Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a transcriptional activator and repressor

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Summary

Cranial placodes, which give rise to sensory organs in the vertebrate head, are important embryonic structures whose development has not been well studied because of their transient nature and paucity of molecular markers. We have used markers of pre-placodal ectoderm (PPE) (*six1*, *eya1*) to determine that gradients of both neural inducers and anteroposterior signals are necessary to induce and appropriately position the PPE. Overexpression of *six1* expands the PPE at the expense of neural crest and epidermis, whereas knock-down of Six1 results in reduction of the PPE domain and expansion of the neural plate, neural crest and epidermis. Using expression of activator and repressor constructs of *six1* or co-expression of wild-type *six1* with activating or repressing co-factors (*eya1* and *groucho*, respectively), we demonstrate that Six1 inhibits neural crest and epidermal genes via transcriptional repression and enhances PPE genes via transcriptional activation. Ectopic expression of neural plate, neural crest and epidermal genes in the PPE demonstrates that these factors mutually influence each other to establish the appropriate boundaries between these ectodermal domains.

Key words: Pre-placodal ectoderm, Neural crest, *foxD3*, *zic2*, *sox2*, *sox3*, *keratin*, *dlx5*, *dlx6*, Cell fate determination, Patterning, *Xenopus*

Introduction

It was discovered over a century ago that the cranial sensory organs of vertebrates arise from discrete areas of thickened ectoderm, called placodes, that form in characteristic positions in the head (Knouff, 1935; LeDouarin et al., 1986). They give rise to the olfactory, lens, auditory-vestibular and lateral line organs, and contribute to the cranial sensory ganglia. Placodes derive from a band of specialized pre-placodal ectoderm (PPE) that surrounds the anterior neural plate. The PPE is competent to form many different placodes that express distinct developmental fates as the result of interactions with their surrounding tissues (Schlosser and Northcutt, 2000; Baker and Bronner-Fraser, 2001).

Several studies indicate that during gastrulation, embryonic ectoderm is separated into fields with distinct fates (neural plate, neural crest, PPE, epidermis; Fig. 1A) in response to different concentrations of BMP. Genes expressed by the presumptive epidermis are positively regulated by BMPs (Suzuki et al., 1997; Feledy et al., 1999; Beanan and Sargent, 2000; Luo et al., 2001a; Tribulo et al., 2003), whereas anti-BMP factors secreted from the organizer and the dorsal midline mesoderm promote neural plate formation (Weinstein and Hemmati-Brivanlou, 1999; Wilson and Edlund, 2001). It has been suggested that signals responsible for establishing the neural plate also establish a lateral neurogenic ectoderm (LNE) that surrounds the neural plate and gives rise to the intervening neural crest and PPE (Baker and Bronner-Fraser, 2001). The best-studied derivative of the LNE, the neural crest, appears to be induced by intermediate concentrations of anti-BMP factors (Morgan and Sargent, 1997; Marchant et al., 1998; Mayor et al., 1999; Mayor and Aybar, 2001; Aybar et al., 2002). In addition, neural crest induction requires signaling pathways that establish the posterior axis of the neural plate (Wnt, FGF, retinoic acid) (LaBonne and Bronner-Fraser, 1998; Chang and Hemmati-Brivanlou, 1998; Mayor and Aybar, 2001; Villanueva et al., 2002; Monsoro-Burq et al., 2003; Glavic et al., 2004a). Whether similar signaling events are involved in establishing the PPE has not been addressed because of a paucity of molecular markers specific for this ectodermal domain.

The LNE also appears to require interactions between the presumptive neural plate and epidermis to initiate the expression of transcription factors that define this border zone and its derivatives (Streit and Stern, 1999; McLarren et al., 2003). For example, Dlx genes are induced by BMP in a concentration-dependent pattern in the epidermis and play a role in positioning the boundaries of the neural plate and the neural crest (Feledy et al., 1999; Beanan and Sargent, 2000; Luo et al., 2001a; Tribulo et al., 2003; McLarren et al., 2003;

Woda et al., 2003). Zic genes are initially expressed throughout the neural plate in response to anti-BMP factors, and as they become restricted to its lateral border they initiate neural crest fates (Nakata et al., 1997; Nakata et al., 1998; Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998). The roles that border genes play to specify the fates of the different ectodermal subdomains remain to be elucidated.

Although placodes have long been recognized as important embryonic structures, their transient nature and the lack of specific molecular markers have made it difficult to study the mechanisms by which they form. Recently, however, markers of the PPE during the initial induction of the placodes have been identified in *Xenopus*. *six1* is homologous to *Drosophila sine oculis*; it is characterized by a homeobox DNA-binding domain and a protein-protein interaction domain called the Six domain. It is initially expressed in a band surrounding the anterior neural plate and later in all neurogenic placodes (Pandur and Moody, 2000). *eya1* is homologous to *Drosophila eyes absent* (*eya*); it functions as a co-factor for Six genes of the Six1/2 and Six4/5 subfamilies (Pignoni et al., 1997; Ohto et al., 1999; Ikeda et al., 2002) and is expressed in a pattern very similar to that of *six1* (David et al., 2001). We have used these markers to demonstrate that gradients of both neural inducer and anteroposterior signals are required for proper PPE formation. Moreover, we show that *six1* expression is required for the establishment of the PPE, and it promotes the PPE at the expense of the neural crest and epidermis by both activating and repressing target gene expression. Finally, we demonstrate that several genes expressed in the embryonic ectoderm mutually influence each other to define its distinct subdomains.

Materials and methods

Expression constructs

The full open-reading frames of *Xenopus six1* and *Drosophila groucho* (*Dgroucho*; LD33829, Berkeley *Drosophila* Genome Project) were cloned into expression vectors (pDH105, pCS2+). To generate a chimeric transactivating *six1* construct, the Six domain plus the homeodomain (SDHD; amino acids 9-183) was amplified by PCR and ligated upstream of the VP16 activation domain in pCS2VP16 (from M. Whitman). To generate a chimeric repressive *six1* construct, the SDHD region was ligated downstream of the Engrailed repressor (EnR) domain in pCS2EnR (from D. Kessler).

RNA microinjection

Transcripts of *six1* (400-600 pg), *six1VP16* (100 pg), *six1EnR* (100 pg), *Dgroucho* (400 pg), *noggin* (5-40 pg) (Smith and Harland, 1992), *chordin* (5-40 pg) (Sasai et al., 1994), *dnWnt8* (500 pg) (Hoppler et al., 1996), *frzb-1* (750 pg) (Wang et al., 1997), *bmp4* (10-50 pg) (Dale et al., 1992), *eya1* (400 pg) (David et al., 2001), *zic2* (100 pg) (Brewster et al., 1998), *foxD3* (25 pg) (Sasai et al., 2001) (from D. Kessler), *dlx5* (200 pg) (Luo et al., 2001a), *dlx6* (200 pg) (Luo et al., 2001a) and *sox2* (200 pg) (Mizuseki et al., 1998) (from T. Grammar) were mixed with *β-galactosidase* (*β-gal*) mRNA (100-200 pg) and microinjected into identified blastomeres with known ectodermal fates (Fig. 1C) as described (Moody, 2000).

Yeast two hybrid analysis

To determine if *Xenopus six1* interacts with *Dgroucho*, the Six domain (amino acids 9-123) was cloned into pGBKT7, and an N-terminal region of *Dgroucho* (amino acids 1-247) was cloned into pGAD424 (Clontech). The yeast strain AH109 was transformed with both vectors and assayed for reporter gene expression according to the MATCHMAKER kit (Clontech).

