Six3 functions in anterior neural plate specification by promoting cell proliferation and inhibiting *Bmp4* expression

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Summary

Although it is well established that *Six3* is a crucial regulator of vertebrate eye and forebrain development, it is unknown whether this homeodomain protein has a role in the initial specification of the anterior neural plate. In this study, we show that exogenous *Six3* can expand the anterior neural plate in both *Xenopus* and zebrafish, and that this occurs in part through Six3-dependent transcriptional regulation of the cell cycle regulators *cyclinD1* and *p27Xic1*, as well as the anti-neurogenic genes *Zic2* and *Xhairy2*. However, *Six3* can still expand the neural plate in the presence of cell cycle inhibitors and we show that this is likely to be due to its ability to repress the expression of *Bmp4* in ectoderm adjacent to the anterior neural plate. Furthermore, exogenous *Six3* is able to restore the size of the anterior neural plate in *chordino* mutant zebrafish, indicating that it has the ability to promote anterior neural development by antagonising the activity of the BMP pathway. On its own, *Six3* is unable to induce neural tissue in animal caps, but it can do so in combination with *Otx2*. These results suggest a very early role for *Six3* in specification of the anterior neural plate, through the regulation of cell proliferation and the inhibition of BMP signalling.

Key words: *Six3*, Cell proliferation, Cell fate, *Bmp4*, *Xenopus*, Zebrafish

Introduction

A complex and well-coordinated sequence of cell movements and inductive signals orchestrate the specification of dorsal ectoderm to form the anterior neural plate (Kuroda et al., 2004). The BMP subfamily of TGFβ proteins plays a key role in specification and patterning of the ectoderm. High levels of BMP signalling drives unspecified ectodermal cells towards an epidermal rather than neural fate, whereas neural tissue differentiates in regions of low BMP activity (Munoz-Sanjuan and Hemmati-Brivanlou, 2002). Cells at the neural plate boundary are likely to be exposed to intermediate levels of BMP activity and differentiate as neural crest, placodes and cement gland (Morgan and Sargent, 1997; Barth et al., 1999; Nguyen et al., 2000; Aybar and Mayor, 2002).

Specification and patterning of the anterior neural plate is crucially dependent upon the spatial localisation of BMP signalling activity. At early stages, BMP genes are expressed throughout the ectoderm, but, before the beginning of gastrulation, transcription is downregulated dorsally, where the neural plate is going to form (Wilson and Edlund, 2001; Stern, 2002; Kuroda et al., 2004). Two subsequent steps appear to regulate BMP expression and activity in the dorsal ectoderm. First, at blastula stages, Wnt and FGF signalling is crucial to suppress the transcription of BMP genes in the dorsal ectoderm (Baker et al., 1999; Bally-Cuif and Hammerschmidt, 2003; Kuroda et al., 2004). Second, by the late blastula stages, BMP antagonists, such as Noggin and Chordin secreted by the organizer, interact with BMPs and prevent their binding to receptors. *Bmp4* transcription is maintained by an autoregulatory loop, where BMP4 protein bound to its receptor stimulates transcription of the *Bmp4* gene (Jones et al., 1992; Hammerschmidt et al., 1996; Piccolo et al., 1997). The activity of BMP antagonists can interrupt this positive-feedback loop.

How different signalling pathways such as Wnt, FGF and BMP are integrated during early ectodermal development is still poorly understood. In anamniotes, it is likely that the activity of Wnt/βcatenin induces dorsal activation of genes that modulate FGF signalling; once activated, FGFs mediate an early restriction of BMP expression and activate the expression of BMP antagonists in the organizer (Furthauer et al., 2004; Tsang et al., 2004; Kudoh et al., 2004). Despite the early repression of BMP activity in presumptive neural territories, BMP signalling maintains the ability to inhibit expression of anterior neural plate genes even during neurulation (Hartley et al., 2001). This is of particular relevance considering that the anterior neural plate is surrounded by non-neural ectoderm and is underlain by anterior mesendoderm, both of which are sources of BMPs.

Persistent suppression of BMP transcription in the anterior neural plate may be maintained by specific transcriptional repressors activated after neural induction (Hartley et al., 2001). This is indeed the case for XBF2 and Xiro1, which act as *Bmp4* transcriptional repressors at early neurula stage, thus ensuring proper neural fate acquisition (Mariani and Harland, 1998; Gomez-Skarmeta et al., 2001).

In addition to suppression of BMP signalling, the rostral neural plate must be protected from the activity of caudalising signals for it to establish anterior forebrain character. Among the signals that promote posterior neural identity are Wnts, and a variety of Wnt antagonists ensure that Wnt activity is suppressed rostrally. The neural plate is patterned along its anteroposterior axis by the graded activity of Wnts, Wnt antagonists and other signals (Wilson and Houart, 2004). This is established by the interplay of Wnt antagonists secreted by the anteriormost neuroectoderm and the underlying mesendoderm, and local sources of Wnt signals in the posterior neuroectoderm, midbrain, diencephalon and mesendodermal tissues (Heisenberg et al., 2001; Kiecker and Niehrs, 2001; Houart et al., 2002). An early event in neural plate patterning is the generation of an anterior region that comprises the presumptive telencephalon, diencephalon and retina. The repression of both Wnt and BMP signalling, together with an enhanced proliferative activity, are crucial for the formation of the anterior neural plate (Zuber et al., 2003; Wilson and Houart, 2004). In fact, embryos with blocked cell proliferation or exaggerated Wnt or BMP signalling display anterior deficiencies (Hammerschmidt et al., 1996; Kim et al., 2000; Houart et al., 2002; Andreazzoli et al., 2003; Zakin and De Robertis, 2004).

Downstream of the signals that subdivide the ectoderm, a variety of transcription factors mediate neural plate patterning. Among these *Bf1*, *Bf2*, *Rx*, *Six3* and *Otx2*, expressed in the anterior neural plate, are crucially involved in the formation of anterior CNS structures (Acampora et al., 1995; Andreazzoli et al., 1997; Mathers et al., 1997; Bourguignon et al., 1998; Mariani and Harland, 1998; Andreazzoli et al., 1999; Loosli et al., 2001; Carl et al., 2002; Lagutin et al., 2003). However, although there is some evidence that links the activity of these transcription factors with those of the Wnt and BMP pathways in the formation of the CNS (Braun et al., 2003; Lagutin et al., 2003), their exact interplay is still scarcely understood.

In this study, we show that *Six3* displays the characteristics expected from an effector of neural inducers involved in specifying and maintaining anterior neural plate properties. *Xsix3* overexpression promotes cell proliferation and inhibits neurogenesis at early neurula stage by activating *Xhairy2*, *Zic2*, *Xrx1* and *Xbf1* and regulating the expression of *p27Xic1* and *cyclinD1*. Furthermore, *Six3* represses BMP expression in both *Xenopus* and zebrafish, and is able to rescue the anterior neural plate defects of *chordino* mutants. The effect of *Xsix3* on *Bmp4* appears to be direct, as suppression occurs even in the absence of protein synthesis and *Xsix3* can bind the *Bmp4* promoter in vitro. Although *Xsix3* efficiently suppresses *Bmp4* expression, we observed that it is unable to induce neural tissue, requiring *Xotx2* for this activity. Taken together with the recent observation that *Six3* is able to repress Wnt expression (Braun et al., 2003; Lagutin et al., 2003), these data indicate *Six3* as a crucial factor for anterior neural plate specification.

Materials and methods

Animals

To obtain embryos, induction of ovulation of pigmented *Xenopus* females, in vitro fertilisation and embryo culture were carried out as described by Newport and Kirschner (Newport and Kirschner, 1982). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Zebrafish embryos were obtained by natural spawning from wild-type and *dino*^{tt250} mutant fish (Schulte-Merker et al., 1997).

In situ hybridization

Whole-mount in situ hybridization experiments on *Xenopus* and zebrafish embryos, and on animal caps, were carried out essentially as described previously (Harland, 1991; Barth and Wilson, 1995). Bleaching of *Xenopus* pigmented embryos was carried out following colour reaction as described by Mayor et al. (Mayor et al., 1995). The templates for the production of in situ hybridization probes for *Xag* (Bradley et al., 1996), *Xcg* (Sive et al., 1989), *Xsix3* (Zuber et al., 2003), *Xrx1* (Casarosa et al., 1997), *rx3* (Chuang et al., 1999), *Zic2* (Brewster et al., 1998), *N-tubulin* (Hartenstein et al., 1989), *elrC* (Perron et al., 1999), *Xash3* (Turner and Weintraub, 1994), *Xbf1* (Bourgouignon et al., 1998), *Xdelta1* and *Xngnr1* (Ma et al., 1996), *Xnotch* (Chitnis and Kintner, 1996), *Xsix1* (Pandur and Moody, 2000), *p27Xic1* (Hardcastle and Papalopulu, 2000), *Xhairy2* (Koyano-Nakagawa et al., 2000), *Sox9* (Spokony et al., 2002), *Xslug* (Mayor et al., 1995), *Xnrp1* (Knecht et al., 1995), *Bmp4* (Fainsod et al., 1994), *bmp4* (Nikaido et al., 1997), *dlx3* (Akimenko et al., 1994), *Sox2* (Misuzeki et al., 1998), *sox3* (Kudoh et al., 2001), *Xk81* (Jonas et al., 1985), *Xwnt8* (Christian et al., 1991), *Xotx2* (Pannese et al., 1995), *otx2* (Li et al., 1994), *hoxb1b* (Eisen and Weston, 1993) and *cyclinD1* (Vernon et al., 2003) have been described previously.

