenopus*	Brugmann et al. (2004), Christophorou et al. (2009)
	BMP	Promotes	Ap2	*Xenopus* zebrafish	Luo et al. (2003), Kwon et al. (2010)
	BMP, Dlx5 (Dlx3b/4b), Gata2/3, Wnt	Promotes	Gata2/3	Chick, *Xenopus*	Wilson et al. (2001), McLarren et al. (2003), Kwon et al. (2010), Pieper et al. (2012)
	FGF, Six1/Eya2	Represses	Gata2/3	Chick	Christophorou et al. (2009), Stuhlmiller and García-Castro (2012)
From blastula to nerula stages	BMP, Dlx5 (Dlx3b/4b), Gata 2/3	Promotes	Foxi1	*Xenopus*	Matsuo-Takasaki et al. (2005), Kwon et al. (2010), Pieper et al. (2012)
	Wnt	Represses	Foxi1	*Xenopus*	Matsuo-Takasaki et al. (2005)
	BMP, FGF, Wnt	Promotes	Irx1	*Xenopus*	Gomez-Skarmeta et al. (1998); Gomez-Skarmeta et al. (1998); Glavic et al. (2004)
	FGF	Promotes	Otx2	Chick,	Wilson et al. (2001), Albazerchi and Stern (2007)
	BMP, Wnt	Represses	Otx2	Chick	Wilson et al. (2001), Albazerchi and Stern (2007)
	Ap2, c-Myc, Dlx5/6 (Dlx3a/4b)[a], FGF, Foxi1, Gata2/3, Irx1, Zic1-5	Promotes	Six1	Chick, medaka, *Xenopus*, zebrafish	Solomon and Fritz (2002); Bellmeyer et al. (2003); Woda et al. (2003), Brugmann et al. (2004), Glavic et al. (2004), Ahrens and Schlosser (2005), Litsiou et al. (2005), Aghaallaei et al. (2007), Hong et al. (2007), Esterberg and Fritz (2009), Sato et al.(2010)[a]; Kwon et al. (2010); Pieper et al. (2012)
	BMP, Foxd3, Msx1[a], Pax3, Pax7, Wnt	Represses	Six1	Chick, *Xenopus*, zebrafish	Brugmann et al. (2004), Ahrens and Schlosser (2005), Litsiou et al. (2005); Philips et al. (2006), Hong et al. (2007), Kwon et al. (2010); Sato et al. (2010)[a]
	Ap2, Dlx5/6 (Dlx3a/4b), FGF, Foxi1, Gata2/3, Six1/Eya2	Promotes	Eya1/2	Chick, medaka, zebrafish	Solomon and Fritz (2002), Litsiou et al. (2005), Christophorou et al. (2009), Esterberg and Fritz (2009), Kwon et al. (2010), Pieper et al. (2012)
	BMP, Wnt	Represses	Eya1/2	Chick, *Xenopus*, zebraafish	Brugmann et al. (2004), Litsiou et al. (2005), Kwon et al. (2010)
	Ap2, Dlx5/6 (Dlx3a/4b), FGF, Foxi1, Gata2/3, Six1/Eya2	Promotes	Six4	Chick, *Xenopus*, zebrafish	McLarren et al. (2003), Litsiou et al. (2005), Christophorou et al. (2009), Esterberg and Fritz (2010), Kwon et al. (2010), Pieper et al. (2012)
	BMP, Wnt	Represses	Six4	Chick, *Xenopus*, zebrafish	Brugmann et al. (2004), Litsiou et al. (2005), Kwon et al. (2010)
	Six1/Eya2	Represses	Foxd3	*Xenopus*	Brugmann et al. (2004)
PPR regionalization	Otx1/2/5	Represses	Gbx2	*Xenopus*	Steventon et al. (2012)
	Otx1/2/5	Promotes	Dmrt4	*Xenopus*	Steventon, et al., 2012
	Pax6[a], Otx1/2/5, Six1/Eya2, Six3[a]	Promotes	Pax6	Chick, mouse	Ashery-Padan et al. (2000)[a]; Liu et al. (2006)[a]; Christophorou et al. (2009), Steventon et al. (2012)
	TGFβ, Dlx5/6 (Dlx3b/4b), FGF, Pax3, Wnt	Represses	Pax6	Chick, mouse	Bhattacharyya et al. (2004), Smith et al. (2005), Bailey et al. (2006); Grocott et al. (2011), Wakamatsu (2011)
	Pax6	Promotes	Six3	Mouse	Ashery-Padan et al. (2000)
	FGF, Pax3, PDGF, Six1/Eya2, Wnt	Promotes	Pax3	Chick	Lassiter et al. (2007), Canning et al. (2008), McCabe and Bronner-Fraser (2008), Christophorou et al. (2009), Dude et al. (2009)
	Pax6	Represses	Pax3	Chick	Wakamatsu (2011)
	Pax3	Promotes	Eya2	Chick	Dude et al. (2009)
	TGFβ	Promotes	Wnt2b	Chick	Grocott et al. (2011)
	FGF, Foxi1	Promotes	Dlx5/6 (Dlx3b/4b)	Chick, zebrafish	Nissen et al. (2003), Solomon et al. (2003), Hans et al. (2004); Litsiou et al. (2005), Bailey et al. (2006), Hans et al. (2007), Pieper et al. (2012)
	Dlx5/6 (Dlx3b/4b), FGF, Wnt	Promotes	Foxi1	*Xenopus*, zebrafish	Nissen et al. (2003), Hans et al. (2004); Philips et al. (2004), Hans et al. (2007), Pieper et al. (2012)
	Pax2, Pax8	Represses	Foxi1	Zebrafish	Padanad and Riley (2011)
	Gbx2, Pax3	Represses	Otx1/2/5	*Xenopus*	Steventon et al. (2012)
	FGF, Foxi1, Wnt	Promotes	Pax8	*Xenopus*, zebrafish	Philips et al. (2001), Leger and Brand (2002), Nissen et al. (2003), Solomon et al. (2003), Hans et al. (2004), Philips et al. (2004); Mackereth et al. (2005); Hans et al. (2007); Park and St Jeannet (2008), Padanad and Riley (2011)
PPR regionalization	Dlx5/6 (Dlx3b/4b), Gbx2, FGF, Six1/Eya2	Promotes	Pax2	Chick, mouse, *Xenopus*, zebrafish	Ashery-Padan et al. (2000); Philips et al. (2001); Leger and Brand (2002); Maroon et al. (2002); Solomon and Fritz (2002); Nissen et al. (2003); Solomon et al. (2003); Wright and Mansour (2003); Hans et al. (2004); Philips et al. (2004); Solomon et al.(2004); Mackereth et al. (2005); Bricaud and Collazo (2006); Hans et al. (2007); Sun et al. (2007); Freter et al. (2008); Christophorou et al. (2009); Padanad and Riley (2011); Steventon et al. (2012)
	Pax3	Represses	Pax2	Chick	Dude et al. (2009)
	Pax2[a]	Promotes	Six1	Zebrafish	Bricaud and Collazo (2006); Sato et al. (2012)[a]

[a] Gene names are colour-coded according to their expression domain as indicated in Fig. 2 using the same colours as in the network figures.

Six and *Eya* genes were initially identified in *Drosophila* as *sine oculis* (*So*) and *eyes absent* (*Eya*), where they are part of the non-linear network of retinal determination genes. *So* and *Eya* loss of function mutations in the fly cause reduction or complete absence of the eye, while their misexpression leads to ectopic eye formation demonstrating their crucial role for fly eye development (Bonini et al , 1993, 1997; Chen et al., 1997; Cheyette et al., 1994; Mardon et al., 1994; Pignoni et al., 1997a, 1997b; Serikaku and O'Tousa, 1994; Weasner et al., 2007). While it was generally assumed that *Drosophila* So acts as a transcriptional activator during eye formation, recent evidence suggests that a key role of So is to repress the antennal selector gene *Cut* (Anderson et al., 2012): misexpression of a constitutive repressor form of So, but not of a constitutive activator, is able to induce ectopic eyes in the antennal disc. Thus, a revised model for retinal determination emerges in which So plays a dual role downstream of the Pax6 homolog Eyeless: together with a yet-to-be-identified co-repressor it inhibits non-retinal fates and promotes eye formation when partnered with Eya. Among the genes activated by So and Eya is the *ski*/*sno* related transcriptional co-factor *Dac* and together they form a regulatory loop to promote each others' expression and retina development (Chen et al., 1997; Davis et al., 1999; Hammond et al., 1998; Mardon et al., 1994; Shen and Mardon, 1997). Likewise, recent vertebrate data suggest a similar mode of action for Six proteins in vertebrate placode development (see below).

As their widespread expression in all sensory placode progenitors suggests, vertebrate Six and Eya proteins not only play important roles in eye formation, but in all other sensory structures in the head. The loss of Six1, Six5, Eya1 and/or Eya4 function causes defects in the eye, the ear, the cranial ganglia and the olfactory epithelium (Chen et al., 2009; Friedman et al., 2005; Konishi et al., 2006; Kozlowski et al., 2005; Laclef et al., 2003; Li et al., 2003; Ozaki et al., 2004; Xu et al., 1999; Zheng et al., 2003; Zou et al., 2004, 2006). Similarly, human mutations in these genes have been associated with Branchio-Oto-Renal syndrome where patients present hearing, renal and branchial defects, with late-onset deafness and lens cataract (Abdelhak et al., 1997; Azuma et al., 2000; Johnson et al., 1999; Ruf et al., 2004; Schonberger et al., 2005; Wayne et al., 2001; Winchester et al., 1999; Zhang et al., 2004). At early developmental stages, *Six* and *Eya* genes play an important role in specifying sensory progenitors at the border of the neural plate. Six1 knock down or misexpression of a constitutive Six1 repressor form leads to the absence of placode progenitors, while misexpression of wild type Six1 promotes PPR identity at the expense of epidermis and neural crest (Brugmann et al., 2004; Christophorou et al., 2009). Like in the fly, Six1 seems to associate with transcriptional repressors or activators: repression of non-placodal fate involves Groucho repressors, while association of Six1 with Eya1/2 favours placode fates. The activation of Six1 target genes is required for normal expression of placode-specific *Pax* genes (Fig. 5), which in turn appear to determine placode identity. This is in contrast to *Drosophila*, where the Pax6 homologue *Eyeless* (*Ey*) acts upstream of *So* and *Eya* and is required for their expression (for review: Donner and Maas, 2004). This inversed regulatory relationship may explain why, unlike in the fly, where So and Eya induce ectopic eyes, misexpression of Six1 and Eya2 in competent non-placodal ectoderm does not induce mature ectopic placodes (Christophorou et al., 2009). With at least three different Pax genes downstream of Six/Eya (Pax2, 3 and 6) additional inputs must be required to provide regional specificity. Together these finding suggest that the Six and Eya network plays a critical role in specifying sensory progenitors and defines their regulatory state, but that additional factors that work in parallel or downstream are required for sense organ formation.