Morpholinos

Morpholino antisense oligonucleotides (MO) were synthesized against two different potential translational start sites in *six1* (5′-GGAAG-GCAGCATAGACATGGCTCAG-3′ and 5′-CGCACACGCAAAC-ACATACACGGG-3′) (Gene-Tools). An equimolar mixture of the two *six1*-MO, or a standard control MO (5′-GGAAGGCAGCATA-GACATGGCTCAG-3′) was microinjected (10-16 ng). A Myc-tagged construct (*six1-myc*) containing the *six1* wild-type 5′UTR was generated to assess morpholino knock-down efficacy by immunofluorescent detection of protein. A rescue construct (*six1-rescue*) was generated by replacing the *six1* wild-type 5′UTR with pCS2+ sequence and changing nine bases in the coding region that would interfere with MO binding without altering amino acid sequence.

Animal cap explants

The animal pole was injected with mRNAs for *noggin* (50 pg), *cerberus* (50 pg) (Bouwmeester et al., 1996), *bmp4* (50 pg), *Wnt8* (50 pg) (Hoppler and Moon, 1998) or constitutively activated *fgfr1* (*cfgfr1*, 50 pg) (Neilson and Friesel, 1996). Animal cap explants were dissected at stages 8.5-9 and cultured in NAM (Messenger and Warner, 1979), in some cases supplemented with recombinant mouse Noggin protein (R & D Systems). Explants were processed for either RT-PCR or in situ hybridization. For each in situ hybridization experiment, control and an entire series of Noggin-treated explants were processed in parallel so that staining intensities could be compared. The intensity of reactivity in experimental caps was compared with control caps, and then sorted into three groups of staining intensity (none, moderate, high). Frequencies of caps in each group were compared between treatments by Chi-square analyses.

RT-PCR

Total RNA from animal cap explants was isolated then subjected to first strand cDNA synthesis using oligo(dT) primers. PCR was performed in the linear range using *six1* primers as described (Pandur and Moody, 2000).

In situ hybridization

Full-length antisense RNA probes for *six1* (Pandur and Moody, 2000), *eya1* (David et al., 2001), *sox2* (Penzel et al., 1997) (from R. Grainger), *sox3* (Zygar et al., 1998), *sox11* (from T. Grammer and R. Harland), *foxD3* (Sasai et al., 2001) (from D. Kessler), *epidermal specific keratin* (Jonas et al., 1989), *dlx5* and *dlx6* (Luo et al., 2001a), and *zic2* (Brewster et al., 1998) were transcribed in vitro, and embryos were processed by standard protocols (Sive et al., 2000). The widths of the expression domains of marker genes were measured in whole-mount preparations of *β-gal* mRNA-injected control embryos and of experimental transcript-injected embryos at 40× with an eyepiece micrometer, as described (Kenyon et al., 2001). Measurements were expressed as differences between injected and uninjected sides of the same embryo, and the mean differences between groups were analyzed by *t*-tests.

Results

six1 is induced by neural inducers in a concentration-dependent manner

six1 expression is initiated during gastrulation and consolidates into a discrete band adjacent to the anterior neural plate that corresponds to the classical description of the PPE (Fig. 1A,B). Consistent with the hypothesis that LNE derivatives are induced by the same anti-BMP factors that establish the neural plate, *noggin* and *cerberus* mRNA-injected animal cap explants express *six1*, whereas neither uninjected nor *bmp4* mRNA injected explants do (Fig. 2A). It has been proposed that neural crest forms in response to an ‘intermediate’ concentration of

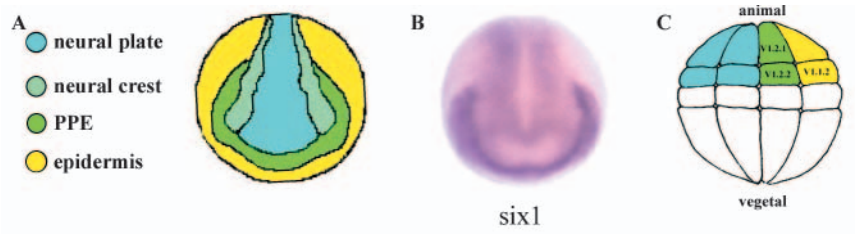

Fig. 1. (A) In the stage 16 *Xenopus* embryo there are four major ectodermal domains: neural plate, neural crest, pre-placodal ectoderm (PPE) and epidermis. (B) The expression pattern of *six1* coincides with the PPE. (C) An idealized depiction of the ectodermal fate of the 32-cell embryo showing four dorsal blastomeres (blue) that contribute significantly to the neural plate, two ventrolateral blastomeres (green) that contribute significantly to the neural crest and PPE, and two ventral blastomeres (yellow) that contribute significantly to the ventral epidermis (from Moody, 1987).

neural inducers (Morgan and Sargent, 1997; Marchant et al., 1998). To test whether this is also true for the PPE, uninjected animal cap explants were cultured in a range of Noggin protein concentrations then subjected to in situ hybridization detection of *six1* expression. Explants were scored as none, moderate or high-expressing in comparison with control explants processed in parallel but cultured in the absence of Noggin. The largest percentage of explants expressing *six1* at high levels was observed in explants cultured in 1-5 ng/ml of Noggin protein, and this expression was drastically reduced as Noggin concentration increased to 25 ng/ml (Fig. 2B). To confirm that this response was specific to Noggin activity as an anti-BMP factor, embryos were first injected with *bmp4* mRNA then explants were cultured in the presence of 1 ng/ml Noggin. *six1* expression was undetected in 66.3% of Noggin-treated BMP-injected explants (n=83), compared with only 3.2% in Noggin-alone treated explants (n=62) (Fig. 2C). These data indicate that *six1* expression is most highly induced at low concentrations of anti-BMP factors.

To determine whether the concentration of Noggin that highly induces PPE genes differs from that for other neurogenic fields, the in situ hybridization assay was repeated using *foxD3* (neural crest), *sox2* (neural plate) and *eya1* (an additional PPE marker) (Fig. 2D). Neither *foxD3* nor *sox2* was induced at the low levels of Noggin that were sufficient for PPE induction, but they began to be highly expressed at higher concentrations (>25ng/ml). *eya1* was highly induced by low levels of Noggin and repressed by high levels, similar to *six1*. These results support the proposal that genes characteristic of the three early neurogenic fields (neural plate, neural crest, PPE) are most highly induced at different concentrations of anti-BMP factors, corresponding to their respective distances from an endogenous source of these factors at the dorsal midline.