Mitotic inhibition with hydroxyurea and aphidicolin treatment

Xenopus embryos were devitellinized at stage 9 and allowed to recover for 30 minutes in 2% Ficoll in 0.1×Marc's modified Ringer (MMR). Stage 10 embryos were then incubated in 0.1×MMR containing 20 mM hydroxyurea and 150 μM aphidicolin (HUA) (Harris and Hartenstein, 1991; Hardcastle and Papalopulu, 2000) until fixation. The effect of hydroxyurea and aphidicolin treatment on cell division was examined as described previously (Andreazzoli et al., 2003).

Embryo microinjections and BrdU incorporation

Capped VP16-*Six3* RNA was generated from a construct containing the full-length *Xsix3* cDNA cloned into *Cla*1/*Xhp*1 sites of CS2-VP16 vectors (Kessler, 1997). To make the GR-*Xsix3* expression construct, the open reading frame of *Xsix3* was PCR amplified using the primers 5′-GCAGATATCATGGTGTTCAGGTCCCCTC-3′ and 5′-GTCCTCGAGTCATACGTCACATTCAGAGTCAC-3′ and inserted into the *Eco*RV/*Xho*1 site of pCS2+/GR kindly provided by Thomas Sargent (see Kodjabachian and Lemaire, 2001). RNAs encoding for *Xsix3* (20, 40 pg), GR-*Xsix3* (65 pg), *Xchh* (1 ng) (Ekker et al., 1995), *Xngnr1* (40 pg) (Ma et al., 1996), *tBR* (250 pg) and *Bmp4* (300 pg) (Kazanskaya et al., 2000) were generated by in vitro transcription using the message machine kit (Ambion, Austin, TX) and co-injected with *lacZ* RNA into one blastomere at the two-cell stage (*Xchh* and *Xngnr1*), or into a dorsal animal blastomere at the four- to eight-cell stage (*Xsix3*, *tBR*, *Bmp4*). β-Galactosidase staining was performed on embryos injected with 200 pg of *lacZ* RNA as previously described (Turner and Weintraub, 1994); X-Gal and salmon gal substrates were used for blue and red staining, respectively (Roche, Biosinth-AG). The optimal concentration of each batch of RNA was identified through the injection of various doses followed by analysis of either the phenotype or the expression of specific markers. Three different *Xsix3* antisense morpholinos were used (Gene Tools): Mo*Xsix3*

targeted against the two pseudoallelic genes of *Xsix3*, *Xsix3.1* and *Xsix3.2* (5′-ACCTGAACACCATGGGATGGCCGG-3′) (Ghanbari et al., 2001); Mo*Xsix3.2* targeted against *Xsix3.2*, the gene that we used for overexpression experiments (5′-CAGCAAAACTAGCGACAGC-GACAGC-3′); and Mo*Six3.1* targeted against *Six3.1* (5′-TGAA-AGAAGCGGCAGCAACACTAGC-3′). Differences in the efficiency of morpholinos were observed; whereas injection of 0.2 mM of Mo*Six3.1* at the two-cell stage does not induce abnormal development, injection of 0.2 mM of both Mo*Xsix3* and Mo*Xsix3.2* leads to a *Xsix3* loss-of-function phenotype. Given that the frequency of affected embryos in the case of Mo*Xsix3* (95%) injection was higher than with Mo*Xsix3.2* (45%), we decided to use Mo*Xsix3* for our studies.

Morpholino oligonucleotides targeted against zebrafish *chordin*, reliably phenocopying the mutant *chordino* phenotype, were injected into one-cell stage embryos at concentrations of 0.15 mM (Nasevicius and Ekker, 2000). In vitro transcription of zebrafish *six3* RNA and injection of 19-74 pg was performed as described (Kobayashi et al., 1998).

S-phase cells were labelled with BrdU essentially according to the protocol of Hardcastle and Papalopulu (Hardcastle and Papalopulu, 2000). To distinguish the injected from the control side of the embryos, we combined immunohistochemistry with in situ hybridization using *Zic2* expression, a gene strongly upregulated upon *Xsix3* overexpression (see below). In these experiments the number of BrdU-positive cells in the injected side was compared with that of the uninjected control side, taking into account also their anteroposterior distribution. Whole-mount TUNEL staining was performed at stage 13, as described by Hensey and Gautier (Hensey and Gautier, 1998).

Animal cap assay and cycloheximide treatment

For *Xenopus* animal cap experiments, capped synthetic *chordin* (150 pg per blastomere) (Sasai et al., 1995), *tBR* (600 pg) (Wylie et al., 1996), *Bmp4* (1 ng), *Xotx2* (250 pg) (Vignali et al., 2000), *Xotx2*-GR (50 pg) (Gammill and Sive, 1997), *Xsix3* (500 pg-2 ng) and GR-*Xsix3* (500 pg-1ng) RNA was injected into one-cell stage embryos and animal caps dissected at stage 9. When sibling control embryos reached stage 14, animal caps were fixed and stored in ethanol at –20°C. To inhibit protein synthesis, animal caps were isolated from GR-*Xsix3* or *Xotx2*-GR injected embryos at stage 9 and aged in high-salt Modified Barth's Saline (MBS). Stage 10.5 caps where then incubated in high-salt containing 10 μM of cycloheximide (CHX). Glucocorticoid receptor (GR) fusion proteins were activated at stage 11, after a 30-minute CHX pre-incubation step, by adding DEX 10 μM into the medium containing CHX. Animal caps were then fixed at stage 15.

Gel mobility-shift assay

GST-Xsix3 protein purification was performed as described (Tessmar et al., 2002). A 315-bp PCR fragment of *Bmp4* promoter was ^{32}P-end-labelled by T4 kinase (Roche). Binding conditions and electrophoresis were as described by Gomez-Skarmeta et al. (Gomez-Skarmeta et al., 2001).

Results

Xsix3 promotes cell proliferation in the neural plate

Overexpression of *Six3* induces forebrain enlargement in zebrafish (Kobayashi et al., 1998), and the expansion and ectopic appearance of retina primordia in *Xenopus* (Bernier et al., 2000) and medaka (Loosli et al., 1999). Additionally, *Six3* influences cell proliferation (Loosli et al., 1999; Bernier et al., 2000; Carl et al., 2002), possibly at a non-transcriptional level (Del Bene et al., 2004). To clarify how *Six3* influences these events, we analysed the consequences of expressing exogenous *Xsix3* on early neural plate development. To gain further insight into the role of *Xsix3* in the control of cell proliferation, embryos overexpressing *Xsix3* were analysed for BrdU incorporation. We found that the anterior neural plate of *Xsix3*-injected embryos displayed a 36% increase in the number of BrdU-positive cells on the injected side compared with the control side (an average of 48.7 positive cells per section in the injected side, n=755 BrdU-positive nuclei, versus 35.7 cells per section in the control side, n=460; P<0.001; Fig. 1A,E). Although at this stage *Xsix3* is expressed exclusively in the anterior neural plate, the posterior neural plate displays a similar significant increase in the rate of BrdU incorporation (34.7%; an average of 18.2 positive cells per section in the injected side, n=473 versus 13.5 cells per section in the control side, n=315; P<0.001; Fig. 1C,E). Thus, the entire neural plate is competent to respond to *Xsix3* proliferative activity.

To analyse the requirement for *Xsix3* function in the control of cell proliferation, we used a loss-of-function approach. In agreement with the work of Kobayashi and colleagues (Kobayashi et al., 2001), we found that a transcriptional activator form of *Xsix3* (VP16-*Xsix3*) acts as a dominant negative. Very low amounts of VP16-*Xsix3* RNA (20-40 pg) injected at the four-cell stage dramatically reduced, and sometimes eliminated, eye formation (94%, n=500; data not shown). This phenotype is efficiently rescued by co-injection of *Xsix3* RNA, indicating that VP16-*Xsix3* specifically antagonises *Xsix3* function (90%, n=150; data not shown). Expression analysis of molecular markers (*Xbf1*, *Xrx1*, *Xpax6*, *Xotx2*, *Xen2* and *Xkrox20*) recapitulated the anterior forebrain truncation phenotypes previously described for *Six3* loss of function in medaka and mouse, or inactivation by dominant-negative variants in zebrafish (data not shown) (Kobayashi et al., 2001; Carl et al., 2002; Lagutin et al., 2003). Moreover, both the anterior and posterior neural plate (ANP and PNP) of VP16-*Xsix3* injected embryos shows a significant decrease of BrdU-positive cells in the injected side compared with the control side (53% and 60% respectively; ANP: an average of 28.5 cells per section in the control side, n=820, versus 13 cells per section in the injected side, n=380; PNP: an average of 13 cells per section in the control side, n=383, versus 5.6 cells per section in the injected side, n=129; P<0.001; Fig. 1B,D,F).