Transcription factors upstream of the *Six* and *Eya* network

How are sensory progenitors positioned at the border of the neural plate? We will analyze the upstream events by dissecting the core transcription factor network involved in the activation of *Six* and *Eya*. The PPR is first identified at neural plate stages, shortly after induction of the central nervous system and after or concomitant with neural crest cell specification. The subdivision of the ectoderm into different domains occurs sequentially starting from pre-gastrula stages, a process that is not very obvious in anamniotes because of their extremely fast development. The "neural plate border" and "binary competence" models have recently been discussed as two opposing models for PPR induction (Pieper et al., 2011; Schlosser, 2006); however, we argue that considering the temporal hierarchy of events unifies both models. Below we review this sequence of events and the molecular cascade that controls them to explain how sensory progenitors are uniquely positioned, surrounding the anterior neural plate.

Among the transcription factors that regulate *Six* and *Eya* gene expression are members of the Dlx family, which play multiple roles in ectodermal patterning. Before we discuss their function it is important to note that the nomenclature and expression/function of specific Dlx family members do not correspond across species. In amniotes for example, *Dlx3* is neural-enriched during gastrulation and is later confined to the olfactory placode (Bhattacharyya and Bronner-Fraser, 2008; Khudyakov and Bronner-Fraser, 2009). Conversely in *Xenopus*, *Dlx3* expression is non-neural and resembles that of *Dlx5*, yet they remain functionally distinct: *Dlx5* is activated downstream of *Dlx3* (Pieper et al., 2012). Amniote *Dlx6* expression overlaps that of *Dlx5*, but its function has yet to be studied within the PPR (Brown et al., 2005). Zebrafish exhibits further differences to both amniotes and *Xenopus*, partly due to gene duplications within the Dlx family. To avoid over-complicating the network model with unresolved cross-species discrepancies, we have elected to treat amniote *Dlx5/6*, *Xenopus Dlx3/5* and teleost *Dlx3b/4b* collectively as "*Dlx5/6 (Dlx3b/4b)*" whereas amniote *Dlx3* is set apart. Accordingly, we acknowledge that critical details of Dlx gene function have been omitted from our present model. Further studies, in particular cross-species analysis of cis-regulatory elements for all Dlx family members, will need to resolve these differences.

Subdivision of the ectoderm by sequential activation of transcription factors

At blastula stages, the embryonic region is characterised by the expression of pre-neural (*Sox3*, *Otx2*, *ERNI*, *Geminin*; Bally-Cuif et al., 1995; Kroll et al., 1998; Papanayotou et al., 2008; Rex et al., 1997; Streit et al., 2000) and non-neural genes (*Dlx* genes, *Gata2/3*, *Msx1*, *Ap2*, *Foxi1/3*; Brown et al., 2005; Hans et al., 2007; Hans et al., 2004; Hoffman et al., 2007; Knight et al., 2003; Li and Cornell, 2007; Luo et al., 2001a, 2001b; Matsuo-Takasaki et al., 2005; McLarren et al., 2003; Ohyama and Groves, 2004; 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) in partially overlapping domains (Fig. 2).

Pre-neural factors are expressed more medially in the chick epiblast, while non-neural factors are enriched laterally. Likewise, in *Xenopus* pre-neural and non-neural factors initially overlap animally, but then become restricted to more dorsal and ventral regions, respectively (Pieper et al., 2012). Although little is known about their regulatory interactions at this stage, some of the signalling inputs have been identified (Fig. 3). *Sox3*, *ERNI* and *Geminin* expression is initiated by FGF signalling, while *Otx2* requires a combination of FGF activation and Wnt and BMP antagonists

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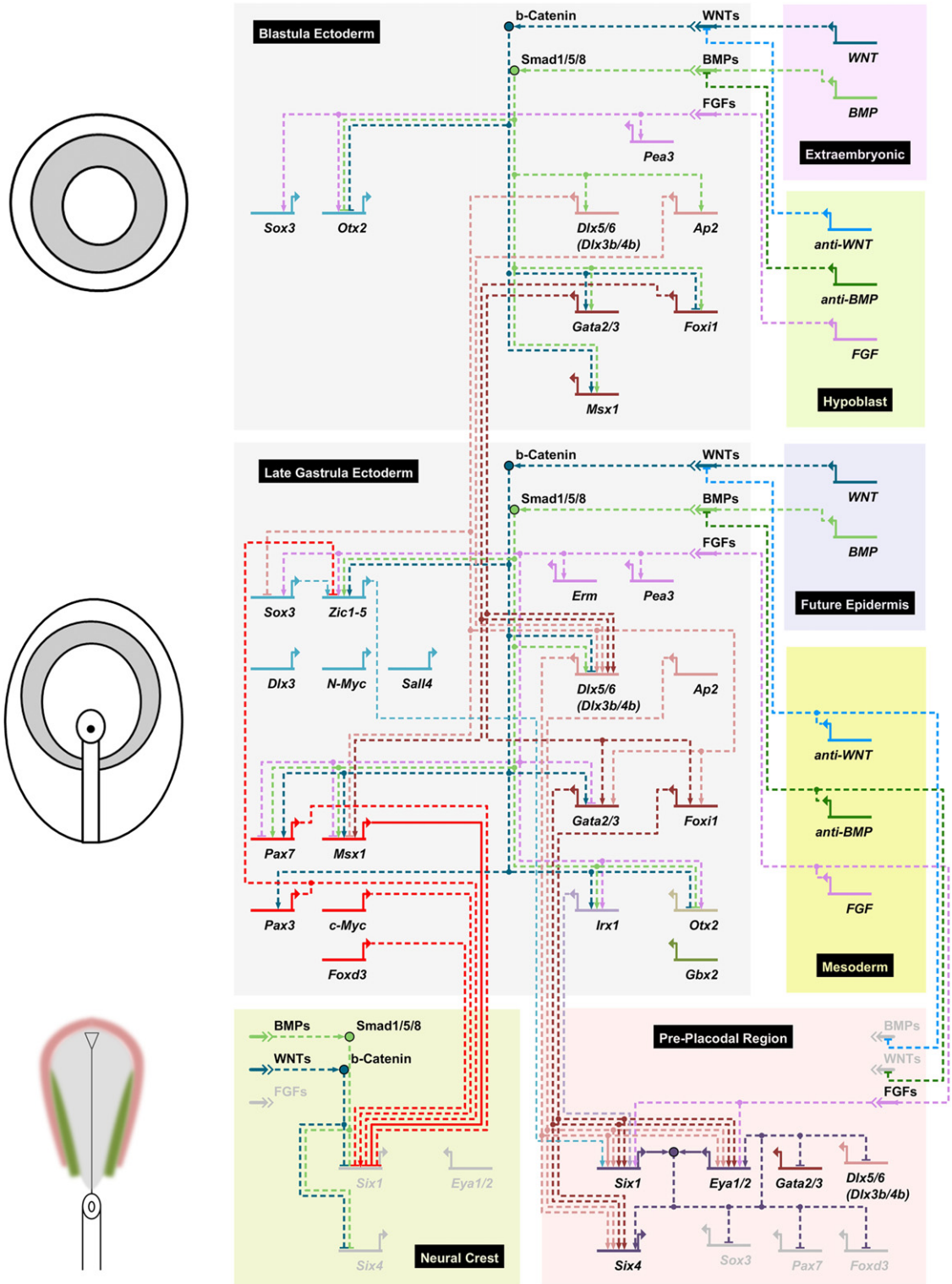

Fig. 3. Gene regulatory interactions establishing the pre-placodal territory. Signalling and transcription factor interactions are shown from blastula and to neurula stages. Diagrams on the left show the corresponding stages in the chick embryo; regions are colour-coded according to the regulatory state described in the network. Neural and non-neural gene expression domains in the pre-streak epiblast (blastula) are established through signals from the hypoblast and extraembryonic region. At gastrula stages, different transcription factors are initiated downstream at the border of the neural plate. At head process stages, Six and Eya genes become expressed in the pre-placodal region, but are repressed in future neural crest cells. Note: as shown in Fig. 2 gene expression domains do not yet form sharp boundaries at this stage. Gene symbols are colour-coded according to their expression profiles summarised in Fig. 2. Progenitor populations (boxes) are colour-coded according to their physical distributions summarised in Fig. 1. In the network, solid lines represent verified direct interactions, while this information is not known for interactions represented in dashed lines. For a neural crest GRN see (Betancur et al., 2010a; Sauka-Spengler and Bronner-Fraser, 2008).