Neural inducers alone are not sufficient to induce six1 in the intact embryo

Consistent with the explant data, increased BMP signaling in the lateral ectoderm of the intact embryo, achieved by injecting doses of *bmp4* mRNA that do not disrupt axial patterning into ventrolateral blastomeres that contribute significantly to the LNE (V1.2.1, V1.2.2; Fig. 1C), reduced endogenous *six1* expression (Fig. 3A; 56% of cases at 20 pg, 67% at 40 pg). To test whether neural inducer alone is sufficient to induce the PPE in the intact embryo, different concentrations of *noggin* or *chordin* mRNA (10 pg, 20 pg and 40 pg) were microinjected into a ventral blastomere (V1.1.2; Fig. 1C) that contributes significantly to the ventral epidermis. These concentrations were used because they induce neural genes in explants but infrequently re-pattern the embryo to produce secondary axes. Two morphologies were observed: a dispersed clone with no obvious patterning defects (Fig. 3B) or an elongated clone associated with a ventrally located putative secondary axis (Fig. 3C,D). Staining with *sox2* confirmed that every embryo with an elongated clone

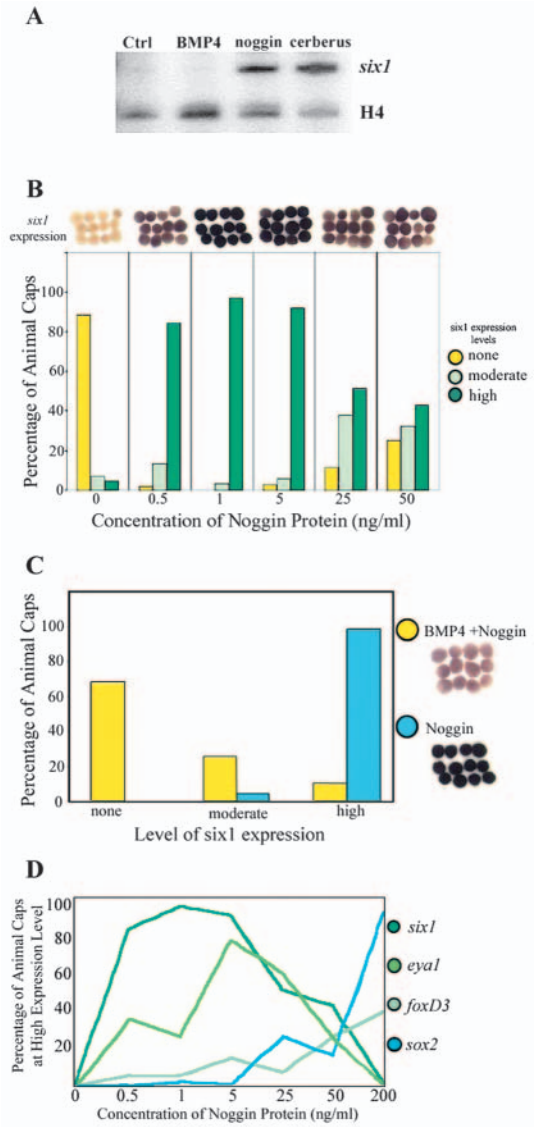

Fig. 2. (A) RT-PCR analysis showing that explants injected with *noggin* or *cerberus* mRNAs express *six1*, whereas control and *bmp4*-injected explants do not. H4, loading control. (B) Explants were cultured in different concentrations of Noggin, and processed for *six1* expression at stage 17. The largest percentage of explants stained at high levels was observed between 1 and 5 ng/ml of Noggin. (C) BMP4 antagonizes the 1 ng/ml Noggin-induction of *six1*. (D) Explants were cultured as in B, and the percentage of explants with high levels of marker gene expression were plotted.

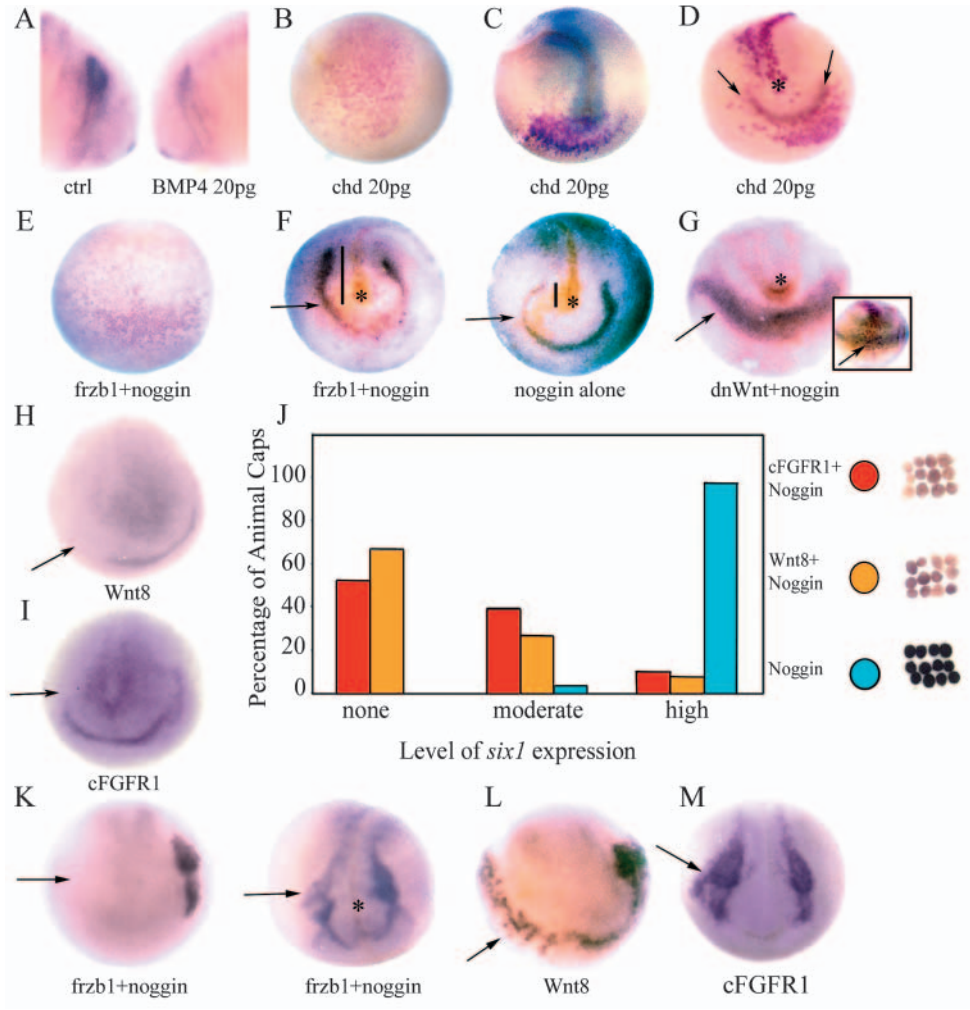

Fig. 3. (A) Expression of *bmp4* in the LNE on one side of embryo (right) reduced *six1* placode expression compared with control, uninjected side (left). (B) Ventral epidermis containing *chordin*-expressing (red) cells in a dispersed pattern; there is no ectopic *six1* expression. (C) Ventral epidermis containing a secondary axis (*sox2*, blue) after ectopic *chordin* expression (red). (D) Ventral epidermis containing a secondary axis/elongated clone (*) after ectopic *chordin* expression (red); ectopic *six1* expression is at its anterior pole (stripe between arrows). (E) When co-injection of *frzb-1+noggin* mRNAs does not form a secondary axis (dispersed red cells), ectopic *six1* is not induced. (F) When co-injection of *frzb-1+noggin* mRNAs forms a secondary axis (left), the ectopic *six1* domain (arrow) extends further posterior (black bar) from the anterior tip of the secondary axis (*), compared with *noggin* alone embryos (right). (G) Co-injection of *dnWnt8+noggin* mRNAs expands the *six1* expression domain (arrows) to encircle both the primary axis (*) and the induced secondary axis (red cells, inset). (H) Wnt8 expression in the LNE (left side) represses *six1* (arrow). (I) cFGFR1 expression in the LNE (left side) represses *six1* (arrow). (J) Explants were injected with either *cfgfr1* or *Wnt8* mRNA and cultured in 1 ng/ml Noggin. The high levels of *six1* expression induced by this concentration of Noggin were significantly repressed by both factors. (K) Expression of *frzb-1+noggin* mRNAs either represses (left) or reduces (right) *foxD3* expression on the treated side (arrows). (L) Wnt8 expression in the LNE (left side) expands *foxD3* (arrow). (M) cFGFR1 expression in the LNE (left side) expands *foxD3* (arrow).

contained ectopic neural tissue (Fig. 3C), whereas none with a dispersed clone did. Regardless of concentration of injected mRNA, in no case was there ectopic *six1* expression associated with a dispersed clone (Fig. 3B), but in every elongated clone, ectopic *six1* expression occurred at its anterior tip (Fig. 3D). Thus, in contrast to explants, in which Noggin alone induces *six1* expression, in whole embryos the additional presence of an axis is necessary for *six1* expression.