To gain insight into how Six3 may act on proliferation in the anterior neural plate, we analyzed the expression of putative Six3 target genes known to be involved in cell proliferation control that are expressed rostrally at early neurula stage. We found that *Xbf1* and *Xrx1* are both ectopically activated in the anterior neural plate (*Xbf1*: 86%, n=45; *Xrx1*: 72%, n=90; Fig. 1H,I) by exogenous *Xsix3*. *CyclinD1*, a positive regulator of the cell cycle expressed in the eye field, is ectopically activated by *Xsix3* (stage 14, 82%, n=34; Fig. 1J), whereas *p27Xic1*, which encodes a cell cycle inhibitor, is repressed by *Xsix3* (100%, n=39; Fig. 1K,L). *CyclinD1* and *p27Xic1* expression is modulated both in the anterior and posterior neural plate (Fig. 1J-L). Thus, *Xrx1*, *Xbf1* and *cyclinD1*, genes encoding factors promoting cell proliferation, are activated, and *p27Xic1*, which inhibits cell proliferation, is repressed by *Xsix3* at early neurula stage; this strongly suggests that *Xsix3* is able to act on the cell cycle machinery already at this early developmental stage.

Ectopic expression of *Xsix3* enlarges the neural plate at the expense of adjacent non-neural tissue

Xsix3 is expressed in the anterior neural plate at early neurula

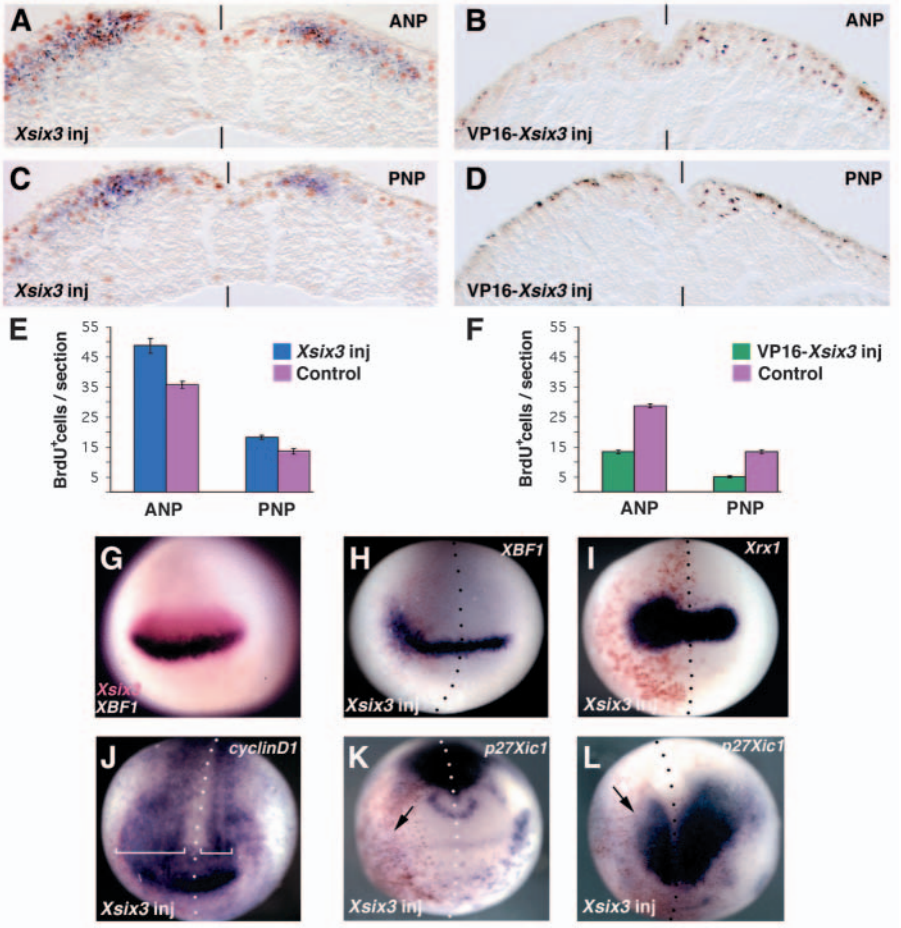

Fig. 1. *Xsix3* promotes cell proliferation in the neural plate. (A-D) Transverse sections of stage 13 embryos injected with either *Xsix3* (A,C) or VP16-*Xsix3* (B,D), processed simultaneously for BrdU incorporation (brown nuclear staining), and *Zic2* (A,C, blue staining) or *lacZ* (B,D, red staining) expression. Sections are at the level of the anterior (A,B) or posterior neural plate (C,D). ANP, anterior neural plate; PNP, posterior neural plate. (E,F) The average number of BrdU-positive cells per section in either the control side (magenta) or the side injected with *Xsix3* (blue) or VP16-*Xsix3* (green). Error bars indicate s.e.m. (G) Double whole-mount in situ hybridization of a stage 13 embryo shows that the *Xsix3* (magenta) and *Xbf1* (purple) expression domains overlap. (H-J) Stage 13 (H,I) and stage 14 (J) embryos injected with *Xsix3* display expansion of the *Xbf1* (H), *Xrx1* (I) and *cyclinD1* (J) expression domains. White brackets indicate the anterior expression domain of *cyclinD1* in the control (right) and injected (left) side. (K,L) *Xsix3* overexpression represses *p27Xic1* both in the anterior, trigeminal ganglion (K, arrow) and in its posterior expression domain (L, arrow) in stage 13 embryos. (G-K) Frontal views, dorsal towards the top; (L) dorsal view, anterior towards the bottom. Red staining represents expression of a co-injected *lacZ* lineage tracer. The injected side of the embryos (to the left of vertical bars or dotted lines representing the midline) is indicated (inj).

stage, raising the possibility that, besides controlling cell proliferation, it might participate in neuroectoderm specification (Zuber et al., 2003). To test this hypothesis, we analysed the expression of neural plate markers in *Xsix3*-injected embryos at early neurula stage. We observed that the general neural markers *Sox2* and *Xnrp1* (Knecht et al., 1995; Kishi et al., 2000) are expanded on the injected side of the embryos (*Sox2*: 77%, *n*=135; *Xnrp1*: 80%, *n*=23; Fig. 2A,B). Notably, ectopic expression of neural markers in the lateral ectoderm was always contiguous to the neural plate.

To examine whether the expression of non-neural markers at the border of the neural plate was also affected, we analysed the expression of the neural crest markers *Xslug* and *Sox9* (Sasai et al., 2001), the cranial placode marker *Xsix1* surrounding the anterolateral neural plate (Pandur and Moody, 2000), and the cement-gland specific marker *Xag* (Sive and Bradle, 1996), which is expressed in the most anterior part of the non-neural ectoderm in a region partially overlapping with *Xsix3* (data not shown). *Xsix3* injection resulted in the reduction or loss of the expression of all of these markers (*Xslug*: 96%, *n*=46; *Sox9*: 93%, *n*=29; *Xsix1*: 93%, *n*=40; *Xag*: 82%, *n*=29; Fig. 2C-F). Moreover, when present, the expression domains of the markers were displaced to a more ventrolateral position (Fig. 2C,D and data not shown). These changes were probably due to the enlargement of the neural plate. This suggests that the expansion of the neural plate occurs, at least in part, by converting non-neural cells at the neural plate border to a neural fate. Complementing the ability of *Xsix3* to promote neural fate, expression of the epidermal marker *Xk81* (Jonas et al., 1989) was also severely blocked in the *Xsix3* injected area (80%, *n*=68; Fig. 2G,H). In contrast to what was observed for the neural markers, *Xsix3*-dependent suppression of *Xk81* occurred also in isolated areas of ventral ectoderm non contiguous with the neural plate (Fig. 2H). These results indicate that *Xsix3* is sufficient to repress epidermal fate but requires a competent territory in order to promote a neural fate.

To test whether the ability of *Xsix3* to expand the anterior neural plate at the expense of non-neural tissue might be a consequence of its proliferative activity, we overexpressed *Xsix3* in embryos in which cell division was blocked by HUA treatment. HUA treatment severely reduced anti-phosphorylated histone H3 (H3P) staining, a marker of cells in mitosis (Fig. 2I,J). Even under these conditions, *Xsix3* is still able to expand *Sox2* expression and to repress the expression of *Xslug*, *Sox9* and *Xk81* (*Sox2*: 69%, *n*=87; *Xslug*: 73%, *n*=29; *Sox9*: 90%, *n*=19; *Xk81*: 76%, *n*=34; Fig. 2K-N). Taken together, these results suggest that the effects of *Xsix3* on markers of neural patterning are at least in part independent of proliferation.