(Albazerchi and Stern, 2007; Papanayotou et al., 2008; Streit et al., 2000; Wilson and Edlund, 2001). Accordingly, the Ets family member *Pea3*, a transcriptional target of FGF signalling, is expressed widely in the embryonic region (Lunn et al., 2007). In amniotes, these signals emanate from the hypoblast (anterior visceral endoderm in mouse), which underlies the embryonic region (for

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review: Stern and Downs, 2012). In contrast, *Dlx5/6*, *Gata2/3*, *Msx1*, *Foxi1* and *Ap2* depend on BMP activity with *Gata2* and *Msx1* also being positively regulated by canonical Wnt signalling, while this pathway inhibits *Foxi1* and *X-Dlx3* (Beanan et al., 2000; Hoffman et al., 2007; Hong and Saint-Jeannet, 2007; Kwon et al., 2010; Matsuo-Takasaki et al., 2005; Pera et al., 1999; Suzuki et al., 1997; Wilson et al., 2001). Accordingly, members of the BMP and Wnt families are expressed in the extraembryonic ectoderm adjacent to the non-neural domain or in the non-neural ectoderm itself (Skromne and Stern, 2001; Streit et al., 1998; Wilson et al., 2001). Recent data from zebrafish define a clear time window for BMP activity (Kwon et al., 2010): *Gata2*, *Foxi1* and *Ap2* require BMP signalling before but not after gastrulation. Thus, prior to gastrulation, antagonistic activity between FGF and Wnt/BMP signalling roughly subdivides the embryonic region into pre-neural and non-neural territories with a large region of overlap (Figs. 2 and 3).

During gastrulation these territories become further subdivided molecularly as new genes are expressed and relative expression boundaries change (Fig. 2). At early gastrula stages, non-neural transcripts form two groups with *Gata2/3* and *Foxi1* being expressed more laterally than *Ap2*, *X-Dlx3* and *Dlx5/6*, whose expression abuts the neural plate (Feledy et al., 1999a; Khudyakov and Bronner-Fraser, 2009; Kwon et al., 2010; Luo et al., 2001b; Pieper et al., 2012; Streit, 2002; Woda et al., 2003). Unlike in fish and Xenopus, in chick *Dlx3* expression is similar to pre-neural genes (Khudyakov and Bronner-Fraser, 2009)). Surprisingly, genes previously considered as neural crest specifiers like *FoxD3* and *N-myc* are transiently coexpressed with pre-neural transcripts before being confined to the neural crest domain (Khudyakov and Bronner-Fraser, 2009) suggesting that at early stages a common regulatory state may define progenitors for both lineages. In addition to *Pea3*, the Ets transcription factor *Erm* is now also present in the forming neural plate and the surrounding ectoderm (Lunn et al., 2007) as are *Zic1-5* (Elms et al., 2004; Elms et al., 2003; Gamse and Sive, 2001; Inoue et al., 2007; Merzdorf, 2007; Mizuseki et al., 1998; Nakata et al., 1997, 1998), *Dlx3* (in chick; Khudyakov and Bronner-Fraser, 2009), *Sall1* (Bohm et al., 2008; Sweetman et al., 2005) and *Spalt4* (or Sall4; Barembaum and Bronner-Fraser, 2007). In *Xenopus*, *Zic1* and *Zic5* are activated at the edge of the neural plate in response to FGF signalling presumably from the underlying paraxial mesoderm (Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2003); in tissue recombination assays paraxial mesoderm can induce *Zic5* in animal caps, but this is blocked in caps injected with dominant negative FGF receptor (Monsoro-Burq et al., 2003). In addition, at intermediate levels of BMP activity Wnt signalling also activates *Zic1* (Hong and Saint-Jeannet, 2007). Thus, different pathways converge on Zic1 (Fig. 3), while nothing is known about the signals that induce *cDlx3*, *Spalt4* and *Sall1* in the neural plate or at its border.

At late gastrula stages, the definitive neural marker *Sox2* is initiated in the neural plate in response to neural inducing signals from the organiser (Rex et al., 1997; Streit et al., 1997; Uchikawa et al., 2003). Neural and non-neural transcripts continue to overlap in a broad territory, named 'the border' of the neural plate (Moury and Jacobson, 1989; Streit and Stern, 1999; Zhang and Jacobson, 1993), and it is in this region that precursors for neural, neural crest, placodes and epidermis are intermingled (Ezin et al., 2009; Fernandez-Garre et al., 2002; Garcia-Martinez et al., 1993; Hatada and Stern, 1994) and *Irx1*, one of the *Six* and *Eya* upstream regulators, is switched on under the influence of BMP and FGF signalling (Bellefroid et al., 1998; Glavic et al., 2002; Gomez-Skarmeta et al., 1998; Goriely et al., 1999; Khudyakov and Bronner-Fraser, 2009) (Figs. 2 and 3).

Simultaneously, distinct anterior–posterior territories are set up in the embryonic region (Fig. 2). *Otx2* and *Gbx2* are among the first genes that roughly separate the embryonic region into rostral and caudal domains with *Otx2* beginning to localise anteriorly and *Gbx2* posteriorly (Acampora et al., 1995; Bally-Cuif et al., 1995; Braun et al., 2003; Broccoli et al., 1999; Gammill and Sive, 2000; Glavic et al., 2002; Li et al., 2009; Millet et al., 1999). Both genes continue to overlap until they form a sharp boundary at early somite stages (Steventon et al., 2012). In chick, *Msx1*, *Pax3* and *c-Myc* expression begins next to the primitive streak, initially widespread encompassing the non-neural ectoderm but then rapidly localising to a few rows of cells lining the posterior neural plate (Bang et al., 1997; Khudyakov and Bronner-Fraser, 2009; Streit and Stern, 1999). Like at pre-gastrula stages, FGF signalling negatively regulates *Msx1* and *Gata2* preventing their expression in more medial, neural territory (Stuhlmiller and García-Castro, 2012). Shortly thereafter, the neural crest specifier *Pax7* is initiated within the Msx1/Pax3 territory (Basch et al., 2006) and over the next few stages, all three genes expand to encompass most of the anterior neural plate in a thin line. Recent evidence in chick suggests that already at gastrula stages posterior Pax7^{+} and anterior Pax7^{-}cells are specified as neural crest cells (Basch et al., 2006; Patthey et al., 2008) indicating that specification of the neural plate border and neural crest may be regulated by different mechanisms along the rostrocaudal axis.

In summary, BMP and Wnt signalling activate early expressed non-neural factors, while FGFs prevent their expression close to the neural plate and initiate pre-neural genes (Fig. 3). As a result, partially overlapping domains of transcription factors define distinct regulatory states within the ectoderm (Fig. 2): neural, epidermal and the border in between. The latter begins to be subdivided molecularly into *Ap2/Dlx3/5/6* positive and negative regions during gastrulation. These dynamic changes highlight the importance of timing when interpreting experimental manipulations as some markers label different cells at different times. There are few, if any systematic studies investigating many transcription factors simultaneously making it difficult to integrate data from different studies and across species. Thus, our knowledge of the regulatory interactions among these factors is still sparse and none of the critical regulator elements have been identified.

Restricting neural fate: repression by non-neural transcription factors

One important function of the early, non-neural genes appears to be the restriction of neural fates by repressing neural markers (Fig. 3). In *Xenopus*, overexpression of Foxi1a represses the neural marker *Sox2*, but promotes non-neural genes like *X-Dlx3* and epidermal keratin (Matsuo-Takasaki et al., 2005). In contrast, loss of Foxi1a leads to *Sox2* expansion and reduction of *Dlx3, Msx1* and epidermal keratin (Kwon et al., 2010; Matsuo-Takasaki et al., 2005). These observations suggest that Foxi1 lies upstream of Dlx3 and Msx1. However, loss- and gain-of-function experiments for Dlx3, Dlx5, Gata2/3, Msx1 and Ap2 suggest more complex regulatory relationships. Misexpression of any of these factors represses neural fate (*Sox2* and/or *-3*), while knock-down or misexpression of dominant negative forms enlarges the neural plate (Feledy et al., 1999a; Linker et al., 2009; Luo et al., 2001b; McLarren et al., 2003; Pieper et al., 2012; Suzuki et al., 1997; Tribulo et al., 2003; Woda et al., 2003). Since these factors are thought to act as transcriptional activators it is likely that their interaction with neural genes is indirect, mediated by yet unknown transcriptional repressors. In addition, they regulate each other: in zebrafish, both Gata3 and Ap2 are required for *Dlx3* expression, while in *Xenopus* Dlx3 and Gata2 regulate their own expression and that of *Dlx5* and *Foxi1a* (Kwon et al., 2010; Pieper et al., 2012). Thus, positive feedback loops reinforce the expression of these transcription factors in the non-neural ectoderm

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possibly making them independent of further signalling input (Fig. 3).

In the posterior non-neural ectoderm, Pax3 is positively regulated by canonical Wnt signalling (Bang et al., 1997; Hong and Saint-Jeannet, 2007) and also antagonises neural specification: its overexpression reduces *Sox2* expression, while Pax3 knock-down expands both *Sox2* and *Zic1* (Hong and Saint-Jeannet, 2007). Interestingly, Zic1 and Pax3 cooperate to promote neural crest cell fates later, while Zic1 alone favours placodal development suggesting that the balance between both factors is important to determine ultimate cell fates.

Thus, repressive action of non-neural genes limits the extent of the neural plate: they suppress neural specific transcription factors and reinforce their own expression (Fig. 3). Whether neural factors in turn repress non-neural fate at these early stages remains to be elucidated. The identification of regulatory modules of each factor will be crucial to determine whether these interactions are direct or indirect.

Transcriptional input into the Six and Eya network

At neurula stages, the expression of Six and Eya family members is first initiated with their PPR-specific expression being regulated by both pre-neural and non-neural transcription factors, together with the earliest known border-specific factor *Irx1* (Figs. 2 and 3). The Dlx family members X-Dlx3 and Dlx5 (and presumably Dlx6, which is co-expressed with Dlx5) continue to play a role in addition to antagonising neural specification (Luo et al., 2001b; McLarren et al., 2003; Pieper et al., 2012; Woda et al., 2003). However, differential expression of the family members suggests a complex role. In Xenopus, *Dlx5* continues to abut the neural plate at early neurula stages like in chick (Beanan and Sargent, 2000; Feledy et al., 1999a; Khudyakov and Bronner-Fraser, 2009; Luo et al., 2001b; McLarren et al., 2003; Streit, 2002), while *Dlx3* (and *Dlx3b/4b* in fish) is absent from the future neural crest domain (Kwon et al., 2010; Luo et al., 2001b; Pieper et al., 2012).