Posteriorizing signals restrict *six1* expression to the head

That *six1* expression was always confined to the anterior pole of both the endogenous axis and the induced secondary axis suggests that signals associated with establishing the anteroposterior neural axis (e.g. Wnts, FGFs) (Gould and Grainger, 1997; Gamse and Sive, 2000) influence where the PPE forms. To test this, endogenous Wnt signaling was reduced in the LNE of the intact embryo by co-expressing either a Wnt1 family antagonist (Frzb-1) or a dominant-negative form of Wnt8 (dnWnt8) with Noggin. Co-expression of *frzb-1+noggin* mRNAs did not induce *six1* in the absence of a secondary axis (Fig. 3E; n=18), but when a secondary axis formed *six1* expression expanded markedly further posterior in every case (Fig. 3F; n=25). Co-expression of *dnWnt8+noggin* mRNAs caused *six1* expression to encircle the entire embryo (Fig. 3G; 84%, n=76), consistent with reports that blocking Wnt signaling anteriorizes the entire embryo (Glinka et al., 1998; Itoh and Sokol, 1999). By contrast, mRNAs encoding molecules that activate Wnt or FGF pathways (Neilson and Friesel, 1996; Fredieu et al., 1997; Pöpperl et al., 1997) repressed *six1* expression, both in whole embryos and in explants cultured in Noggin (Fig. 3H-J). Consistent with reports that posteriorizing factors play a positive role in neural crest induction, *foxD3* expression was reduced after *frzb-1+noggin* mRNA injections (Fig. 3K), and expanded after *Wnt8* or *cfgfr1* mRNA injections (Fig. 3L,M). These data demonstrate that like neural crest genes, normal *six1* expression is modulated by both neural inductive and posteriorizing factors. However, unlike the neural crest, PPE formation is positively influenced by the former and negatively regulated by the latter. Therefore, the relative levels of these factors likely influence whether a LNE cell expresses a neural crest versus placodal fate.

six1 expression expands placode gene expression at the expense of epidermis and neural crest, and is necessary for PPE formation

At neural plate stages, *sox2/sox3* neural plate expression domains are separated from the *six1/eya1* placodal domains by

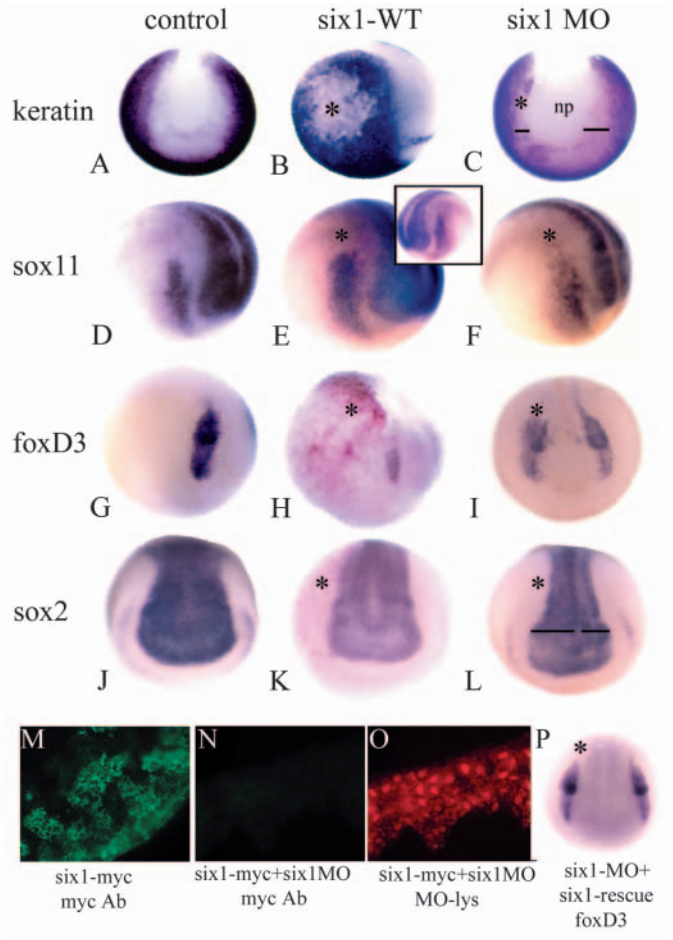

Fig. 4. Neural plate stage control expression patterns (A,D,G,J). (B,E,H,K) Overexpression of *six1-WT* (B) represses *keratin*, (E) increases the placodal domain of *sox11* (inset, control side), (H) represses *foxD3*, but (K) has no significant effect on *sox2* expression. The asterisks indicate the injected sides. (C,F,I,L) Six1-MO knock-down (C) expands the *keratin* domain closer to the border of the neural plate [np; compare bars on injected (*) versus control sides], (F) reduces the placodal domain of *sox11*, (I) increases the width of *foxD3* domain, and (L) expands *sox2* (bars indicate distance from midline). Quantitation of changes is presented in Table 1. (M) Injection of *six1-myc* results in protein expression detected by Myc antibody (green). (N) No protein (green) is detected when *six1*-MO is co-injected. (O) Same section as in N showing presence of lysamine-tagged *six1*-MO. (P) Injection of *six1-rescue* mRNA restores normal *foxD3* domain on *six1*-MO injected side (*).

the intervening *foxD3/slug* neural crest domain (Schlosser and Ahrens, 2004; Glavic et al., 2004b). Epidermis-specific *keratin* is expressed lateral to the *six1* domain (Fig. 4A) and another PPE marker, *sox11*, is expressed in the neural plate and in lateral crescents that overlap with those of *six1/eya1* (Fig. 4D). To test whether *six1* regulates the expression of any of these genes, wild-type *six1* (*six1-WT*) was overexpressed in the LNE region by mRNA injection into ventrolateral blastomeres. *keratin* expression was significantly repressed at the sites of increased *six1* expression (Fig. 4B; 86.8% of embryos; Table 1). By contrast, genes expressed in the PPE domain were expanded; *sox11* (75%) and *eya1* (81%) domains were significantly larger than in controls (Fig. 4E; Table 1). The *foxD3* domain was significantly reduced (Fig. 4H; 73.7%; Table 1). These changes in gene expression in the lateral ectoderm did not significantly alter the extent of the neural plate domain, as defined by the size of the expression domains of *sox2* or *sox3* (Fig. 4K; Table 1). These results demonstrate that elevated *six1* expression in the lateral ectoderm promotes PPE genes at the expense of epidermal and neural crest genes, but has minimal direct effect on the neural plate domain.