To rule out the possibility that the observed effects on neural plate specification might be a secondary consequence of an early repression of Wnt gene expression by *Xsix3* (Lagutin et al., 2003), we injected a Dexamethasone (DEX)-inducible form of the *Xsix3* construct. In these experimental conditions, activation of GR-Xsix3 at mid-gastrula stages is able to

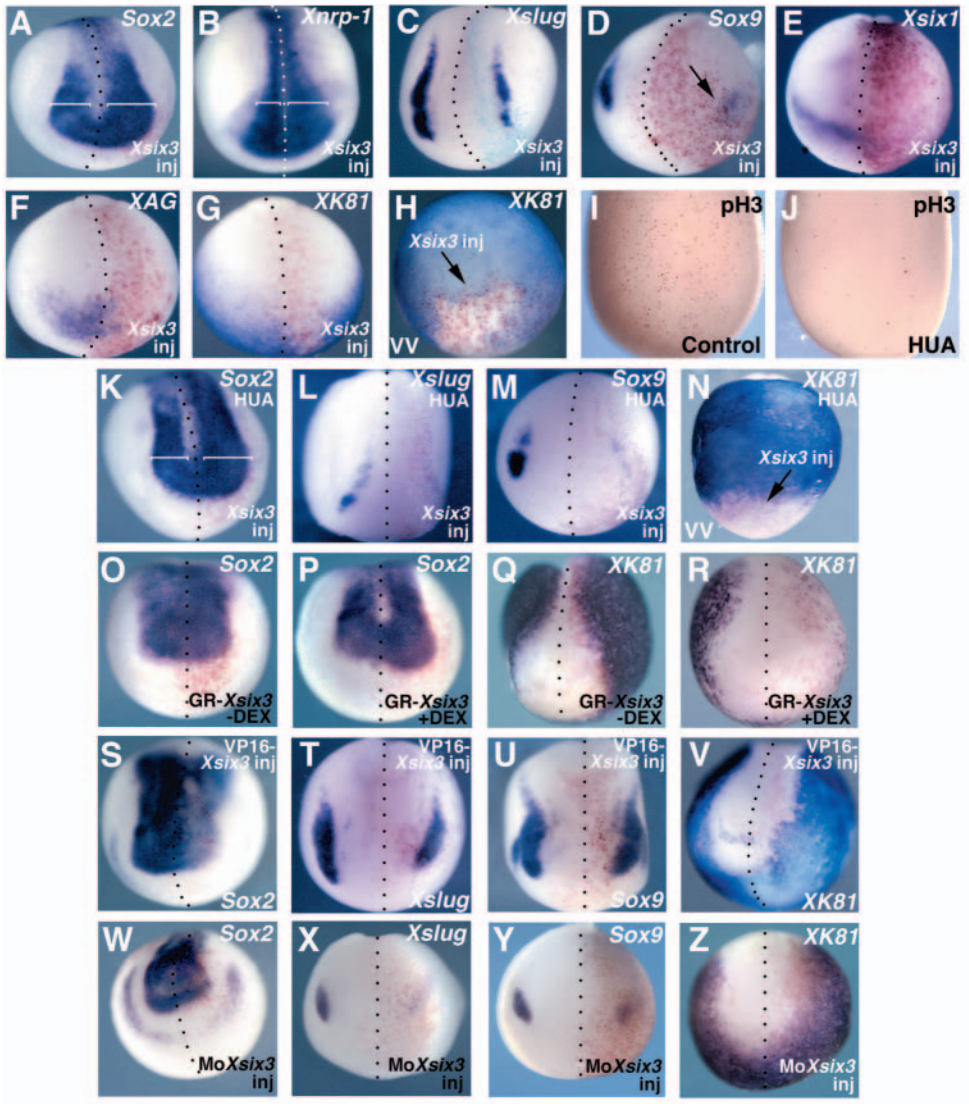

Fig. 2. Alterations of the size of the neural plate in *Xsix3* gain- and loss-of-function experiments. (A-H,K-Z) Embryos injected unilaterally with *Xsix3* (A-H,K-N), GR-*Xsix3* (O-R), VP16-*Xsix3* (S-V) or Mo*Xsix3* (W-Z), and analysed at early neurula stage. The probes used are indicated in each panel. Red staining in A,B,D-H,K-Z and turquoise staining in C represent expression of the co-injected *lacZ* lineage tracer. The injected side of the embryos (to the right of dotted lines representing the midline) is indicated (inj). (A-G,K-M,O-Z) Frontal views, dorsal towards the top; (H,N) ventral views (VV), anterior towards the bottom. (H,N) Arrows indicate *Xk81* repression caused by *Xsix3* overexpression. (I,J) Embryos untreated (I) or treated (J) with HUA, brown nuclear staining representing anti-phosphorylated histone H3 staining; dorsal views, anterior towards the bottom. (K-N) Embryos injected with *Xsix3* and treated with HUA. (A,B,K) White brackets indicate the anterior expression of *Sox2* (A,K) or *Xnrp1* (B) in *Xsix3*-injected (right) and control (left) sides.

promote *Sox2* and to repress *Xk81* expression (*Sox2*: 100%, *n*=91; *Xk81*: 90%, *n*=90; Fig. 2P,R). In the absence of DEX, the fusion protein GR-Xsix3 is inactive or only slightly active, and embryos do not display significant *Sox2* and *Xk81* expression alterations (*Sox2*: 89% normal expression, 11% slightly expanded, *n*=80; *Xk81*: 85% normal expression, 15% slightly reduced, *n*=59; Fig. 2O,Q). These results suggest that *Xsix3* has a role in the initial specification of the neural plate.

To further analyse the requirement for *Xsix3* function in anterior neural plate specification, we used two different loss-of-function strategies. In VP16-*Xsix3*-injected embryos, we observed repression of *Sox2* and expansion of *Xk81* in the prospective anterior neural plate (*Sox2*: 72%, *n*=42; *Xk81*: 81%, *n*=32; Fig. 2S,V). The neural crest markers analysed are still expressed, although they are localized closer to the midline (*Xslug*: 77%, *n*=27; *Sox9*: 83%, *n*=31; Fig. 2T,U). Thus, conversion of presumptive neural plate cells towards an epidermal fate is likely to contribute to the *Six3* loss-of-function phenotype.

As an independent loss-of-function approach, we injected an antisense morpholino (Mo*Xsix3*). Virtually all the Mo*Xsix3*-injected embryos show eye and anterior head defects similar to those observed in VP16-*Xsix3*-injection experiments (95%, *n*=130, data not shown). However, the penetrance of Mo*Xsix3* phenotypic alterations is milder than that observed with VP16-*Xsix3*, as we never observed a complete loss of eye structures in Mo*Xsix3*-injected embryos. The phenotype is efficiently rescued by co-injection of *Xsix3* RNA (data not shown and Fig. 5Q). As for VP16-*Xsix3*, overexpression of Mo*Xsix3* reduces *Sox2* and expanded *Xk81* expression at early neurula stages (*Sox2*: 100%, *n*=25; *Xk81*: 80%; *n*=75; Fig. 2W,Z). Similarly, the expression of the neural crest markers *Sox9* and *Xslug* is delocalised in a more dorsal position, even though in this case a reduced expression is also observed (*Xslug*: 100, *n*=35; *Sox9*: 100%, *n*=28; Fig. 2X,Y). Altogether, these results indicate that *Xsix3* acts as a transcriptional repressor in the control of anterior neural plate specification.

Xsix3 suppresses differentiation of primary neurons

In *Xenopus*, a subset of neuroectodermal cells leave the cell cycle and start to differentiate at the end of gastrulation, giving rise to primary neurons (Hartenstein, 1989). As *Xsix3* promotes neural plate expansion, we tested its involvement in the specification of primary neurons.

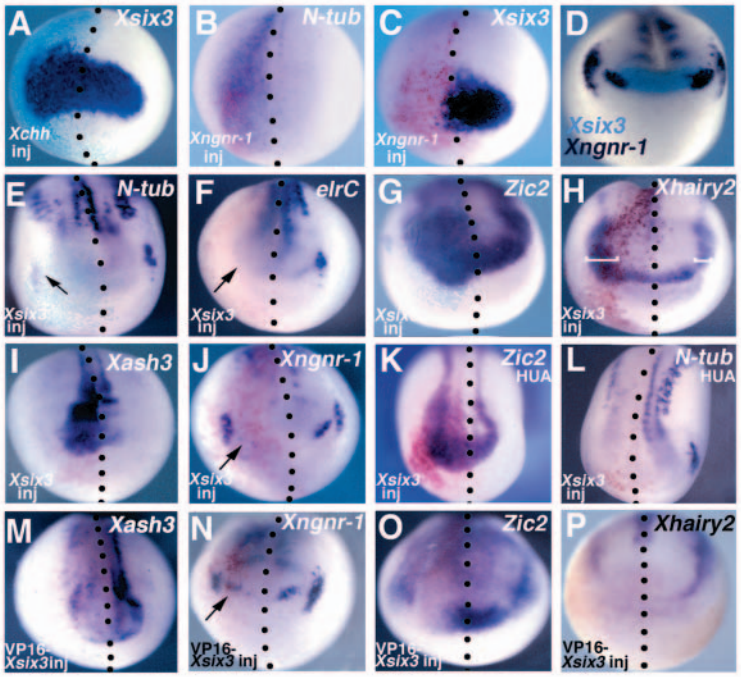

Fig. 3. *Xsix3* regulates the expression of genes that control cell differentiation, and its expression is controlled by Hedgehog and Neurogenin signalling. (A-C) Embryos injected unilaterally with *Xchh* (A) and *Xngnr1* (B,C) display ectopic expression of *Xsix3* (A) and *N-tubulin* (B), and repression of *Xsix3* (C). (D) Comparison of the expression of *Xsix3* (light blue) with that of *Xngnr1* (purple) in a stage 13 embryo. (E-K) Embryos injected with *Xsix3* display repression of *N-tubulin* (E, the arrow indicates the repressed expression in the trigeminal ganglion), *elrC* (F) and *Xngnr1* (J), and ectopic expression of *Zic2* (G), *Hairy2* (H) and *Xash3* (I). (K,L) Embryos injected with *Xsix3* and treated with HUA show expansion of *Zic2* (K) and repression of *N-tubulin* (L). (M,O,P) Embryos injected with VP16-*Xsix3* display reduction of the *Xash3* (M), *Zic2* (O) and *Xhairy2* (P) expression domains. (J,N) Arrows indicate the anterior boundaries of *Xngnr1* in the injected side of the embryo. (A-P) Frontal views, dorsal towards the top. Red staining in B,C,F-P and turquoise staining in A,E represent expression of the co-injected lacZ lineage tracer. The injected side of the embryos (to the left of dotted lines representing the midline) is indicated (inj). (H) White brackets indicate the anterior expression of *Xhairy2* in *Xsix3*-injected (left) and control (right) sides.