Misexpression of *Dlx5* in chick or *Dlx3* in *Xenopus* represses neural crest fates, while promoting the expression of the pre-placodal markers *Six1/4* and *Eya1*. In contrast, Dlx3 knock-down or misexpression of a dominant negative form results in the loss of pre-placodal and crest markers. Similarly, in zebrafish the absence of *dlx3b* and *-4b* function (b380 mutants or morphants) causes the loss of PPR markers and a reduction of olfactory, trigeminal and otic placodes, while dlx3b overexpression leads to an enlarged PPR (Esterberg and Fritz, 2009; Kaji and Artinger, 2004; Solomon and Fritz, 2002). Thus, Dlx proteins are required for PPR specification and promote PPR specific gene expression. Indeed, recent studies implicate X-Dlx3 as competence factor for sensory progenitors: Dlx3 function is required for PPR induction by FGF and BMP antagonists (Pieper et al., 2012). In agreement with these findings, Dlx5 activates *Six1* expression by directly binding to its anterior PPR enhancer (Six1-14; Sato et al., 2010). Together, these experiments implicate Dlx family members as important upstream regulators of Six genes and mediators of placodal development.

The function of Dlx proteins during neural crest cell specification appears to be more complex. First, different Dlx family members show slightly different expression patterns in *Xenopus*, with *Dlx5* encompassing epidermal, placodal and crest territories, while *Dlx3* is absent from neural crest cells (Luo et al., 2001a). Second, while Dlx5, but not Dlx3 has been implicated in neural crest cell formation (Feledy et al., 1999b; Luo et al., 2001a), a recent study shows that both gain and loss of Dlx3 function reduce neural crest markers (Pieper et al., 2012), while in fish, Dlx3b/4b may control neural crest cell formation in a non cell autonomous manner (Kaji and Artinger, 2004). These observations suggest that a fine balance of Dlx protein function is required for normal crest development. This balance may be achieved through interaction with Msx1 proteins, which show partially overlapping expression. Msx and Dlx proteins can form heterodimers to modulate their action as transcriptional repressors or activators (Zhang et al., 1997). Thus, Dlx protein function may differ depending on the amount of Msx1 present. In addition, as mediator of BMP signalling and epidermal specification, Msx1 inhibits PPR fate: in the absence of Dlx3b/4 function, knock-down of MsxB, C and E in zebrafish restores placode development (Phillips et al., 2006). In agreement with this, Msx1 binds to the anterior PPR enhancer of Six1 and negatively regulates its expression.

Two recent studies in zebrafish and *Xenopus* have identified Ap2, Foxi1 and Gata2/3 as important regulators and potential competence factors for pre-placodal genes (Kwon et al., 2010; Pieper et al., 2012). Knock-down of one or more of these factors leads to loss of *Six1/4* and *Eya1* expression, while overexpression alone or in combination results in ectopic expression of PPR specifiers. Importantly, like Dlx3 the presence of these factors is required for PPR induction by FGF signalling in combination with BMP antagonists (see below) providing strong evidence for their role as competence factors. Thus, while Ap2 and Dlx family members are required for both PPR and neural crest cell specification, Foxi1 and Gata2/3 only regulate placodal fate. Thus, although the genes that specify neural crest and placode precursors are regulated differentially they also share some transcriptional input. In summary, members of the Foxi1, Gata, Dlx and Ap2 family play a role in demarcating the boundary between neural and non-neural ectoderm and are critical regulators of PPR fate (Fig. 3).

Much less is known about the role of other pre-neural and non-neural factors in regulating PPR specific transcripts. In Xenopus, Sox3 represses epidermal character, while promoting neural plate identity by inducing *Sox2*; both Sox proteins positively regulate neural *Zic1* and *Geminin* expression (Rogers et al., 2009). Placode-specific genes have not been investigated. In medaka, misexpression of Sox3 results in the formation of ectopic placodes within the PPR and may promote PPR gene expression, although this has not been examined systematically (Köster et al., 2000).

As discussed above, the three transcription factors *Pax3*, c-*Myc* and *Msx1* are first expressed along the posterior neural plate and later in neural crest cells. All three promote neural crest cell formation, but play different roles in placode specification. While c-Myc is required for the development of both neural crest and PPR as shown in knock-down studies in *Xenopus* (Bellmeyer et al., 2003), Msx1 and Pax3 negatively regulate PPR specific genes (Hong and Saint-Jeannet, 2007). In zebrafish, sensory progenitors depend on Dlx3b/4b function; however, their specification is rescued when MsxB, C and D are knocked down in Dlx3b/4b mutants (Esterberg and Fritz, 2009; Kaji and Artinger, 2004; Phillips et al., 2006; Solomon and Fritz, 2002). In agreement with this observation, Msx1 negatively regulates the anterior PPR Six1 enhancer (Sato et al., 2010). As a direct target of BMP signalling (Maeda et al., 1997; Suzuki et al., 1997; Yamamoto et al., 2000) Msx1 may mediate placode inhibition by BMPs (see below). Likewise, overexpression of Pax3 represses *Six1* in the PPR and as a canonical Wnt target (Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005), Pax3 may mediate its activity to repress placode formation (see below). It therefore seems likely that at early gastrula stages, when *Pax3* and *Msx1* are present in the posterior non-neural ectoderm, they restrict *Six1* expression to the head ectoderm, while at neurula stages, when both are present in the neural folds, where neural crest cells are located, they prevent *Six1* expansion into the crest territory.

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At the neural plate border, *Pax3* and *Zic1* are expressed in partially overlapping domains and their balance may control the decision between placodal versus neural crest fates (Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005). In the absence of Zic1 function, *Six1* expression is lost, but only in the lateral PPR (Hong and Saint-Jeannet, 2007), while Zic1 overexpression shows conflicting results. Whereas some studies show enhanced *Six1* expression in the PPR (Hong and Saint-Jeannet, 2007; Li et al., 2009), others report *Six1* loss (Brugmann et al., 2004). Thus, Zic1 function is context dependent and appears to switch from a positive to a negative regulator of placode precursor specification depending on the presence of other transcription factors like Pax3.

Finally, *Irx1*, a member of the TALE family of homeodomain proteins, is expressed in the PPR just prior to the onset of *Six1/4* and *Eya1/2* and later in the placodes themselves (Glavic et al., 2002; Gomez-Skarmeta et al., 1998; Goriely et al., 1999; Khudyakov and Bronner-Fraser, 2009). In *Xenopus*, overexpression of Xiro1 enhances *Six1* expression, while Xiro1 loss reduces it (Glavic et al., 2004), suggesting that Irx1 is a positive regulator of *Six1*. Since Irx1 itself is activated by FGF and BMP signalling (Bellefroid et al., 1998; Glavic et al., 2004), but slightly later than the BMP-dependent placode competence factors (Gata2/3, Foxi1, Dlx3, Ap2), it is possible that Irx1 is a downstream mediator of these factors. Alternatively it may cooperate with Foxi1: while *Foxi1* expression initially encompasses the entire non-neural ectoderm at neural stages it becomes confined to the PPR concomitant with the onset of Irx1.

In summary (Fig. 3), Gata, Foxi1, Dlx and Ap2 proteins have been implicated as competence factors required cell autonomously for the expression of PPR specific genes of the Six and Eya families. Likewise, Irx1 and Zic1 positively regulate *Six1* expression, while the neural crest specifiers Pax3 and Msx1 repress its transcription. So far only Dlx and Msx proteins have been shown to interact directly with the Six1 anterior PPR enhancer to provide positive and negative input, respectively. However, overall the regulatory interactions that control PPR-specific expression of Six1/4 and Eya1/2 remain poorly understood.

Stabilising sensory progenitor fate: positive feedback loops and repression of alternative fates

Once Six and Eya genes are initiated in the PPR they act to stabilise the system by promoting sensory progenitor fate and repressing non-placodal character (Fig. 3). Misexpression of Six1 and Eya2 induces ectopic expression of another Six family member, *Six4*, as well as *Eya2* itself (Christophorou et al., 2009). Unlike Six1, the Six4 protein contains a transactivation domain in addition to the homeo- and six-domain (Kawakami et al., 1996; Kawakami et al., 2000) and may therefore activate target genes independent of other co-activators. Thus, in a positive feedback loop Six and Eya proteins promote their own expression, although it is unclear whether they do so by directly binding to their enhancers or via other factors like Six4.

Simultaneously, they repress genes characteristic for other cell fates including their own competence factors. For example, while Gata3 and Dlx5 are necessary for initiating *Six1* and *Eya1* in the PPR (Kwon et al., 2010; Pieper et al., 2012), once expressed Six1 and Eya1/2 repress both genes cell autonomously to prevent cells from adopting non-placodal fate (Brugmann et al., 2004; Christophorou et al., 2009). In addition, *Dlx5* and *Gata3* are induced ectopically in neighbouring cells suggesting that the Six/Eya complex activates a signalling pathway cell autonomously, which in turn regulates gene expression in neighbouring tissue. Whether Six/Eya activate a transcriptional repressor or whether Six1 associates with a co-repressor (see above) to shut down Dlx5 and Gata3 transcription is currently unknown. However, in analogy to So activity in the fly eye it is possible that a repressor function of Six1 is key for the manifestation of placodal fate. Likewise, misexpression of Six1 alone or in combination with Eya2 represses the neural markers *Sox2* and *Sox3* as well as the neural crest specific genes *Pax7* and *FoxD3* in a cell autonomous manner.

Thus, a model emerges in which pre-neural and non-neural upstream factors activate *Six* and *Eya* expression next to the anterior neural plate to specify sensory progenitors (Fig. 3). A positive feedback loop of the Six/Eya complex subsequently ensures that once expressed these genes become independent of this upstream input, while cell autonomous repression of neural, non-neural and neural crest fate stabilises placode progenitor identity.