To test whether Six1 is required for the formation of the PPE, two morpholino antisense oligonucleotides that span *six1* translation start sites were injected into ventrolateral blastomeres to locally reduce endogenous *six1* translation. The PPE markers *sox11* (86.4%) and *eya1* (95.5%) were significantly reduced (Fig. 4F; Table 1), compared with uninjected controls and to control MO-injected embryos. By contrast, the domains of *keratin* (95.5%) and *foxD3* (80%) were significantly expanded (Fig. 4C,I; Table 1). Lateral reduction of Six1 also significantly enlarged the neural plate domains of *sox2* (90.9%) and *sox3* (95.5%) (Fig. 4L; Table 1). Protein translated from an injected myc-tagged *six1* mRNA was repressed in the presence of *six1*-MO (Fig. 4M-O), demonstrating the efficacy of the knock down. Furthermore, the *six1*-MO expansion of *foxD3* expression reverted to control levels by the co-injection of a *six1-rescue* mRNA (Fig. 4P). These results demonstrate that Six1 is necessary for the expression of PPE genes, and that in its absence neighboring ectodermal domains expand.

six1 affects ectodermal genes via both transcriptional activation and repression

Six1/2 type factors likely function as both transcriptional activators and repressors depending upon the presence of co-factors (Silver et al., 2003). To determine whether the above effects are via transcriptional activation or repression, both activating (*six1VP16*) and repressive (*six1EnR*) constructs were made. The *keratin* expression domain was dramatically repressed by *six1VP16* (Fig. 5A; 96.3%; Table 1). The lateral expression of *six1EnR* also caused a significant reduction in *keratin* expression (Fig. 5C; 100%, Table 1). PPE genes (*sox11,* 95.5%; *eya1,* 75%) were significantly expanded by *six1VP16*, and significantly reduced (68.8% and 90%, respectively) by *six1EnR* (Fig. 5E,G; Table 1). The *foxD3* expression domain was significantly expanded by *six1VP16* (90.9%), and significantly reduced by *six1EnR* (Fig. 5I,K; 81.8%; Table 1). A neural plate gene (*sox2*) was unaffected by either construct expressed in the lateral ectoderm (Table 1). These results predict that: (1) *keratin* expression is repressed by Six1 both directly and indirectly because the *six1-WT* and both activating and repressing constructs reduce its domain; (2) PPE genes are transcriptionally activated by Six1 because the effect of *six1VP16* mimics *six1-WT* and the effect of *six1EnR* is the reverse; (3) *foxD3* is transcriptionally repressed by Six1 because the effect of *six1EnR* mimics *six1-WT* and the effect of *six1VP16* is the reverse.

These predictions are supported by co-expressing *six1-WT* with known co-factors that have either activating or repressive functions. The interaction between Six and Eya proteins to cooperatively regulate transcription has been well documented with both *Drosophila* and vertebrate proteins (Pignoni et al., 1997; Ohto et al., 1999; Ikeda et al., 2002; Silver et al., 2003). The interaction between vertebrate Six and Eya proteins is thought to be necessary to recruit Eya proteins into the nucleus

Table 1. Differences in expression domain sizes in response to altered levels of *six1*

Target gene	Tissue marked	Control	six1-WT	six1VP16	six1-WT + eya1-WT	six1EnR	six1-WT + Dgroucho	Six1-MO	Control MO
keratin	Epidermis	2.3% (*n*=19)	29.8%† (*P*<0.001, *n*=38)	304.7%† (*P*<0.001; *n*=27)	250.9%† (*P*<0.001; *n*=21)	190.8%† (*P*<0.001; *n*=23)	76.5%† (*P*<0.01; *n*=22)	25.6%* (*P*<0.001; *n*=22)	4.4%† (NC; *n*=53)
sox11	Placode	0.8% (*n*=29)	38.4%* (*P*<0.001; *n*=24)	55.2%* (*P*<0.001; *n*=22)	28.5%* (*P*<0.001; *n*=22)	23.1%† (*P*<0.001; *n*=16)	37.6%† (*P*<0.001; *n*=24)	23.0%† (*P*<0.001; *n*=22)	0.8%† NC; *n*=44)
eya1	Placode	0.1% (*n*=38)	34.4%* (*P*<0.001; *n*=21)	27.4%* (*P*<0.001; *n*=16)	Not determined	35.5%† (*P*<0.001; *n*=20)	Not determined	26.8%† (*P*<0.001; *n*=22)	1.7%† (NC; *n*=22)
foxD3	Neural crest	0.9% (*n*=39)	21.2%† *P*<0.001; *n*=38)	67.4%* (*P*<0.001; *n*=22)	125.8%* (*P*<0.001; *n*=20)	29.0%† (*P*<0.001; *n*=44)	33.6%† (*P*<0.001; *n*=22)	25.4%* (*P*<0.001; *n*=20)	5.4%† (NC; *n*=48)
sox2	Neural plate	0.1% (*n*=37)	0.1%† (NC; *n*=46)	1.9%* (NC; *n*=20)	0.9%† (NC; *n*=23)	0.01%* (NC; *n*=16)	9.6%* (*P*<0.01; *n*=21)	32.4%* (*P*<0.001; *n*=22)	0.7%† (NC; *n*=20)
sox3	Neural plate	0.1% (*n*=40)	4.9%* (NC; *n*=24)	Not determined	Not determined	Not determined	Not determined	57.2%* (*P*<0.001; *n*=22)	0.3%† (NC; *n*=26)
zic2	Lateral patch	2.1% (*n*=27)	25.2%* (*P*<0.001; *n*=26)	254.4%* (*P*<0.001; *n*=22)	69.9%* (*P*<0.001; *n*=21)	11.3%* (NC; *n*=19)	19.3%* (NC; *n*=22)	30.7%* (*P*<0.001; *n*=20)	3.6%† (NC; *n*=19)
dlx5	Epidermal border	0.5% (*n*=34)	17.9%† (*P*<0.001; *n*=14)	Not determined	Not determined	Not determined	Not determined	22.1%† (*P*<0.001; *n*=26)	0.5%† (NC; *n*=19)
dlx6	Epidermal border	0.02% (*n*=22)	12.3%† (*P*<0.001; *n*=27)	29.6%† (*P*<0.001; *n*=15)	23.5%† (*P*<0.001; *n*=22)	35.8%† (*P*<0.001; *n*=20)	17.2%† (*P*<0.001; *n*=22)	26.8%† (*P*<0.001; *n*=25)	0.9%† (NC; *n*=20)

Measurements are expressed as the mean percent difference between uninjected and injected sides of embryos. For control embryos, it is the mean differences between left and right sides.

Percent differences from experimental embryos were compared with control embryos using Student's *t*-test.

NC, no significant change (*P*>0.01).

*An increase in expression domain size.

†A decrease in expression domain size.

(Ohto et al., 1999). Sequences in the SD/HD region target the complex to DNA and the Eya conserved N-terminal domain causes the complex to activate transcription (Pignoni et al., 1997; Xu et al., 1997; Kawakami et al., 2000). We co-expressed *six1-WT* and *eya1-WT* mRNAs in ventrolateral blastomeres, and observed that for every marker gene examined results were identical to those obtained with the *six1VP16* construct (Fig. 5B,F,J; Table 1). The transcriptional co-repressor Groucho also is unable to bind to DNA directly (Courey and Jia, 2001) and requires protein-protein interactions to assert its repressive activity. In yeast two-hybrid analyses, the vertebrate homologue Six3 has been shown to physically interact via the SD region with Groucho-related (Grg) proteins in mouse and in zebrafish (Kobayashi et al., 2001; Zhu et al., 2002). Although Six3/Six6 homologs are distinct from other Six related factors as they do not interact with Eya type proteins, the *Drosophila* Six1/2 type homologue Sine oculis does interact with Groucho in yeast two-hybrid analyses (Giot et al., 2003) (K.L.K. and F.P., unpublished). As there are multiple Grg proteins in vertebrates and no biochemical data are available for those specific to *Xenopus*, we determined that the Six domain of *Xenopus* Six1 does interact with *Drosophila* Groucho using yeast two-hybrid analysis (data not shown). Thus, we used *Drosophila* Groucho for our functional assays in *Xenopus* embryos. We co-expressed *six1-WT* and *Dgroucho-WT* mRNAs in ventrolateral blastomeres and observed that for every marker gene examined results were identical to those obtained with the *six1EnR*