First, we investigated the effects of positive and negative regulators of cell differentiation on *Xsix3* expression. *Cephalic hedgehog* (*chh*) induces proliferation and delays differentiation in *Xenopus* anterior neural plate (Franco et al., 1999). Injection of *Xchh* strongly expands the *Xsix3* expression domain at the early neurula stage (75%, *n*=45; Fig. 3A). Conversely, *Xngnr1*, which encodes a factor promoting neurogenesis in the anterior neural plate (Ma et al., 1996) – as shown by its ability to induce ectopic *N-tubulin* expression (100%, *n*=29; Fig. 3B) – strongly repressed *Xsix3* (100%, *n*=24; Fig. 3C).

To test the effects of *Xsix3* on neurogenesis, we analyzed the expression of *Xngnr1* and the neuronal differentiation markers *N-tubulin* and *elrC* in *Xsix3*-injected embryos. *Xsix3* overexpression represses *Xngnr1* in the most anterior semicircular stripes of expression, corresponding to the presumptive olfactory placodes and part of the telencephalon (38%, *n*=53, Fig. 3J, arrow), and in the midbrain-hindbrain boundary (100%, *n*=53, Fig. 3J). At early neurula stage, *N-tubulin* and *elrC* are expressed in primary neurons organized into three longitudinal domains on each side of the posterior neural plate, which will give rise to motoneurons, interneurons and sensory neurons, and in the trigeminal ganglia (Chitnis et al., 1999; Perron et al., 1999). *Xsix3* injection causes a downregulation of *N-tubulin* (90%, *n*=50; Fig. 3E) and *elrC* (91%, *n*=23; Fig. 3F), both in the trigeminal ganglion (Fig. 3E,F, arrows) and in the posterior stripes. To understand which step of the neurogenesis cascade can be affected by *Xsix3*, we examined the effects of *Xsix3* injection on the antineurogenic genes *Zic2* (Brewster et al., 1998), *Xhairy2* (Dawson et al., 1995) and *Xash3* (Chitnis and Kintner, 1996). *Xsix3*-injected embryos showed an expansion of *Zic2* (83%, *n*=108; Fig. 3G), *Xhairy2* (82%, *n*=43; Fig. 3H) and *Xash3* (69%, *n*=26; Fig. 3I) along the anterior lateral neural plate. These results suggest that *Xsix3* reduces primary neuron formation by upregulating the prepattern antineurogenic genes *Zic2* and *Xhairy2*. The fact that *Xsix3* overexpression results in the repression of *N-tubulin*, and the expansion of the *Zic2* and *Xhairy2* expression domains, even in embryos in which cell division was blocked (*Zic2*: 78%, *n*=75; *N-tubulin*: 80%, *n*=20; Fig. K,L and data not shown) suggests that the effect of *Xsix3* on neuronal differentiation is not dependent on cell proliferation. These data indicate that *Xsix3* inhibits neuronal differentiation through the activation of the antineurogenic factors *Zic2*, *Xhairy2* and *Xash3*, and/or through the repression of neurogenic factors such as *Xngnr1*.

To determine the requirement for *Xsix3* function in the control of neurogenesis, we investigated the expression of the same markers in VP16-*Xsix3*-injected embryos. In the case of *Xash3*, *Zic2* and *Xhairy2*, we observed a repression of their expression domains (100%; *Xash3*: *n*=25; *Zic2*: *n*=69; *Xhairy2*: *n*=35; Fig. 3M,O,P). Despite this, *Xngnr1* expression in VP16-*Xsix3*-injected embryos was not significantly increased (Fig. 3N). These results suggest that the effects observed in *Xsix3* loss of function are mainly independent of precocious neurogenesis.

Xsix3 inhibits epidermal fate but requires *Xotx2* to induce neural markers in animal caps

As *Xsix3* can expand the neural plate, we tested whether *Xsix3* is able to induce neural tissue independently of mesoderm by performing animal cap assays. Control uninjected caps showed no expression of *Sox2*, *Xnrp1*, *Xash3*, *Xhairy2* and *Zic2* (0% in all caps; *Sox2*: *n*=79; *Xnrp1*: *n*=25; *Xash3*: *n*=27; *Zic2*: *n*=55; *Xhairy2*: *n*=60; Fig. 4), whereas they did express *Xk81* (100%, *n*=61; Fig. 4). None of these markers was induced in *Xsix3*-injected caps (*Sox2*: *n*=129; *Xnrp1*: *n*=30; *Xash3*: *n*=35; *Zic2*: *n*=90; *Xhairy2*: *n*=90; Fig. 4). By contrast, *Xsix3* is able to strongly suppress expression of the epidermal marker *Xk81* (83%, *n*=78; Fig. 4). These results indicate that *Xsix3* negatively regulates the expression of the epidermal marker *Xk81* and that it requires additional factors, absent in animal caps, to activate neural markers in the ectoderm. Among these,

Xotx2, encoding a factor crucial for anterior neural plate formation, is co-expressed with *Xsix3* in the presumptive anterior neuroectoderm at early neurula stage (stage 12.5) (Zuber et al., 2003). Similar to *Xsix3*, overexpression of *Xotx2* in animal caps strongly suppresses the expression of the epidermal marker *Xk81*, but it does not activate (or only weakly activates) expression of general neural markers (100%; *Sox2*: *n*=20; *Xnrp1*: *n*=35; Fig. 4) (Vignali et al., 2000). However, co-injection of *Xsix3* and *Xotx2* mRNA strongly activates the expression of both *Sox2* and *Xnrp1* (*Sox2*: 88%, *n*=60; *Xnrp1*: 82%, *n*=60; Fig. 4). These results suggest that *Xotx2* and *Xsix3* may function in a synergistic way in anterior neural plate specification.

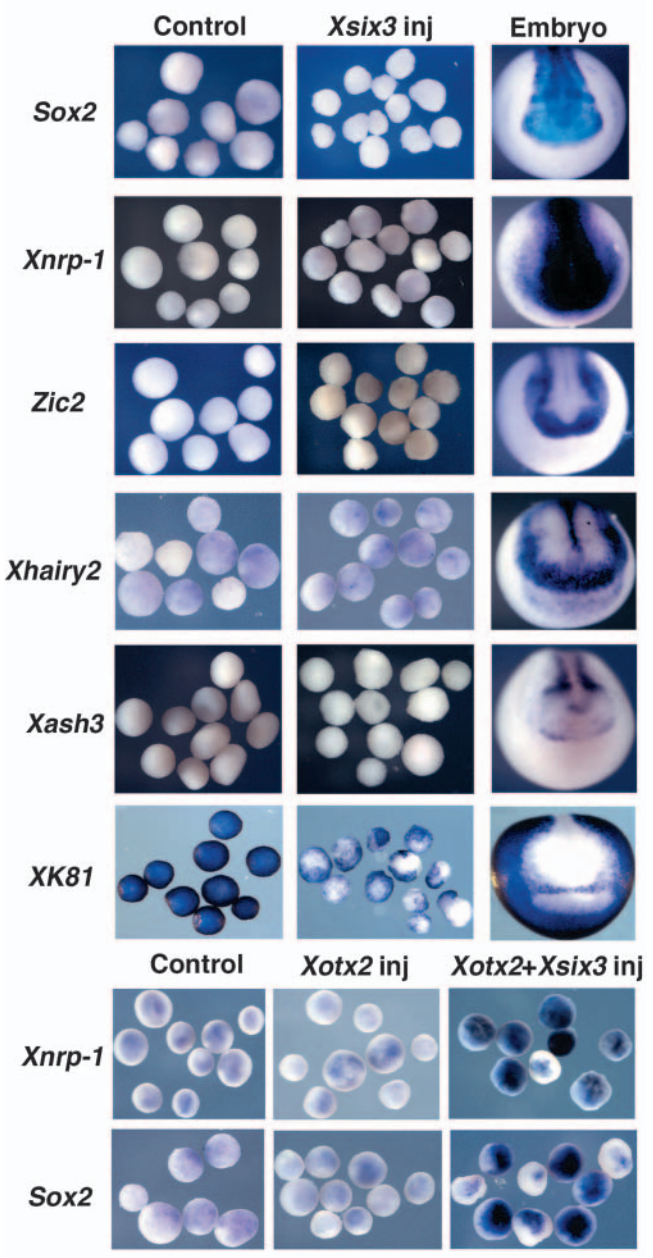

Fig. 4. *Xsix3* represses epidermal fate but requires *Xotx2* for the induction of neural markers in animal caps. Animal caps from control uninjected embryos (Control) or embryos injected with either *Xsix3*, *Xotx2*, or a mixture of the two RNAs, as indicated at the top, were dissected at stage 9, cultured to stage 14 and analysed for the expression of the genes indicated to the left. The column to the right (Embryo) shows expression of the analysed genes in control embryos.