Signalling events upstream of the core PPR gene network

Signalling input into the Six and Eya network

A number of signalling pathways have been implicated in PPR specification and they appear to act sequentially during this process (Fig. 3). PPR inducing signals emanate from the underlying mesoderm and the adjacent neural plate: when grafted ectopically either tissue can induce *Six1*, *Six4* and *Eya2* (Ahrens and Schlosser, 2005; Litsiou et al., 2005). Both tissues express different members of the FGF family: *FGF4* and *FGF8* are present in the chick mesoderm, while *FGF8* is found in the anterior neural plate in *Xenopus* (Ahrens and Schlosser, 2005; Ohuchi et al., 2000; Shamim and Mason, 1999). As discussed above, FGF signalling promotes the expression of pre-neural genes (*Sox3*, *ERNI*, *Geminin*) prior to gastrulation and may continue to do so at the border of the neural plate. The future placode territory receives FGF signalling as evidenced by expression of the FGF targets *Pea3* and *Erm* as well as the presence of phosphorylated Erk (pErk) (Khudyakov and Bronner-Fraser, 2009; Lunn et al., 2007; Stuhlmiller and García-Castro, 2012). FGF signalling prevents the expansion of PPR-repressing factors (*Msx1*, *BMP4*) towards the neural plate (Stuhlmiller and García-Castro, 2012), thus providing a favourable environment for PPR specification. In addition, FGF8 is sufficient to induce *Eya2*, but not any other PPR specifier (Litsiou et al., 2005).

Loss-of-function approaches show that FGF signalling is necessary to establish the Six/Eya network within the PPR. In *Xenopus* FGF8 knockdown or misexpression of a dominant negative FGF-receptor prevents *Six1* expression (Ahrens and Schlosser, 2005; Brugmann et al., 2004), while in chick inhibition of FGF signalling abolishes the PPR-inducing ability of the mesoderm (Litsiou et al., 2005). The presence of pErk in future sensory progenitors from gastrula stages onwards suggests that FGF is an early signal in the cascade of events leading to PPR specification. Thus, FGFs clearly play an important role in sensory progenitor specification, but alone are not sufficient to induce all components of the core PPR network. While several studies implicated the FGF pathway in neural crest cell induction (LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997; Monsoro-Burq et al., 2003, 2005; Stuhlmiller and García-Castro, 2012; Villanueva et al., 2002), a recent study in chick investigated the temporal aspects: FGF/MAPK signalling is required early for neural crest cell specification (Stuhlmiller and García-Castro, 2012). This raises the possibility that a primary role of FGF signalling may be to induce a 'border state', in which cells are competent to give rise to neural, neural crest and placodes, and thus poise the embryonic ectoderm for other signals that subsequently differentiate between these fates.

In contrast to FGF two other signalling pathways, canonical Wnt and BMP, negatively regulate the core PPR network. In chick *Wnt6* is expressed in the trunk ectoderm (Garcia-Castro et al.,

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2002; Schubert et al., 2002), while *Wnt8c* emanates from lateral and post-otic mesoderm (Litsiou et al., 2005). Together, they limit the lateral and posterior extent of the PPR thus restricting sensory progenitors to the head ectoderm next to the neural plate. In chick and frog, misexpression of Wnt antagonists (Crescent, Frzb1) leads to lateral and posterior expansion of pre-placodal *Six1*, *Six4* and *Eya2* (Brugmann et al., 2004; Litsiou et al., 2005) but cannot elicit ectopic expression of these genes away from the endogenous PPR. Conversely, activation of the pathway by misexpression of Wnt8c, Wnt8 or constitutively active β-catenin abolishes the expression of all three genes (Brugmann et al., 2004; Litsiou et al., 2005).

Similar to Wnts, high levels of BMP activity suppress the core PPR network. *BMP4* and *BMP7* are expressed in most of the non-neural ectoderm (Fainsod et al., 1994; Streit et al., 1998), but antagonists from the underlying mesoderm and the PPR itself (Chapman et al., 2004; Esterberg and Fritz, 2009; Ogita et al., 2001; Rodriguez Esteban et al., 1999) block their activity to allow PPR specific gene expression. Recent results from zebrafish demonstrate that while BMP activity is required early to initiate the expression of competence factors, BMP signalling must be completely blocked for the PPR to be specified (Kwon et al., 2010). In chick, misexpression of the cell-autonomous BMP antagonist Smad6 causes an expansion of *Six4* and *Eya2*, but not of *Six1*, into the future epidermis (Litsiou et al., 2005). In *Xenopus* however, inhibition of BMP signalling by noggin or a dominant negative receptor does expand *Six1*, while BMP4 overexpression inhibits its expression (Ahrens and Schlosser, 2005). This apparent discrepancy in *Six1* regulation may be due to species-specific differences, to the particular antagonists used or to a difference in timing of the experiments. Regardless, these findings do agree that *Six1*, *Six4* and *Eya2* are inhibited by BMPs, although like with Wnt/β-catenin, inhibition of BMP alone is insufficient to induce an ectopic PPR (Ahrens and Schlosser, 2005; Litsiou et al., 2005).

Together these data demonstrate that sensory progenitors must be protected from inhibitory Wnt and BMP signalling. The underlying mesoderm provides both a favourable environment in the form of FGF and protective signals: it secretes FGFs and the Wnt and BMP antagonists *Cerberus* and *DAN* (Chapman et al., 2004; Ogita et al., 2001; Rodriguez Esteban et al., 1999; Shamim and Mason, 1999), while the PPR itself expresses the BMP antagonist *Crossveinless 2* (*Cv2*) (Esterberg and Fritz, 2009). Therefore, the primary role of canonical Wnt and BMP signalling is to suppress the core PPR network within the prospective epidermis and trunk ectoderm, while antagonists facilitate its expression by local reduction of BMP and Wnt activity.

Whether Six1, Six4 and Eya2 are directly activated or inhibited by FGF, Wnt and BMP signalling remains to be elucidated. The only PPR enhancer identified so far regulates *Six1* expression in anterior sensory progenitors and does not contain binding sites for downstream effectors of these signals (Sato et al., 2010). BMP-dependent *Six1* repression is likely to be mediated by the BMP effector Msx1, which directly binds to this enhancer. It is therefore possible that the loss of *Six1* disrupts the positive feedback loop that maintains *Eya2* and activates *Six4*. Therefore, *Six4* and *Eya2* may be indirect targets of BMP signalling with Eya2 being induced by FGF. Together these observations suggest that combinatorial activity of FGF and Wnt and BMP antagonists is required to activate the complete set of PPR specific genes. Indeed, combined overexpression of FGF8 and noggin induces ectopic *Six1* in the ventral ectoderm of *Xenopus* embryos (Ahrens and Schlosser, 2005), and misexpression of both Smad6 and Crescent together with exposure to exogenous FGF8 induces *Six4* in chick (Litsiou et al., 2005). Interestingly, in the latter experiment, when FGF signalling is inhibited shortly after initial exposure *Six4* expression continues unimpeded (Litsiou et al., 2005). Thus, transient FGF activity is sufficient to promote PPR specification supporting the idea that one of the main functions of FGF is to prime the tissue for further signalling input (see above) and that once expressed the Six/Eya network rapidly becomes independent of activating external signals.

Signals differentiating sensory placode and neural crest progenitors

At early neurula stages, cells at the edge of the neural plate appear to remain in an unstable, multi-potent state and retain the ability to respond to local signals and to differentiate accordingly. Placodal (*Six1/Six4/Eya2*) and neural crest (*Pax7*, *Msx1*, *FoxD3*) transcripts partially overlap (Fig. 2). Yet, they mutually repress each other: Six1 represses *Pax7* and *FoxD3*, while Pax7 and Msx1 repress *Six1* (Fig. 3) (Brugmann et al., 2004; Christophorou et al., 2009; Sato et al., 2010). It is possible that mutual repression prevents further specification until changes in the signalling landscape tip the balance and allow the two populations to diverge. In support of this idea, BMP and Wnt pathways appear to recapitulate their earlier activities to promote this process.

Recently a two-step model has been proposed for neural crest cell induction with the second phase requiring canonical Wnt and BMP signalling (Patthey et al., 2008; Steventon et al., 2009; Steventon and Mayor, 2012). In agreement with this, misexpression of Wnt antagonists expands *Six1, Six4* and *Eya2* at the expense of the neural crest specifier *Pax7* (Litsiou et al., 2005). However, PPR transcripts never encroach into the definitive neural crest territory possibly due to elevated BMP activity. In contrast, activation of Wnt signalling expands *Pax7* into the PPR, but not into the future epidermis, while repressing *Six1, Six4* and *Eya2*. Thus, in this context canonical Wnt may not only induce *Pax7* directly, but also indirectly by removing otherwise suppressive *Six1* and thus allow *Pax7* expansion within the PPR.

In summary, at the edge of the neural plate the level of BMP and Wnt signalling determines whether cells adopt neural crest or placodal fate. High levels of BMP and Wnt activity promote neural crest cell formation, while both pathways must be repressed for sensory progenitors to be specified.