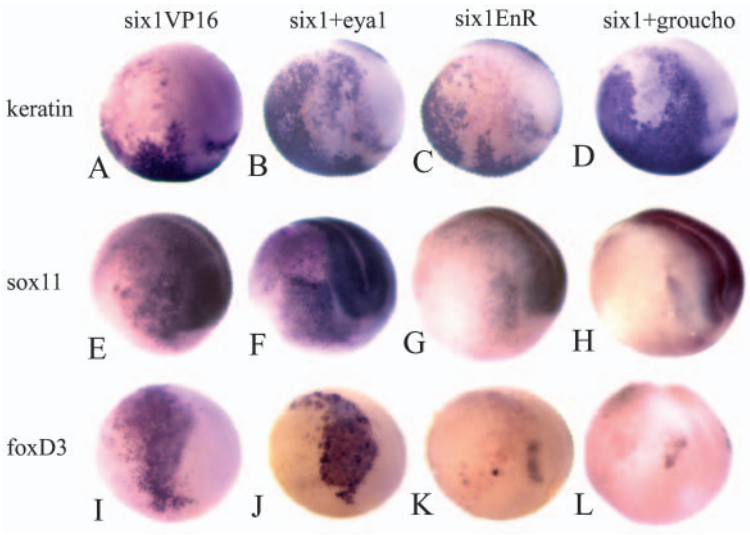

Fig. 5. Constructs that cause transcriptional activation (six1VP16; six1+eya1) reduce *keratin* expression (A,B), expand the placodal domain of *sox11* (E,F) and expand the *foxD3* domain (I,J). Constructs that cause transcriptional repression (six1EnR; six1+groucho) reduce *keratin* expression (C,D), reduce *sox11* placodal expression (G,H) and reduce the *foxD3* domain (K,L).

construct (Fig. 5D,H,L; Table 1). These data indicate that Six1 functions in the embryo as both a transcriptional activator and repressor, depending upon with which co-factor it is able to interact. In *Xenopus*, both *eya1* and at least three Grg genes are expressed in domains that overlap with endogenous *six1* expression (Choudhury et al., 1997; Molenaar et al., 2000; David et al., 2001), but relative levels of protein expression or functional activity are not known.

Subdomains within the LNE depend upon mutual gene interactions

It has been proposed that interactions between the border of the neural plate and the non-neural ectoderm are crucial for establishing fates within the LNE (Luo et al., 2001a; McLarren et al., 2003; Woda et al., 2003). We investigated whether expanded *six1* expression affects genes whose domains lie at the border between the PPE and neural plate (*zic2*) or the border between the PPE and epidermis (*dlx5*, *dlx6*). Increased *six1-WT* expression significantly expanded the patch of *zic2* that is expressed lateral to the neural plate (Fig. 6A,B; 73.1%; Table 1), without affecting the neural plate domain (data not shown). This effect was mimicked by both the *six1VP16* construct (100%) and co-injection of *six1-WT+eya1-WT* mRNAs (85.7%), but not by the *six1EnR* construct or co-injection of *six1-WT+Dgroucho* mRNAs (Table 1). These results indicate that Six1 positively regulates the lateral *zic2* domain via transcriptional activation. However, reduction of Six1 protein by *six1*-MO injections also expanded *zic2* expression (Fig. 6C; 90%; Table 1), indicating that other factors that are antagonized by Six1 probably also positively regulate *zic2* (see below). Increased *six1-WT* expression had two effects on Dlx expression. First, it caused the lateral stripes of *dlx5* and *dlx6* expression to be located further laterally (Fig. 6E; 71.4% and 70.4%, respectively; Table 1). Second, the *dlx5* (78.6%) and *dlx6* (51.9%) stripes either had gaps of full repression or were diffuse and less intense compared with the uninjected, control side (Fig. 6E). The *six1VP16* construct (93.3%), co-injection of *six1-WT+eya1-WT* mRNAs (90.0%), the *six1EnR* construct (80.0%) and co-expression of *six1-WT+Dgroucho* mRNAs (65.0%) all caused a phenotype similar to *six1-WT* for *dlx6* (Table 1). Both *dlx5* (84.6%; Table 1) and *dlx6* (Fig. 6F; 100%; Table 1) were moved more laterally or repressed by reduction of Six1 by MO injection, suggesting that other factors antagonized by Six1 also negatively regulate the *dlx5/6* genes (see below).

The LNE comprises several subdomains of a number of transcription factors (Schlosser and Ahrens, 2004; Glavic et al., 2004b), many of which are affected by altering the levels of *six1* expression. To understand further the regulatory interplay that subdivides the LNE, we investigated the effects of expanded expression of some of these genes on neighboring expression domains. Expression of *sox2* in the lateral ectoderm repressed *six1* expression (Fig. 6G; 88%, *n*=17). Expanded expression of *foxD3* repressed *six1* expression (Fig. 6H; 92%, *n*=39), caused ectopic *sox2* expression (not shown, 100%, *n*=16), repressed *keratin* expression (not shown, 100%, *n*=21), repressed *dlx5* expression (not shown, 73%, *n*=15) and caused ectopic *zic2* expression (Fig. 6I; 76.9%, *n*=13). Increased *zic2* expression also repressed *six1* expression (Fig. 6J; 68.3%, *n*=41), and expanded *foxD3* expression (Fig. 6K; 77.8%, *n*=18). Increased expression of *dlx5* repressed *six1* expression (Fig. 6L; 84.6%, *n*=78). These data demonstrate a dynamic interplay between the transcription factors that regulate subdomain identity within the LNE and along its boundaries (Fig. 7).

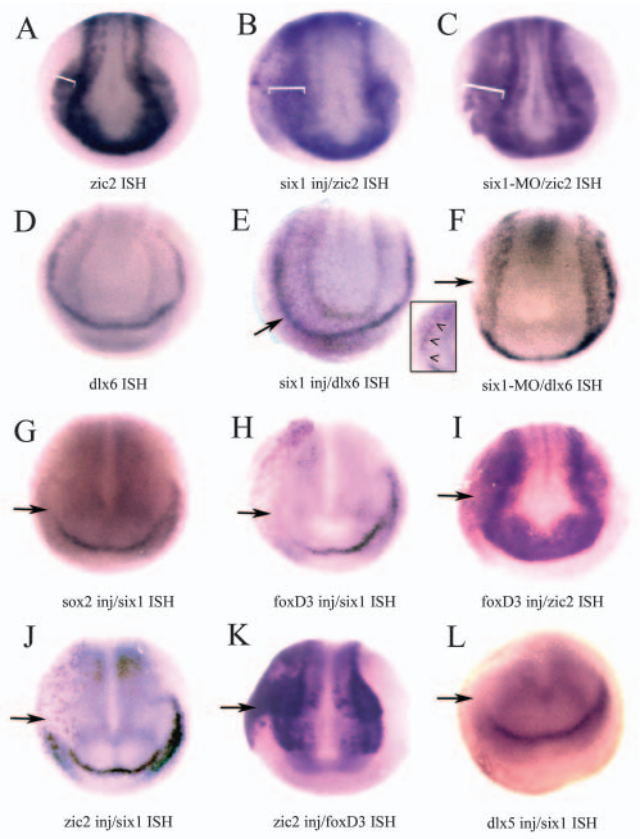

Fig. 6. (A) Control *zic2* expression; bracket indicates LNE domain. (B) Overexpression of *six1-WT* expands the *zic2* LNE domain (bracket). (C) Six1 knock-down expands the *zic2* LNE domain (bracket). (D) Control *dlx6* expression. (E) Overexpression of *six1-WT* moves the *dlx6* stripe laterally (arrow), and in some cases eliminates expression in places (arrowheads, inset). (F) Six1 knockdown reduces the lateral *dlx6* stripe (arrow). (G) Overexpression of *sox2* reduces *six1* (arrow). (H,I) Overexpression of *foxD3* (H) reduces *six1* (arrow) and (I) expands *zic2* LNE expression (arrow). (J,K) Over-expression of *zic2* (J) reduces *six1* (arrow) and (K) expands *foxD3* (arrow). (L) Over-expression of *dlx5* reduces *six1* (arrow). mRNAs were injected on left-hand side; right-hand side is internal control.