Mutual antagonism between *Xsix3* and *Bmp4*

As *Xsix3* inhibits the expression of the epidermal marker *Xk81*, we asked whether this effect may result from a downregulation of the expression of the epidermalizing factor BMP4 (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). At blastula stage, *Bmp4* is ubiquitously expressed and becomes restricted to the ventrolateral regions of the embryo during gastrulation. At this stage, *Bmp4* and *Xsix3* display mainly complementary expression domains, with *Xsix3* being activated in the anterior neural plate concomitant with repression of *Bmp4* in the same domain.

Overexpression of *Xsix3* resulted in repression of *Bmp4* expression both in the embryo (71%, *n*=49; Fig. 5A) and in animal caps (71%, *n*=138; Fig. 5C). Control uninjected caps displayed normal *Bmp4* expression (100%, *n*=39; Fig. 5B).

To elucidate whether *Bmp4* and *Xsix3* might antagonise each other, we analysed the effects that the overexpression of each of these genes exert on the other. We observed that *Bmp4* overexpression leads to a strong reduction of *Xsix3* expression (88%, *n*=26; Fig. 5D). Conversely, interfering with BMP signalling by injection of either *tBR*, a dominant-negative BMP receptor, or *chordin* mRNA (Weinstein and Hemmati-Brivanlou, 1999) induced a strong activation of *Xsix3* both in animal caps (control: 0%, *n*=30; *chordin*: 100%, *n*=39; *tBR*: 100%, *n*=45; Fig. 5G,H,I) and in the anterior neural plate of the embryo (*tBR*: 92%, *n*=26; Fig. 5E). Conversely, both VP16-*Xsix3* and Mo*Xsix3* injection leads to expansion of *Bmp4* expression in the presumptive anterior neural plate (VP16-*Xsix3*: 45%, *n*=50; Mo*Xsix3*: 77%, *n*=27; Fig. 5J,K). Additionally, TUNEL analysis showed that both *Bmp4*- and VP16-*Xsix3*-injected embryos displayed an anterior accumulation of apoptotic nuclei (Fig. 5L,M).

To analyse whether the effects of *Xsix3* loss of function are a consequence of BMP4 expansion in the anterior neural plate, we tested whether interfering with BMP signalling can counteract the reduction of the anterior neural plate in Mo*Xsix3*-injected embryos. To achieve this, we analysed the expression of *Zic2* (a gene expressed both in the anterior and posterior neural plate that is strongly modulated by *Xsix3*), in Mo*Xsix3*/*tBR* co-injected embryos. Injection of Mo*Xsix3* alone repressed anterior *Zic2* expression (81%, *n*=73; Fig. 5N). Conversely, Mo*Xsix3*/*tBR* co-injected embryos showed a complete or partial rescue of the *Zic2* expression domain (65%, normal expression; 10% slightly reduced expression, 25% expanded expression; *n*=124; Fig. 5P). None of the co-injected embryos showed the strong expansion of *Zic2* seen for *tBR* alone (100%, *n*=60; Fig. 5O). As a control, a similar rescue is observed when Mo*Xsix3* is co-injected with *Xsix3* (83%, normal expression; 17% slightly expanded; *n*=68; Fig. 5Q). Taken together, these results indicate a mutual antagonism between *Xsix3* and *Bmp4*.

six3 rescues the anterior alterations in zebrafish *chordino* mutants

To analyse whether *six3* could counteract the effects of BMP dependent modulation of the neural plate size in vivo, we took

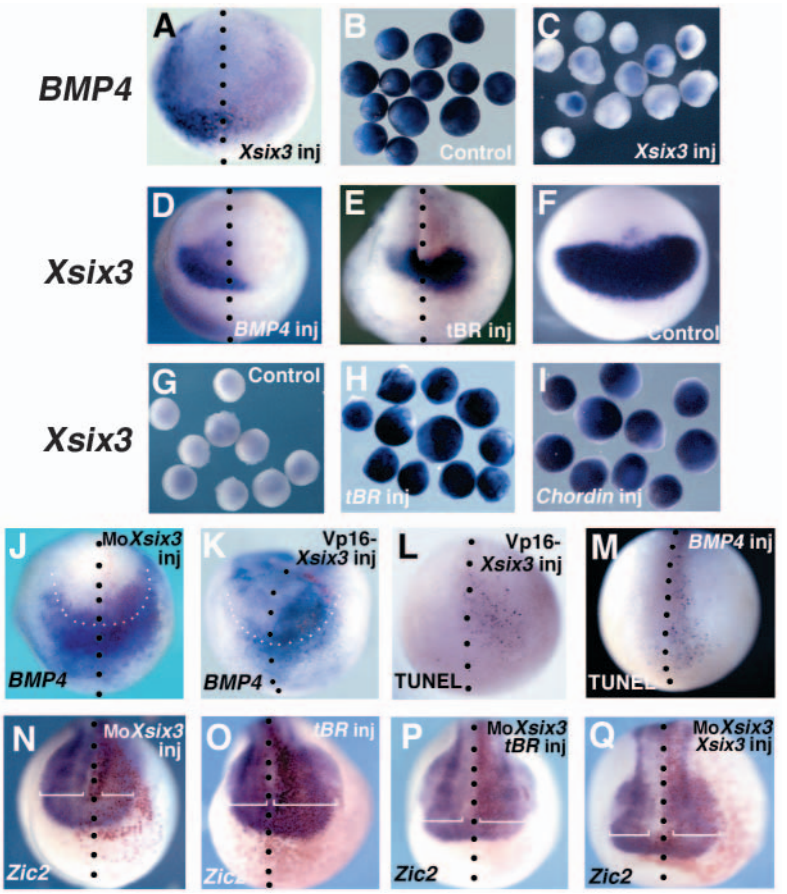

Fig. 5. *Xsix3* and *Bmp4* repress each other. (A-Q) Control and injected embryos, and animal caps, were analysed at stage 13 for the expression of the genes indicated. (A-C) *Xsix3* overexpression represses *Bmp4* expression in embryos (A) and animal caps (C), whereas uninjected caps (B) strongly express *Bmp4*. (D) *Xsix3* is repressed in embryos injected with *Bmp4*. (E) *tBR* injection expands the *Xsix3* expression domain. (G-I) Animal caps injected with *tBR* (H) or *Chordin* (I) strongly express *Xsix3*, whereas uninjected caps (G) do not express *Xsix3*. (J,K) Mo*Xsix3*- and VP16-*Xsix3*-injected embryos display anterior expansion of *Bmp4* (the white dots indicate the anterior neural plate border). (L,M) VP16-*Xsix3* and *Bmp4* injection induces apoptosis (TUNEL staining) in the anterior neural plate. (N,O) Mo*Xsix3* reduces (N) and *tBR* expands (O) the domain of *Zic2* expression. (P,Q) *Zic2* repression is rescued in embryos co-injected either with Mo*Xsix3* and tBR (P, compare with N), or with Mo*Xix3* and *Xsix3* (Q, compare with N).

advantage of zebrafish *chordino* mutants/morphants. These mutants (Hammerschmidt et al., 1996) carry a mutation in the *chordin* gene, a known antagonist of BMP signalling. The mutation causes the enlargement of tail structures, alterations in trunk development and a reduction of anterior central nervous system structures, including the eye (Fig. 6B) (Schulte-Merker et al., 1997). Analysis of *chordino* mutants/morphants at early neurula stages (90% epiboly) shows a reduced anterior neural plate (*rx3*, *otx2*, *sox3*) and expanded epidermal (*bmp4*, *dlx3*) markers, a phenotype similar to VP16-*Xsix3*-injected frog embryos (Fig. 6F,J,N,R,V).

To investigate whether Six3 activity can compensate for the loss of Chordin function, we overexpressed *six3* in wild-type and *chordino* mutant/morphant embryos. Injection of *six3* mRNA into wild-type zebrafish embryos results in a consistent expansion of both brain (Kobayashi et al., 1998) and eye structures, as reported for *Xenopus* and medaka (Bernier et al., 2000; Loosli et al., 1999) (Fig. 6C, Table 1). The development of posterior structures remained unaffected (Fig. 6C). At early neurula stages, *six3* misexpression resulted in the anterior lateral and ventral expansion of the neural markers *rx3*, *otx2* and *sox3*, together with a mild reduction of the epidermal fate markers *bmp4* and *dlx3*, whereas the expression of the posterior neural plate marker *hoxb1b* remained largely unaffected (Fig. 6G,K,O,S,W). These data suggest that *six3* is able to specifically expand the anterior neural plate without affecting anteroposterior patterning.