Integrating FGF, BMP and Wnt signalling

How are these pathways integrated to generate distinct cell fates at the border of the neural plate? Extracellular BMP signals are transduced to the nucleus by Smad1/5/8 proteins following their phosphorylation by active receptor complexes (Massague, 1998; Wu and Hill, 2009). However, these receptor-regulated Smads are also targeted by other kinases including mitogen activated protein kinase (MAPK) and glycogen synthase kinase 3 (GSK3), which are effectors of FGF and canonical Wnt signalling, respectively (Fuentealba et al., 2007; Kretzschmar et al., 1997; for review: Eivers et al., 2008, 2009). Therefore, Smad1/5/8 are important hubs for integrating these and other signalling pathways suggesting that they also hold the key for signal integration during sensory progenitor specification. The mechanisms of how different pathways converge on Smads have been reviewed extensively elsewhere (Eivers et al., 2008, 2009). Briefly, in response to BMP signalling Smad1/5/8 are activated through phosphorylation at the C-terminal MH2 domain and subsequently accumulate in the nucleus, where they modulate gene expression together with other co-factors. Smad1/5/8 phosphorylation by MAPK largely occurs in the linker region and may prevent their accumulation in the nucleus thus inhibiting their transcriptional activity. In addition, this 'primes' them for further inhibitory phosphorylation by GSK3, which targets them for degradation via subsequent ubiquitination. Since GSK3 is inhibited by canonical Wnt signalling, Wnt activation effectively stabilises

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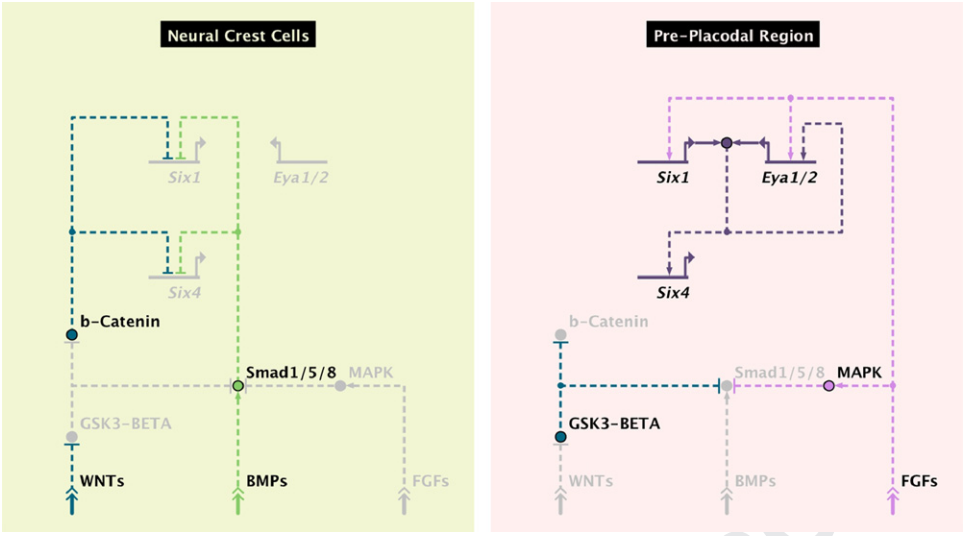

Fig. 4. Integration of signalling pathways in placode and neural crest progenitors. The possible mode of FGF, BMP and Wnt signal integration in the neural crest and placode territory.

Smad1/5/8. This synergy between the BMP and Wnt pathway is consistent with their role in sensory progenitor specification: both suppress the PPR network (Fig. 4). Conversely, FGF/MAPK signalling initiates the inhibitory cascade and opposes BMP signalling consistent with its positive role in PPR specification and activation of the PPR network (Fig. 4). Thus, activation of FGF signalling in the PPR cooperates with extracellular BMP and Wnt antagonists to inhibit both pathways and to generate a signalling environment that favours activation of the Six/Eya network and consequently sensory progenitor specification.

Regionalisation of the PPR

Although the PPR appears to be a homogeneous territory with uniform *Six/Eya* gene expression and a universal lens 'ground state', rostro-caudal patterning is already well under way at the time of its induction. Among the earliest regionally restricted genes are *Otx2* and *Gbx2* (Acampora et al., 2001; Acampora et al., 1995; Bally-Cuif et al., 1995; Li et al., 2009; Simeone et al., 1992; Simeone et al., 1993; Tour et al., 2001; von Bubnoff et al., 1996) (Fig. 2); both transcripts overlap initially, but form a boundary later separating otic from maxillomandibular trigeminal progenitors (Steventon et al., 2012). This boundary is established by mutual repression at the transcriptional level and Otx2/Gbx2-mediated cell sorting to sharpen the boundary (Steventon et al., 2012). A similar mechanism acts in the neural plate to establish the mid-hindbrain boundary (Broccoli et al., 1999; Glavic et al., 2002; Hidalgo-Sanchez et al., 2005; Joyner et al., 2000; Katahira et al., 2000; Li and Joyner, 2001; Millet et al., 1999; Wassarman et al., 1997), suggesting that Otx2 and Gbx2 are part of a general mechanism that allocates rostro-caudal identity across the entire ectoderm.

From neurula stages onwards, the induction of different transcription factors in distinct rostro-caudal domains demarcates the subdivision of the placode territory, first into larger regions contributing to multiple placodes and later into individual placodes each with a unique transcription factor code. These changes in gene expression have recently been reviewed extensively elsewhere (Schlosser, 2006). Here we summarise the earliest steps of anterior–posterior patterning with particular focus on the regulation and role of paired box family transcription factors, the Pax genes (Fig. 5). At some point during placode development (differing depending on species) the combined expression of *Pax6*, *Pax3* and *Pax2/8* spans the entire placode territory suggesting that they play a key role in allocating regional identity to placode progenitors. While none of the regulatory elements that control Pax gene expression in PPR sub-domains have been identified, all require direct or indirect transcriptional input from the Six and Eya network (Christophorou et al., 2009) again highlighting the important role of these genes for placode formation.

The anterior PPR: adenohypophysis, olfactory and lens progenitors

Surprisingly, the apparent uniform expression of *Six1* is regulated by at least two different enhancers, with the anterior PPR enhancer (Six1-14; Sato et al., 2010) encompassing adenohypophyseal, olfactory and lens precursors (Bhattacharyya et al., 2004; Dutta et al., 2005; Kozlowski et al., 1997; Pieper et al., 2011). Activation of this enhancer occurs at neurula stages within a broader *Otx2* domain, just prior to or concomitant with the initiation of *Pitx3* (Dutta et al., 2005) and *Six3* (Liu et al., 2006) within the Six1-14 domain and with *Pax6* (Bailey et al., 2006; Li et al., 1994) in a slightly larger territory, which initially also seems to include trigeminal precursors. This territory of overlapping gene expression in the anterior PPR contains cells with identical developmental potential and can give rise to any anterior placode if exposed to appropriate signals (Fig. 5). Such signals arise from surrounding tissues to induce distinct placodal fates locally. Hedgehog signalling from the midline promotes anterior pituitary character, while repressing lens and olfactory fates: in the absence of hedgehog the latter expand, whereas ectopic activation represses lens formation (Cornesse et al., 2005; Dutta et al., 2005; Herzog et al., 2004; Karlstrom et al., 1999; Kondoh et al., 2000; Sbrogna et al., 2003; Varga et al., 2001; Zilinski et al., 2005). FGFs from the anterior neural ridge promote olfactory identity, while repressing lens (Bailey et al., 2006) and lens fate appears to require prolonged BMP exposure from within the ectoderm itself, as well as later FGF and BMP from the optic vesicle (Faber et al., 2001; Faber et al., 2002; Furuta and Hogan, 1998; Sjodal et al., 2007; Wawersik et al., 1999).

Otx2 plays a crucial role in defining the anterior and intermediate (see below) PPR by repressing Gbx2 (Fig. 5; see above). In

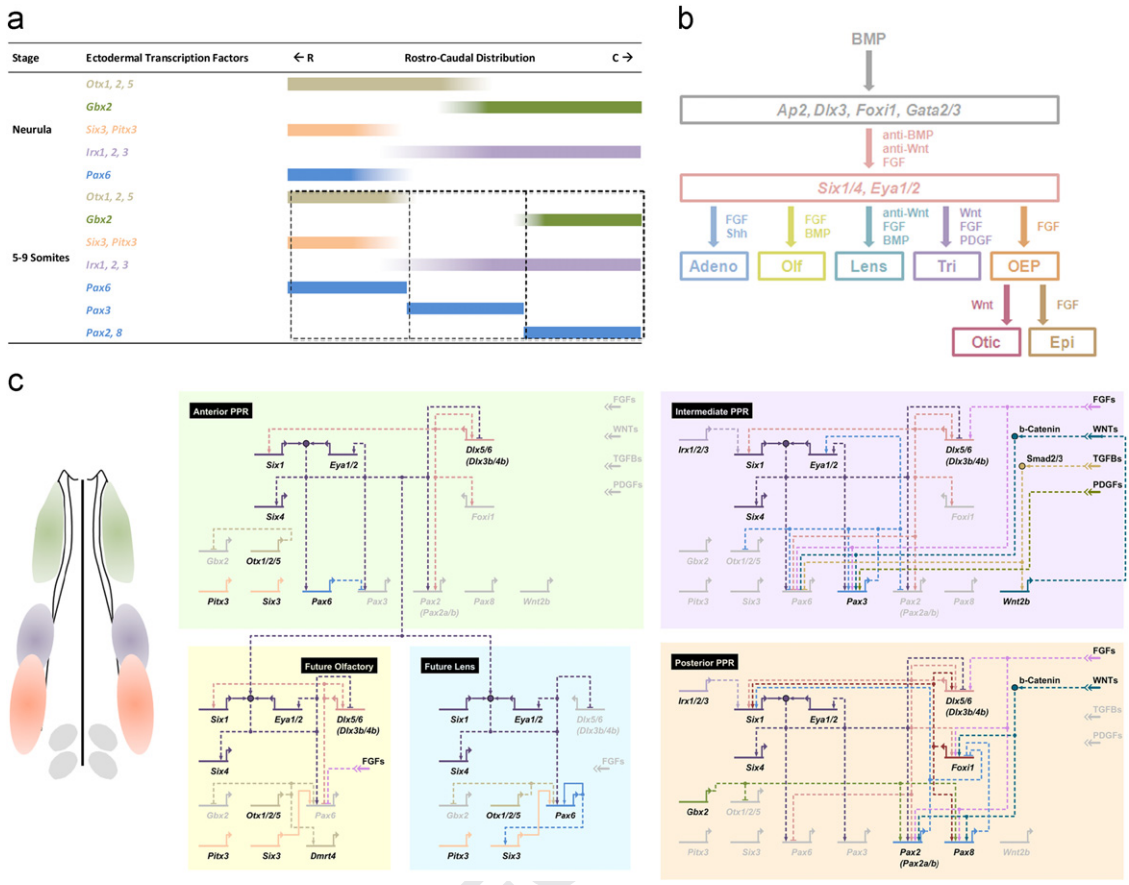

Fig. 5. Anterior–posterior patterning of the PPR. (A) Diagram showing differential gene expression along the rostro-caudal axis at neurula and 5-9 somite stages. See the main text for detailed description and references. Hatched boxes indicate the regulatory states described in the networks in (C). Note: the precise boundaries of Pax gene expression have not been mapped. (B) Summary of signalling pathways implicated in the induction of distinct placodes from the PPR. Adeno: adenohypophysis; Olf: olfactory; Tri: ophthalmic trigeminal; OEP: otic-epibranchial territory; Epi: epibranchial. (C) Gene regulatory networks defining the anterior PPR (green) and its subdivision into olfactory (yellow) and lens (blue) precursors, the intermediate (opV; purple) and posterior (light orange) PPR. Left: diagram of a 5-somite stage chick embryo with colour-coded regions for the regulatory states shown in the networks.

addition, in *Xenopus* activation of Otx2 target genes is required for the early specification of olfactory, lens and trigeminal precursors: misexpression of a constitutive repressor form of Otx2 prevents the expression of molecular markers characteristic for each placode (Steventon et al., 2012).