Discussion

Three potential mechanisms have been proposed for subdividing the embryonic ectoderm into neural plate, neural crest, PPE and epidermis. First, cells are exposed to different levels of BMP and axial signaling. Second, different domains express transcription factors that strongly promote one fate over another. For example, Sox2 promotes a neural stem cell fate (Graham et al., 2003), FoxD3 promotes a neural crest fate (Dottori et al., 2001; Sasai et al., 2001) and AP-2 promotes an epidermal fate (Luo et al., 2002). Third, interactions between the presumptive neural plate and epidermis may create a border zone environment that is conducive for neural crest and PPE fates (Baker and Bronner-Fraser, 1997; Feledy et al., 1999;

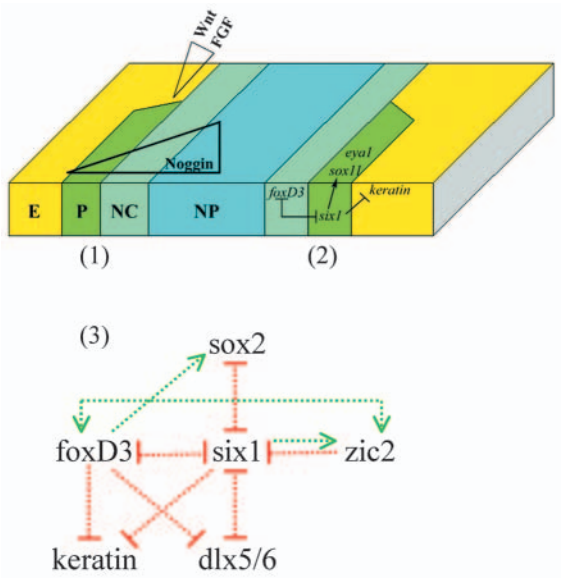

Fig. 7. Model of the three steps involved in PPE formation. (1) The embryonic ectoderm is separated into presumptive epidermis (E), pre-placodal (P), neural crest (NC) and neural plate (NP) domains in response to a combination of a dorsoventral gradient of BMP-antagonizing factors (Noggin) and a gradient of posteriorizing signals (Wnt, FGF). (2) *six1* promotes the expression of placode genes at the expense of neural crest and epidermal genes. (3) A network of gene interactions further defines the ectodermal subdomains. Green arrows indicate that the expression of the downstream gene is enhanced, and red bars indicate that it is reduced. Lines are broken to indicate that interactions may be direct or include intermediary genes.

Knecht and Bronner-Fraser, 2002; McLarren et al., 2003; Bastidas et al., 2004). For example, pieces of neural plate transplanted to ventral epidermis induce neural crest and placodal markers at their margins (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; McLarren et al., 2003; Woda et al., 2003; Glavic et al., 2004b).

We investigated the potential roles of these mechanisms in PPE specification. Placodes have long been recognized as important embryonic anlage for cranial sensory structures, but the mechanisms by which the early, transient PPE that gives rise to all individual placodes is induced and segregated from neighboring ectodermal domains have not been elucidated. Our findings support the involvement of all three mechanisms in PPE specification (Fig. 7): (1) gradients of both neural inductive and anteroposterior signaling are necessary to induce and appropriately position the PPE; (2) *six1* acts as a placodal fate specifying gene that positively regulates the expression of other PPE markers, and negatively regulates neural crest and epidermal identities: and (3) interactions between several transcription factors expressed in the LNE border zone modulate the relative sizes of the different ectodermal subdomains.

Induction of PPE and neural crest by differential responses to neural inducing and anterior-posteriorizing factors

The lateral neurogenic ectoderm (LNE), which comprises neural crest and PPE, is located between the neural plate and the epidermis in an intermediate region of a proposed gradient of BMP activity (Weinstein and Hemmati-Brivanlou, 1999; Wilson and Edlund, 2001). It has been shown that neural crest markers are induced by concentrations of neural inducers lower than those required for neural plate markers (Morgan and Sargent, 1997; Marchant et al., 1998), and we demonstrate that even lower concentrations elicit the highest levels of expression of two placodal markers (*six1*, *eya1*). This differential responsiveness to Noggin concentrations in explants supports the idea that an endogenous gradient of neural inducing signals provides a first step in separating the embryonic ectoderm into its different domains (Fig. 7). However, in the intact embryo, in which several signaling pathways intersect to influence the fate of the cell, intermediate BMP levels are not sufficient to induce either neural crest or placode fates. The neural crest, which is absent from the most anterior pole of the neural plate but extends to its posterior tip, requires Wnt and FGF signaling (LaBonne and Bronner-Fraser, 1998; Chang and Hemmati-Brivanlou, 1998; Mayor and Aybar, 2001; Villanueva et al., 2002; Monsoro-Burq et al., 2003; Glavic et al., 2004a). These pathways are thought to act both to posteriorize the axis and to directly promote neural crest fate (Monsoro-Burq et al., 2003; Lewis et al., 2004). We demonstrate that these same signals antagonize *six1* expression, and propose that their posterior expression restricts placode formation to the head (Fig. 7). In the intact embryo, the expression of endogenous Wnt and FGF antagonists in anterior regions may additionally contribute to restricting placode formation (Leyns et al., 1997; Glinka et al., 1998; Piccolo et al., 1999; Bradley et al., 2000; Nutt et al., 2001; Zhang et al., 2001; Yamaguchi, 2001; Tsang and Dawid, 2004).

These data indicate that PPE and neural crest formation require the combined activities of neural inducing and anterior-posteriorizing factors. However, different concentrations of BMP antagonists favor one fate over the other, and posteriorizing factors promote neural crest over placodal fate. Thus, it is likely that the LNE is initially competent to give rise to both placodal and crest derivatives, and the acquisition of the specific fate of a LNE cell results from local concentrations of the pertinent signaling pathways. This hypothesis is consistent with: (1) observations that although *six1*/*eya1* are most highly expressed at Noggin concentrations lower than those for *foxD3*, there is significant overlap in the two dose-response curves; (2) fate maps showing that otic placode precursors are intermingled with future neural crest precursors (Streit, 2002); and (3) observations that the expression domains of some neural crest and placodal marker genes partially overlap (McLarren et al., 2003; Schlosser and Ahrens, 2004; Glavic et al., 2004b).

six1 acts as a placodal fate specifying gene

Studies in vitro have previously shown that Six proteins can function as transcriptional activators and repressors (Kobayashi et al., 2001; Ohto et al., 1999; Silver et al., 2003; Li et al., 2003). However, only evidence for repression has been documented in vivo, specifically for the related factors Optx2/Six6 and Six3 (Zuber et al., 1999; Kobayashi et al., 2001). We find that placodal fate specification by Six1 requires both activating and repressing activities. Analyses of *six1-WT* and *six1-MO* injected embryos show that Six1 positively regulates the expression of PPE factors and negatively

regulates other ectodermal genes (Fig. 4). By using Six1VP16 and Six1EnR constructs, we further demonstrate that these effects result from both activating and repressing activities of Six1 in vivo (Fig. 5). These activities may be mediated, at least in part, by the association of Six1 with the transcriptional activator Eya1 or transcriptional repressors of the groucho family (Fig. 5). Whether endogenous Six1 functions as an activator or repressor is probably determined by the relative levels and activities of the different co-factors within *six1*-expressing cells.