In *chordino* mutants/morphants, *six3* injection resulted in a highly specific rescue of anterior defects in a concentration-dependent manner, whereas the trunk maintained the ventralized morphology typical for the loss of *chordin* gene function (Fig. 6D, Table 1). At early neurula stages, we observed the rescue of *rx3*, *otx2* and *sox3* expression, whereas *hoxb1b* expression in the posterior neural plate remained largely unaffected. Moreover, *six3* was able to suppress the expanded expression of *bmp4* and *dlx3* caused by the lack of *chordin* function in the anterior region of co-injected embryos (Fig. 6H,L,P,T,X). These data indicate that *six3* can act independently of the BMP inhibitor Chordin, in repressing epidermal fate anteriorly, to specifically promote anterior neural plate development.

Xsix3 directly represses *Bmp4* transcription

Because *Xsix3* works as a transcriptional repressor, we investigated whether *Bmp4* might be a direct target of *Xsix3* and could therefore be repressed by GR-*Xsix3* in the absence of protein synthesis. To achieve this, the protein synthesis inhibitor cycloheximide (CHX) was added to GR-*Xsix3*-injected animal caps before DEX activation.

Injection of GR-*Xsix3* resulted in repression of *Bmp4* expression in animal caps treated with DEX (88%, *n*=62; Fig. 7A), as well as in animal caps treated with both CHX and DEX (90%, *n*=120; Fig. 7A). By contrast, control caps injected with GR-*Xsix3* in the absence of DEX displayed normal *Bmp4* expression (100%, *n*=60; Fig. 7A). Similar results were observed in GR-*Xsix3*-injected caps following CHX but not DEX treatment, suggesting that CHX alone had no effect on *Bmp4* expression (100%, *n*=65; Fig. 7A). As a positive control for the efficiency of CHX treatment, we injected *Xotx2*-GR and analysed the expression of *Xcg* and *Xag*, which are known to be direct and indirect *Xotx2* targets, respectively (Gammill and Sive, 1997). As expected, although DEX treatment of injected embryos activated both *Xcg* and *Xag*, addition of DEX and CHX led to the activation of *Xcg* but not of *Xag* (Fig. 7B).

To directly test the interaction of Xsix3 with the regulatory regions of the *Bmp4* gene, we performed an electrophoretic mobility shift assay (EMSA; Fig. 7C). We found that the Xsix3-GST fusion protein binds to a ^{32}P-labelled 315 bp fragment of the *Bmp4* promoter, which displays putative binding sites for

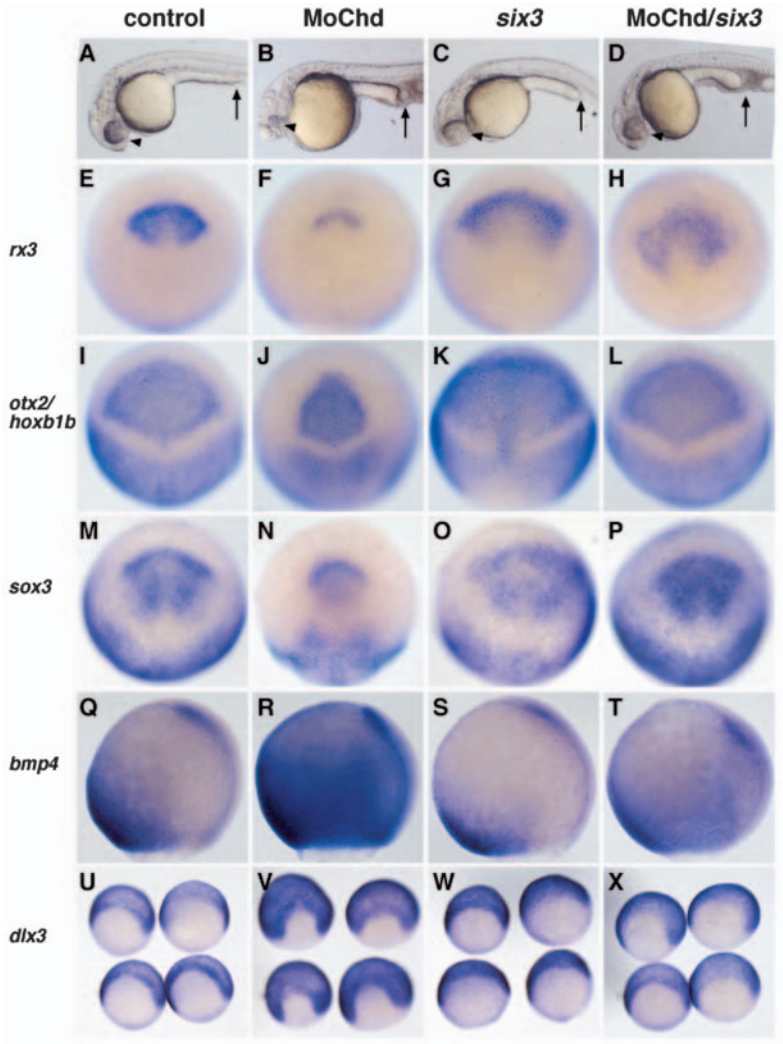

Fig. 6. *six3* rescues *chordin* loss of function. (A-D) Lateral view of 24 hpf zebrafish embryos. Arrowheads indicate the eye/brain size in control (A), and in embryos injected with *MoChd* (B), *six3* (C), or with both *six3* and *MoChd* (D). Arrows indicate the posterior region, which is expanded in embryos injected with *MoChd*, or co-injected with *MoChd* and *six3*. (E-X) Early neurula embryos (90% epiboly) treated as in A-D and analysed for expression of the genes indicated. (E-P,U-X) Dorsal-anterior views, vegetal towards the bottom; (Q-T) lateral views, anterior towards the top.

homeodomain-containing proteins and was previously shown to interact with Xiro1 (Gomez-Skarmeta et al., 2001). The Xsix3-GST protein binds to the *Bmp4* promoter fragment in a concentration-dependent manner forming several complexes. This binding, which is not observed with the GST protein alone, is specifically competed by an excess of the unlabelled fragment (Fig. 7C). Thus both in vivo and biochemical assays suggest that Xsix3 directly represses *Bmp4* transcription.

Discussion

In this study, we show that exogenous *Six3* expands the anterior neural plate in zebrafish and *Xenopus*. In part, this is due to increased proliferation of neural progenitor cells. However, we show that *Six3* can still induce neural plate expansion in the presence of cell cycle inhibitors. Accordingly, we indicate that *Xsix3* acts as a transcriptional repressor in the specification of the anterior neural plate and we identify *Bmp4* as a direct downstream target of *Six3*. Gain- and loss-of-function experiments show that *Six3* represses *Bmp4* expression, thus promoting neural fate at the expense of neural crest and epidermis. Altogether, these data indicate that *Six3* plays a crucial role in controlling cell fate specification and cell proliferation in the anterior neural plate.

Xsix3 promotes cell proliferation and delays cell differentiation in the neural plate

The ability of Six3 to induce proliferation and inhibit neuronal differentiation may involve interactions with different cofactors in the neural plate. Despite the anterior restricted expression of *Xsix3*, both anterior and posterior regions of the neural plate are competent to respond to *Xsix3*. This suggests that cofactors required for *Xsix3* proliferative activity are expressed throughout the neural plate. The recently isolated Six3 cofactors can be grouped into two categories underscoring different mechanisms of action for Six3. One group includes the groucho family, NeuroD and ATH3/ATH5, which contribute to the specificity of *Six3* transcriptional activity (Kobayashi et al., 2001; Zhu et al., 2002; Tessmar et al., 2002; Lopez-Rios et al., 2003). The second category of Six3 interactors is represented by Geminin. Six3 displaces the DNA replication-inhibitor Geminin from Cdt1 resulting in activation of cell proliferation in a transcriptional-independent manner (Del Bene et al., 2004). Both types of interactors are expressed along the entire neural plate thus being potentially available for cooperation with injected *Xsix3*. Although the Six3/Geminin complex may contribute to the enhanced cell proliferation elicited by Six3, this protein is likely to control cell proliferation and neurogenesis by also acting as a transcription factor. Indeed, *Xsix3* misexpression expands the expression of *Zic2*, *Xhairy2* and *cyclinD1*, while repressing that of *p27Xic1*, *N-tubulin* and *elrC*. These effects are unlikely to be explained as a consequence of cell proliferation induced by Six3/Geminin interaction. Indeed, *Xsix3* is able to regulate the expression of the same genes even when cell proliferation is blocked (Fig. 3K,L and data not

Table 1. Concentration-dependent rescue of loss of *chordin* function by *Six3* overexpression

	Wild type	Small eye	Big eye/brain	Anterior rescue	Others	*n*
MoChd (1 nl; 0.15 mM)	5%	85%	0%	–	10%	40
Six3: 19 pg	90%	0%	0%	–	10%	49
+ MoChd (1 nl; 0.15 mM)	9%	38%	3%	38%	12%	34
Six3: 37 pg	16%	0%	77%	–	7%	57
+ MoChd (1 nl; 0.15 mM)	13%	22%	0%	48%	17%	23
Six3: 74 pg	25%	0%	57%	–	18%	67
+ MoChd (1 nl; 0.15 mM)	10%	10%	48%	15%	17%	52
+ MoChd (2 nl; 0.15 mM)	2%	7%	54%	18%	19%	107
Six3: 74 pg in dino mutant	0%	26%	63%	11%	0%	19