The paired box transcription factor *Pax6* is the earliest Pax gene expressed in the PPR (Bailey et al., 2006; Li et al., 1994; Zygar et al., 1998). In its absence the lens and olfactory placodes fail to thicken and their development is severely impaired (Ashery-Padan et al., 2000; Collinson et al., 2000; Grindley et al., 1997; Quinn et al., 1996). The signals that induce *Pax6* in the anterior PPR are currently unknown, and despite extensive cis-regulatory studies no pre-placodal enhancer has been identified within the Pax6 locus. It is clear however that Six1 plays a critical role in either *Pax6* initiation or in its maintenance: misexpression of a constitutive repressor form of Six1 prevents anterior *Pax6* expression (Christophorou et al., 2009) (Fig. 5). Whether Pax6 is a direct target of Six1 or is regulated by an intermediary protein remains to be elucidated.

During the segregation of lens and olfactory progenitors, Dlx5 and Pax6 may play antagonistic roles. Although initially co-expressed at pre-placodal stages, *Pax6* and *Dlx5* expression separates into two mutually exclusive domains, the future lens and olfactory placodes, respectively (Bhattacharyya et al., 2004). FGF8 from the anterior neural ridge suppresses Pax6 transiently in the olfactory region, while promoting *Dlx5* expression (Bailey et al., 2006). Conversely, misexpression of exogenous Dlx5 in the lens territory leads to loss of *Pax6* (Bhattacharyya et al., 2004). Thus, Dlx5 overexpression actively suppresses *Pax6* and may lead to transient downregulation of Pax6 in the olfactory placode.

Within the early lens placode Pax6 activates its own transcription as well as other targets, however this autoregulation does not appear to be essential at pre-placodal stages in the mouse (Ashery-Padan et al., 2000). As the lens placode forms Pax6 is directly activated by the Six family member Six3, which interacts with the Pax6 lens placode enhancer (Pax6-EE; Liu et al., 2006). As Six3 and Pax6 are already coexpressed at pre-placodal stages it is tempting to speculate that Six3 has also an earlier role in Pax6 regulation.

Equally important is the question of how *Pax6* is restricted to the anterior PPR (Fig. 5). While it is normally absent from the epibranchial and otic territory (Li et al., 1994), explant studies in chick demonstrated that the entire PPR has an intrinsic 'bias' towards *Pax6* expression: culturing the posterior PPR ex vivo leads to a rapid upregulation of *Pax6* and ultimately results in lens formation (Bailey et al., 2006). This observation suggests that in vivo signals extrinsic to the PPR actively suppress *Pax6* to prevent ectopic lens formation. Two strong candidates for this role are Wnt and FGF signalling. Within the neural plate and its border Wnt signalling establishes posterior identity (Carmona-Fontaine et al., 2007; Heisenberg et al., 2001; Kim et al., 2000; Li et al., 2009; Patthey et al., 2008; van de Water et al., 2001;

Villanueva et al., 2002; for review: Houart et al., 2002) although a direct role (rather than indirect through patterning of the neural tube) in the early subdivision of the placode territory has not yet been established. However, in support of Wnt involvement the Wnt target genes *Gbx2* and *Irx1*-3 (Braun et al., 2003; Gomez-Skarmeta et al., 2001; Itoh et al., 2002; Kiecker and Niehrs, 2001; Li et al., 2009; Rhinn et al., 2009) are expressed in the posterior PPR, with *Gbx2* abutting *Otx2* and *Irx1-3* complementary to *Six3* expression. Like in the neural plate, these Wnt responsive factors pattern the PPR through repression of their anterior counterparts as we have recently shown for Otx2 and Gbx2 (Steventon et al., 2012). In addition, *Pax3* starts to be expressed in the ophthalmic trigeminal placode as *Pax6* is lost from this domain and represses *Pax6* transcription and vice versa (Wakamatsu, 2011). Like *Gbx2* and *Irx1-3*, *Pax3* is activated by canonical Wnt signalling (Canning et al., 2008; Lassiter et al., 2007) and all three factors may participate in the *Pax6* restriction. At later stages, the Wnt pathway continues to downregulate *Pax6* and confines it to the prospective lens placode (Grocott et al., 2011; Smith et al., 2005). Thus, canonical Wnt signalling may be an important negative regulator of *Pax6*, first to confine its expression to the anterior PPR and later to the lens territory (Fig. 5). In turn, Wnt antagonists from the hypoblast (anterior visceral endoderm in mouse; for review: Stern and Downs, 2012) and the mesendoderm underlying the anterior PPR protect this territory, thus allowing the expression of *Pax6* and other anterior PPR genes.

While also implicated in anterior–posterior patterning of the neural tube, within the placode territory FGF signalling appears to control a different process: the suppression of *Pax6* and simultaneous induction of individual placodes. FGFs mediate the induction of multiple placodes including the olfactory, trigeminal, epibranchial and otic (see below; 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) (Fig. 5). They actively promote the expression of placode-specific genes and simultaneously suppress *Pax6*. In the presence of FGF8, posterior PPR explants fail to initiate *Pax6* expression, suggesting that FGF activity normally prevents inappropriate *Pax6* expression. In summary, Wnt and FGF pathways may cooperate to restrict Pax6 to the anterior-most PPR. While Wnt continues to inhibit *Pax6* at lens placode stages (Grocott et al., 2011; Smith et al., 2005), FGF from the optic vesicle later promotes its expression and lens character (Faber et al., 2001; Vogel-Hopker et al., 2000).

The posterior PPR: otic and epibranchial precursors

Prior to the Six/Eya network *Gbx2* is already expressed in the posterior ectoderm and subsequently localises to the posterior placode territory abutting *Otx2* anteriorly (Acampora et al., 1995; Bally-Cuif et al., 1995; Braun et al., 2003; Broccoli et al., 1999; Gammill and Sive, 2000; Glavic et al., 2002; Li et al., 2009; Millet et al., 1999). Shortly thereafter, at neurula stages members of the Irx family become confined to the posterior PPR, with their anterior limit rostral to *Gbx2* (Bellefroid et al., 1998; Glavic et al., 2002; Gomez-Skarmeta et al., 1998; Goriely et al., 1999) (Fig. 5). Members of two gene families, Dlx (Dlx3b/4b in fish, Dlx5/6 in chick) and Foxi genes (Foxi1 in fish, Foxi3 in chick and mouse), are initially expressed in the non-neural ectoderm and throughout the PPR, but now become rapidly confined posteriorly (Brown et al., 2005; Nissen et al., 2003; Ohyama and Groves, 2004; Solomon and Fritz, 2002; Solomon et al., 2003a, 2003b). These transcription factors form a network of interactions regulating both each other and the onset of *Pax2*, *Pax8* and *Sox3* (Hans et al., 2004; Kwon et al., 2010; Nissen et al., 2003; Padanad and Riley, 2011; Solomon et al., 2003a). Together, the latter factors label a posterior equivalence group of cells (posterior placode area or PPA), all of which can generate otic, epibranchial and lateral line placodes (for review: Ladher et al., 2010; Schlosser, 2010). Like in the anterior PPR spatially and temporally controlled signalling events segregate these different fates over time.

Gbx2 is among the earliest factors to promote posterior PPR identity and appears to play a dual role (Steventon et al., 2012; Fig. 5): it represses *Otx2* early and provides positive input for *Pax8* and *Pax2* later. In *Xenopus*, Gbx2 knock-down leads to *Otx2* expansion, while misexpression of Gbx2 and of a constitutive repressor results in *Otx2* loss suggesting that Gbx2 acts as transcriptional repressor (Steventon et al., 2012). However, Gbx2 switches to an activator during otic specification: Gbx2 constitutive repressor and Gbx2 knock-down lead to a loss of otic *Pax8* and *Pax2*. Gbx2 alone cannot induce *Pax2/8* suggesting that other cofactors are required. These findings highlight that transcription factor action is highly dependent on the cellular context and available cofactors.

Studies in mouse, zebrafish and chick show that Pax2 and Pax8 function is critical for normal ear development (Bouchard et al., 2010; Burton et al., 2004; Christophorou et al., 2010; Mackereth et al., 2005; Torres et al., 1996) and for the formation of some epibranchial neurons (Nechiporuk et al., 2007). Pax2 knockout mice show severe malformations of the cochlea and the endolymphatic duct as well as absence of the saccule (Burton et al., 2004; Torres et al., 1996). While Pax8 mutant mice do not show an ear phenotype, Pax2/Pax8 double mutants arrest ear development at the vesicle stage highlighting their important role at early stages (Bouchard et al., 2010). Likewise, in humans PAX2 mutations are associated with sensorineuronal deafness (Favor et al., 1996; Sanyanusin et al., 1995; Schimmenti et al., 1997). The fact that birds appear to have lost Pax8 due to chromosomal rearrangements allows the investigation of Pax2 function directly: in chick Pax2 knock-down impairs early otic specification as evidenced by the loss of early otic markers (Christophorou et al., 2010). In zebrafish, both Pax2 and Pax8 cooperate during otic vesicle development: in the absence of Pax8, Pax2a and Pax2b a small otic placode is induced, but degenerates completely over time (Mackereth et al., 2005). Together, these findings suggest that Pax2 and 8 play an important role in specification of otic cells from the PPR, as well as during later ear development.