Definition of sub-domains within the embryonic ectoderm

Our studies also predict complex in vivo interactions between *six1* and other ectodermal genes. *sox2* and *sox3* are both induced in presumptive neural ectoderm by neural inductive signaling, and promote stabilization of a neural fate (Mizuseki et al., 1998; Penzel et al., 1998). Later, they are both expressed in neural stem cells (Graham et al., 2003). We show that endogenous *six1* expression in the LNE precedes *sox2/3* placodal expression, indicating that *sox2/3* are not upstream of *six1*. *six1* overexpression in the LNE has no significant effect on *sox2/3* neural plate expression, indicating that its effects in this border domain are cell autonomous and not due to intermediate signaling. However, when *six1* is reduced in the lateral ectoderm, *sox2/3* expression expands laterally. This could be a secondary result of the expansion of *foxD3*, which in turn expands *sox2/3* (Sasai et al., 2001) (Fig. 5) and/or a mutual antagonism between *six1* and *sox2/3*. The latter possibility is supported by the observations that *six1* expression in the neural plate dramatically represses *sox2/3* (data not shown) and that expression of *sox2* in the LNE represses *six1*. At later stages *sox2/3* are expressed in placodal domains that presumably overlap with *six1* expression. How these genes interact at this later phase of placode development remains to be determined.

foxD3 is required for neural crest formation, and both explant and in vivo studies show that it induces neural crest and neural plate marker genes (Sasai et al., 2001). We show that *foxD3* and *six1* have a mutually antagonistic relationship; the over-expression of one gene causes the repression of the other. Conversely, the reduction of Six1 causes the *foxD3* domain to expand, and the reduction of *foxD3* by *six1* overexpression causes expansion of other placodal markers. It is not yet known whether the interactions between *foxD3* and *six1* are direct or indirect; because *six1* can repress both *foxD3* and *sox2* expression domains and *foxD3* can expand *sox2/3* domains (Sasai et al., 2001) (data herein), the interaction could be via *sox2/3* regulation.

The *zic1, zic2* and *zic3* genes, which are likely to be functionally redundant, are first expressed throughout the entire presumptive neural epithelium and then become restricted to the lateral border of the neural plate and neural crest (Nakata et al., 1997; Nakata et al., 1998; Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998; LaBonne and Bronner-Fraser, 1999). The Zic genes appear to be important for the initial phase of both neural plate and neural crest development, and all three can induce ectopic expression of neural crest markers. In explants, *zic1* induces *foxD3* and *slug*, and *foxD3* induces *zic1* and *zic2*, leading to the proposal that Zic genes act upstream of *foxD3* and *slug* to initiate neural crest fate, and that Zic gene and *foxD3* expression is maintained in the neural crest by mutual interactions (Sasai et al., 2001). We corroborate a mutual positive interaction between *zic2* and *foxD3* by in vivo expression assays (Fig. 6). However, in the LNE *foxD3* and the Zic genes do not have identical expression patterns (Sasai et al., 2001) (herein), indicating that they also may be interacting through intermediary genes. We demonstrate a similarly complex interaction between *six1* and *zic2*. Overexpression of *six1* expands the *zic2* lateral domain, but reduction of Six1 also expands it. We propose that the former phenotype is caused by activation/maintenance of *zic2* by *six1*, whereas the latter phenotype is most probably caused by the expansion of *foxD3*, which subsequently expands the *zic2* domain.

Members of the Dlx gene family represent some of the earliest genes expressed at the border between the neural plate and epidermis (Feledy et al., 1999; Luo et al., 2001a; Luo et al., 2001b; Beanan and Sargent, 2000). In chick, *dlx5* is expressed at the neural/non-neural border, overlapping with *eya2* and *six4* in the pre-placodal thickening where it is proposed to create a border zone in which lateral neurogenic fates can be expressed (McLarren et al., 2003). In *Xenopus* there is a low level of *dlx5/6* expression along the border of the neural plate, but the most intense stripe is adjacent to *six1* expression along the anterior neural ridge, and overlapping with the lateral edge of the crescent of *six1* PPE expression (Luo et al., 2001a; Luo et al., 2001b) (herein). In chick, *dlx5* overexpression results in a weak upregulation of *six4* expression (McLarren et al., 2003), whereas in *Xenopus* wild-type *dlx5*/*dlx6* both strongly reduce *six1* expression (this study), and activator Dlx constructs cause a loss of *six1* expression (Woda et al., 2003). It is not clear whether these differences are due to species differences in the precise patterning of the embryonic ectoderm, as has been proposed for neural induction (Aybar and Mayor, 2002), or due to the fact that Six1 and Six4 belong to different subclasses of the Six gene family (Kawakami et al., 2000).

Regardless, it is clear that in frog *six1* has two effects on *dlx5/6* expression. Most prominently, overexpression of *six1* in the LNE pushes the *dlx5/6* stripe laterally away from the neural plate midline. This phenotype is probably due to *six1* causing an expansion of the PPE (*eya1*, *sox11*) and reduction of epidermis (*keratin*), resulting in the formation of a new border between the expanded LNE and the epidermis. This interpretation is consistent with the effects of *six1* overexpression on *keratin*, and further suggests that the effect is not due to movement of the neural plate border because the *sox2/3* domains do not change. Likewise, *dlx5/6* negatively regulate *six1* expression. A previous study also demonstrated a mutual regulation between Dlx genes and *six1* (Woda et al., 2003): inhibition of endogenous Dlx activity relocated the *six1* expression domain more laterally, whereas activation relocated it more medially. The second effect of *six1* is complete repression of *dlx5/6* expression in those cells expressing *six1*. This may result from Six1 either repressing *dlx5/6* gene expression or causing changes in gene expression in the affected cells that secondarily create an environment that is not compatible with *dlx5/6* expression. Interestingly, *foxD3* overexpression has similar effects on *dlx5/6*, which could be direct, or, unlike *six1*, could be due to the expansion of *sox2/3*. We further observed paradoxically similar *dlx5/6* phenotypes

by activator and repressor *six1* construct expression and *six1-MO* injections. We predict that these can be explained by *six1* effects on *foxD3*. The injection of *six1VP16*, *six1-WT+eya-WT* and *six1*-MO may indirectly reduce *dlx5/6* by expansion of *foxD3*, whereas *six1-WT* alone and *six1EnR* constructs may directly reduce *dlx5/6*. These results support the proposal that *dlx5/6* contribute to forming the LNE border zone (McLarren et al., 2003; Woda et al., 2003), and additionally demonstrate that they do so by participating in a complex interplay with several genes expressed in adjacent domains. It will be important to determine the precise molecular interactions between these various gene pathways to fully understand their roles in specifying LNE fates.

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