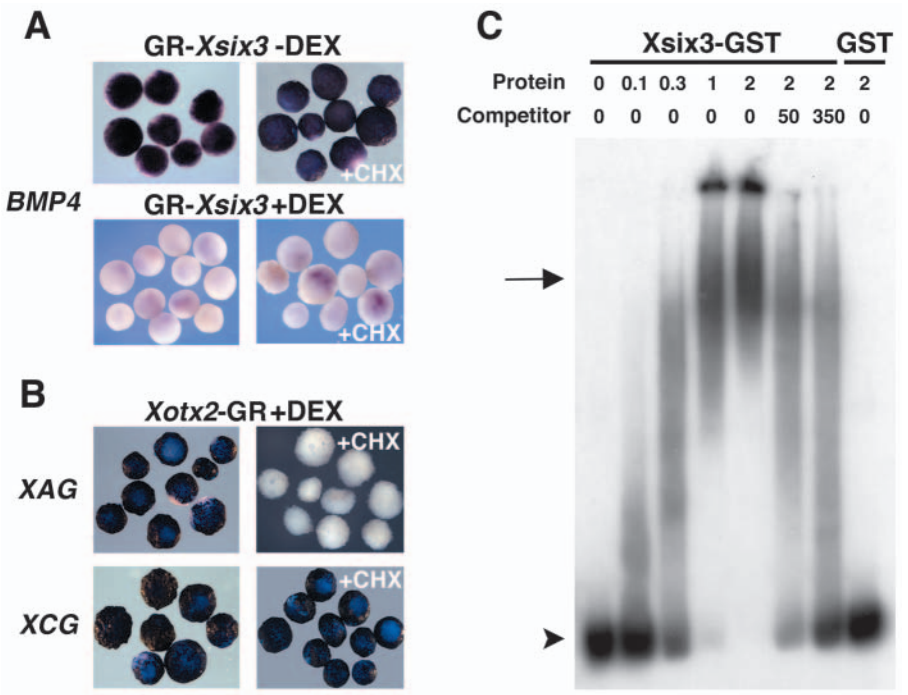

Fig. 7. *Xsix3* directly represses *Bmp4* transcription. (A,B) Animal caps were analysed at stage 13 for the expression of the genes indicated to the left. (A) *Bmp4* repression in GR-*Xsix3*-injected animal caps treated either with dexamethasone (DEX) alone, or with both DEX and cycloheximide (CHX), compare with control with no DEX treatment shown above. (B) Control of CHX treatment. *Xotx2*-GR-injected animal caps treated with DEX alone, or with both DEX and CHX. (C) Xsix3 binds to a *Bmp4* promoter fragment containing homeodomain-binding sites. Xsix3-GST, but not GST alone, binds to the labelled *Bmp4* promoter to form various complexes in a concentration-dependent manner. Addition of an excess of unlabelled *Bmp4* promoter fragment (50- to 350-fold) significantly inhibits the binding. The arrow indicates the major complex formed with the highest protein concentration. The arrowhead indicates the free probe. The amount of added protein is indicated in micrograms.

shown). Reduction of cell proliferation in VP16-*Xsix3*-injected embryos provides additional evidence that *Xsix3* regulates cell proliferation at the transcriptional level.

Six3 represses *Bmp4* expression

A prerequisite for dorsal ectoderm to acquire neural fate is the inhibition of BMP signalling, and one of the ways in which this is accomplished is through the transcriptional inhibition of BMP genes in the nascent neural plate (reviewed by Bally-Cuif and Hammerschmidt, 2003). Prior to gastrulation, *Bmp4* is expressed throughout the embryo, but begins to be cleared from the prospective neural plate soon after *Xsix3* activation at gastrula stage, which would be consistent with a role for *Xsix3* in repressing *Bmp4* at these early stages (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Zuber et al., 2003). Indeed, our overexpression experiments in both embryos and animal caps provide compelling evidence for a mutual antagonism between *Six3* and *Bmp4*. In addition, the reduction of neural plate size elicited by loss of *Xsix3* function is significantly alleviated by attenuation of BMP signalling. Moreover, our study in zebrafish shows that *six3* is able to compensate for the lack of the BMP antagonist *chordin*, to specify the size of the anterior neural plate. Because *chordin* is not required for *six3* induction (data not shown), it is likely that these two genes act in parallel to exclude *Bmp4* from the anterior neural plate.

Bmp4 transcriptional repression is not sufficient to elicit neural induction

Although *Six3* is able to expand the expression domains of the neural markers *Xsox2*, *Xnrp1*, *otx2* and *sox3*, we never observed ectopic expression of these genes in the ventral ectoderm, where *Six3* represses the epidermal marker *Xk81*. Furthermore, we show that in animal caps *Xsix3* does not activate the expression of neural markers, despite being able to repress epidermal markers. This suggests that *Xsix3* activity alone is not sufficient to respecify non-neural ectoderm to a neural fate. Indeed, we found that neural markers are induced only if *Xotx2* is co-injected with *Xsix3*, indicating that these two genes cooperate in neural fate determination. These data suggest that an additional step besides *Bmp4* transcriptional inhibition might be required to drive unspecified ectodermal cells towards a neural fate. A number of genes expressed in the presumptive neuroectoderm and involved in promoting neural fate (e.g. *Xanf1*, *Opl* and *Sox2*) have been described (Kuo et al., 1998; Mizuseki et al., 1998; Ermakova et al., 1999). These genes share the inability to induce neural tissue in animal caps. Because for most of these genes their effect on *Bmp4* expression has not been tested, at the moment it is not clear how common is the ability of repressing *Bmp4* without inducing neural fate in competent ectoderm. However, it still remains to be clarified whether the ability of factors like *Xiro2*, *Soxd* and *Zic3* to neuralise competent ectoderm is simply the result of the suppression of *Bmp4* expression, as has been previously reported for *Geminin* and *Xiro1* (Nakata et al., 1997; Gomez-Skarmeta et al., 1998; Gomez-Skarmeta et al., 2001; Mizuseki et al., 1998; Kroll et al., 1998). Moreover, even though *Xiro1* can directly repress *Bmp4*, Gomez-Skarmeta and co-workers suggested that this protein may repress additional uncharacterized factors to neuralise the ectoderm (Gomez-Skarmeta et al., 2001).

Xsix3 controls anterior neural fate, at least in part, independently from *Geminin*

The recently demonstrated interaction between Six3 and Geminin opens the possibility that *Xsix3* may play a role in neural fate determination in a Geminin-dependent way. However, Six3 and Geminin activities in the neural plate appear to be distinct. Geminin is a coiled-coil protein, with two separable functional domains, one of which neuralises ectoderm, whereas the other is involved in the inhibition of DNA replication (Kroll et al., 1998; McGarry and Kirschner,

1998). Thus, Geminin and Six3 have antagonistic functions in the control of cell proliferation; by contrast, they share the ability to repress *Bmp4* and to promote neural plate expansion in *Xenopus*. However, unlike *Xsix3*, *Geminin* has the ability to induce neural tissue in animal caps. Moreover, differently from the anterior restricted effects of *Xsix3*, *Geminin* induces neural tissue of posterior but not anterior character in animal caps. Finally, although the DNA-binding activity of Six3 is dispensable for the interaction with Geminin, mutagenesis of Six3/Groucho binding sites, which impairs the transcriptional repressor activity of Six3, completely abolishes its in vivo function (Kobayashi et al., 2001; Zhu et al., 2002). Altogether, these data indicate that *Six3* may function in different pathways either acting through the interaction with Geminin, or controlling the transcription of key regulators of proliferation and anterior neural plate specification.

A crucial role for *Six3* in anterior neural plate specification

Six3 plays a dual role in the anterior neural plate controlling proliferation and neurogenesis, and protecting the anterior neuroectoderm from the ventralizing activity of BMPs. The fate of amphibian presumptive neuroectoderm is reversible during early gastrula stages and can be changed to epidermis by transplantation to the ventral side (Spemann, 1938). Although by the end of gastrulation, the neuroectoderm has little competence left to form epidermis in transplants, epidermalising factors surrounding the neural plate maintain their ability to inhibit neural plate genes (Hartley et al., 2001). *Six3* is activated by the neural inducers Noggin, Chordin and β-catenin, and begins to be expressed at a high level at mid-gastrula stage, when it is likely to modulate the responsiveness of neuroectodermal cells rather than the initial fate decisions of uncommitted ectodermal cells (Bernier et al., 2000; Zuber et al., 2003; Kuroda et al., 2004) (this work). Thus, by repressing *Bmp4* expression, *Xsix3* might maintain the competence of neuroectodermal cells to form the anterior neural plate.

These data, together with the observation that *Six3* also counteracts Wnt signalling (Lagutin et al., 2003) and promotes cell proliferation, suggests that *Six3* links cell specification and proliferation to maintain and refine anterior identity.

We dedicate this paper to Giovanni Riccio.

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