Although initially thought to be otic inducers, more recent evidence implicates members of the FGF family as key signals to induce the PPA (Fig. 5). FGFs from the head mesoderm and the hindbrain are required and sufficient to induce the otic placode in fish, chick and mouse (for review: Barald and Kelley, 2004; Ladher et al., 2000; Ladher et al., 2010; Leger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Ohyama et al., 2007; Phillips et al., 2001; Phillips et al., 2004; Riley and Phillips, 2003; Schimmang, 2007; Wright and Mansour, 2003), but have more recently also been implicated in epibranchial placode induction (Freter et al., 2008; Nechiporuk et al., 2007; Nikaido et al., 2007; Sun et al., 2007). The precise nature of the FGF ligands involved differs between species, with FGF3 and -8 being required in zebrafish, FGF3 and -10 in mouse and FGF3 and -19 in chick. Prolonged exposure of PPA cells to FGFs promotes epibranchial fates, while repressing otic character (Freter et al., 2008; Nechiporuk et al., 2007). Instead, cells close to the neural tube are exposed to hindbrain-derived canonical Wnt signalling and adopt otic fate, while epibranchial fate is suppressed (Freter et al., 2008; Ladher et al., 2000; Ohyama et al., 2006). Thus, a model emerges in which FGFs initially induce a posterior placode equivalence group, from which otic and epibranchial identity is established depending on length of FGF exposure and on the presence or absence of Wnt

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(Fig. 5). While this model holds true in amniotes and Wnt activity also promotes otic identity in *Xenopus* (Park and Saint-Jeannet, 2008), the role of Wnt signalling in zebrafish otic induction is still under debate (Phillips et al., 2004). Induction of lateral line placodes has so far remained elusive.

While at gastrula stages *Foxi1* and *Dlx3b/4b* are under the control of BMP signalling (see above), they are controlled by FGFs in the PPA (Hans et al., 2007; Hans et al., 2004; Nissen et al., 2003), where they promote each other's expression in a positive feedback loop: exogenous FoxI1 induces Dlx3b and vice versa, while Dlx3b, like Dlx5 in chick (McLarren et al., 2003), also regulates its own expression (Aghaallaei et al., 2007; Solomon and Fritz, 2002). In zebrafish *Dlx3b* expression depends on Foxi1 function (Solomon et al., 2003a), while in *Xenopus* Foxi1 depends on Dlx3 activity (Pieper et al., 2012). Thus, Foxi1 and Dlx3b/4b regulate each other in the PPA, where they synergise to promote Pax gene expression and consequently posterior placode specification. Downstream of Foxi1 cells activate *Pax8* and *Sox3*; accordingly zebrafish Foxi1 mutants lose the earliest PPA gene *Pax8* as well as the slightly later expressed *Pax2* (Hans et al., 2007; Nissen et al., 2003; Solomon et al., 2003a). In contrast, Dlx3b/4b controls *Pax2*, but not *Pax8* (Hans et al., 2007; Hans et al., 2004; Mackereth et al., 2005; Padanad and Riley, 2011; Solomon and Fritz, 2002; Solomon et al., 2004; Sun et al., 2007): in the absence of Dlx3b/4b function *Pax8* expression remains normal while *Pax2* is lost. Thus, FGF regulates the two Pax genes that demarcate the PPA using two independent pathways. Once activated, Pax2 and Pax8 cooperate to suppress *Foxi1* as a prerequisite for otic specification and to promote otic fate synergistically (Mackereth et al., 2005; Padanad and Riley, 2011) (Fig. 5).

Like Pax6 anteriorly, *Pax2* expression in the PPA requires the activation of Six1 target genes: its expression is lost after misexpression of a constitutive repressor form of Six1 or after Six1 knockdown (Bricaud and Collazo, 2006; Christophorou et al., 2009). However, thereafter Pax2 controls Six via a recently identified otic specific enhancer (Sato et al., 2012), suggesting that a positive feedback loop between Six1 and Pax2 locks cells in an otic state. In contrast, other Pax proteins negatively regulate *Pax2*: exogenous Pax3 suppresses *Pax2* expression in chick otic placode (Dude et al., 2009). Thus, mutual repression between Pax genes patterns the placode territory to define subgroups of cells with distinct developmental potential (Fig. 5).

The intermediate PPR: trigeminal precursors

Pax3 is the earliest marker of the prospective ophthalmic trigeminal (opV; profundal in anamniotes) placode, where its expression is initiated at the 8 somite stage in chick and slightly earlier in *Xenopus* (Dude et al., 2009; Pieper et al., 2011; Schlosser and Ahrens, 2004; Stark et al., 1997; for review: Schlosser, 2006). Before the onset of *Pax3* expression in chick, at least some opV precursors are *Pax6* positive (compare anterior position of opV-fated cells; Xu et al., 2008) and *Pax6* expression (Bailey et al., 2006; Bhattacharyya et al., 2004), while some maxillomandibular trigeminal (mmV; trigeminal in anamniotes) precursors arise from the $Pax2^{+}$ territory (Xu, 2008). Most of the mmV however, does not seem to express any Pax gene. In contrast in Xenopus, mmV/trigeminal precursors initially express *Pax6* reflecting their location anterior to the profundal placode at early stages (Pieper et al., 2011). Thus, due to the lack of molecular markers for the mmV/trigeminal placode little is known about its specification and the molecular interactions involved.

In the opV territory, the onset of *Pax3* coincides with the disappearance of *Pax6* in agreement with the mutual repression between these factors (Wakamatsu, 2011). Like the other Pax genes in the placode territory, *Pax3* transcription requires the activation of Six1 target genes since misexpression of a constitutive repressor form of Six1 prevents its expression (Christophorou et al., 2009). Additionally, Pax3 controls its own expression and positively feeds back onto *Eya2* (Dude et al., 2009): misexpression of exogenous Pax3-Engrailed fusion protein (which suppresses Pax3 targets) leads to the loss of endogenous *Pax3* and *Eya2*. Finally, misexpression of Pax3 in the posterior PPR represses the otic/epibranchial marker *Pax2* (Dude et al., 2009) suggesting that indeed cross-repressive interactions between different Pax genes are critically involved in rostro-caudal patterning of the placode territory.

Neighbouring tissues control *Pax3* induction in the opV and in particular the neural tube has been implicated (Canning et al., 2008; Stark et al., 1997), although signals from migrating neural crest cells cannot be excluded. Again, neural tube-derived canonical Wnt signalling is thought to play a role in *Pax3* induction and Wnt activity is required for its maintenance. Wnts appear to cooperate with the FGF pathway (Canning et al., 2008; Lassiter et al., 2007; Shigetani et al., 2008) and PDGF signalling has also been implicated, but is not sufficient to induce Pax3 in competent ectoderm (McCabe and Bronner-Fraser, 2008). Additionally in chick, *Pax3* induction next to the dorsal neural tube and its subsequent lateral expansion correlates with the onset of neural crest cell migration making them a potential source of opV inducing signals. Indeed, neural crest derived TGF-β signalling activates *Wnt2b* expression in the overlying ectoderm including in the *Pax3* domain (Grocott et al., 2011). Although TGF-β alone cannot induce *Pax3* in competent anterior PPR explants it is possible that a combination of TGF-β/Wnt2b and FGF/PDGF is required. Thus, multiple pathways appear to converge to induce trigeminal identity via Pax3 activation (Fig. 5). However, without the identification of Pax3 enhancer regions it remains unclear whether they directly control its expression or act via intermediate targets. In the future we will need to understand how these signals are integrated intracellular.

In summary, subdivision of the placode territory occurs sequentially with the establishment of multiplacodal domains. Within these domains cells have equivalent developmental potential and can give rise to any placode if exposed to appropriate signals. Following the expression of the Six and Eya network, Pax genes mediate this initial subdivision into anterior, intermediate and posterior placodal areas by mutual repression. While Six and Eya target activation is required for all Pax genes, irrespective of their rostro-caudal location, other factors must cooperate to impart regional identity and to induce Pax genes in specific locations. Good candidates for this role are regionally restricted factors like Otx2, Gbx2, Irx1-3 and Six3 in analogy to their role in the neural tube.

Conclusion

In the last decade or so, many of the transcription factors and signals that influence sensory placode development have been identified. The GRN presented here reveals their temporal hierarchy and how both signals and transcription factors are repeatedly used first to specify the PPR and then to subdivide it into placode cells with unique identity. Over time the developmental potential of ectodermal cells becomes progressively restricted and cross-repressive interactions and positive feedback loops are critically important to segregate and stabilise different fates, respectively. In particular, the repeated use of FGF – first as a 'border' inducing signal, then as PPR inducer and finally as inducer for most placodes – demonstrates how the regulatory state of each cell population and its developmental history

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determines the ultimate outcome. The next challenge will be to determine direct FGF targets and how the same signal is interpreted at each stage.

While the GRN presented here allows us to predict new interactions and loss- and gain-of-function phenotypes, it clearly demonstrates our lack of knowledge with respect to the cis-regulatory mechanisms involved. With the notable exception of Six1 and a few otic genes (Barembaum and Bronner-Fraser, 2010; Betancur et al., 2010b; Saigou et al., 2010; Sato et al., 2010), none of the regulatory elements that control spatial and temporal gene expression in sensory progenitors have been identified. These will be crucial to understand how signalling and transcription factor inputs are integrated to control cell fate decisions. Finally, it is surprising that Six and Eya co-factors (except for the expression of putative co-factors; Neilson et al., 2010) and downstream targets have not been reported in vertebrates. Their identification will be important to understand not only how these factors control the development of diverse placodes, but also how mutations in the Six/Eya pathway in humans leads to congenital abnormalities in sense and other organs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.06.028.

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