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Eya1 and *Six1* promote neurogenesis in the cranial placodes in a *SoxB1*-dependent fashion

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ABSTRACT

Genes of the *Eya* family and of the *Six1/2* subfamily are expressed throughout development of vertebrate cranial placodes and are required for their differentiation into ganglia and sense organs. How they regulate placodal neurogenesis, however, remains unclear. Through loss of function studies in *Xenopus* we show that *Eya1* and *Six1* are required for neuronal differentiation in all neurogenic placodes. The effects of overexpression of *Eya1* or *Six1* are dose dependent. At higher levels, *Eya1* and *Six1* expand the expression of *SoxB1* genes (*Sox2*, *Sox3*), maintain cells in a proliferative state and block expression of neuronal determination and differentiation genes. At lower levels, *Eya1* and *Six1* promote neuronal differentiation, acting downstream of and/or parallel to *Ngnr1*. Our findings suggest that *Eya1* and *Six1* are required for both the regulation of placodal neuronal progenitor proliferation, through their effects on *SoxB1* expression, and subsequent neuronal differentiation.

Introduction

The cranial placodes, which contribute to the cranial ganglia and sense organs of the vertebrate head, originate from the pre-placodal ectoderm that surrounds the anterior neural plate (reviewed in Baker and Bronner-Fraser, 2001; Bailey and Streit, 2006; Schlosser, 2006). Genes of the *Eya* family and the *Six1/2* and *Six4/5* subfamilies are specifically expressed in this pre-placodal ectoderm and continue to be expressed later in various placodes (Pandur and Moody, 2000; Ghanbari et al., 2001; David et al., 2001). Six proteins bind directly to DNA to regulate gene transcription; interactions with various cofactors including Eya modulate their activity (Pignoni et al., 1997; Ohto et al., 1999; Ikeda et al., 2002). Mutants or morphants of *Six1* and *Eya1* in mice, humans and zebrafish display similar developmental deficits affecting multiple placodal derivatives (Xu et al., 1999, Zheng et al., 2003; Li et al., 2003; Laclef et al., 2003; Zou et al., 2004; Ozaki et al., 2004; Friedman et al., 2005; Kozlowski et al., 2005; Bricaud and Collazo, 2006; Ikeda et al., 2007). In *Xenopus*, *Six1* is required for the formation of the pre-placodal ectoderm (Brugmann et al., 2004). While these mutant phenotypes suggest that *Six1* and *Eya1* play a central role in the regulation of placodal neurogenesis the underlying mechanisms remain poorly understood.

Regulation of neurogenesis is particularly well studied in the *Xenopus* neural plate (reviewed in Bertrand et al., 2002). Neuronal differentiation is initiated by neuronal determination (proneural) genes coding for basic helix loop helix (bHLH) transcription factors, such as the neurogenin related gene *Ngnr1* (Ma et al., 1996). These are transiently expressed in proliferating neural progenitors, and promote cell cycle exit and the expression of bHLH neuronal differentiation genes such as *NeuroD* (Ma et al., 1996; Farah et al., 2000). Proneural proteins also induce expression of ligands of the Notch pathway such as Delta1, which prevent neighboring cells from adopting a neuronal fate (lateral inhibition; Chitnis et al., 1995; Ma et al., 1996). Cell cycle exit is required

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for the progression of neuronal differentiation and is regulated by inhibitors of cyclin dependent kinases including $p27^{Xic1}$ (Hardcastle and Papalopulu, 2000; Carruthers et al 2003). $p27^{Xic1}$ also promotes neuronal differentiation more directly, possibly by stabilizing Ngnr1 protein (Vernon et al., 2003; Nguyen et al., 2006). Neuronal differentiation genes then activate batteries of neuron-specific genes including *N-Tubulin* (Lee et al., 1995). However, neuronal differentiation occurs only in a small subpopulation of neural plate cells; most cells are kept in a proliferative progenitor state where differentiation is blocked by various transcription factors including Sox1, Sox2 and Sox3, members of the SoxB1 subfamily of HMG box containing transcription factors (Bylund et al., 2003; Graham et al., 2003). In addition, *SoxB1* genes bias lineage choices of progenitor cells towards a neural or neuronal fate (Mizuseki et al., 1998; Pevny et al., 1998; Kishi et al., 2000; Zhao et al., 2004; Kan et al., 2004, 2007; Wang et al., 2006).

Neurogenic placodes express many of the same genes that regulate neurogenesis in the neural plate (Schlosser and Northcutt, 2000; Abu-Elmagd et al., 2001; Vernon et al., 2003; Schlosser and Ahrens, 2004). *Ngn1*, *Ngn2* and *NeuroD* act as neuronal determination and differentiation genes, respectively, in some placodes (Ma et al., 1998; Fode et al., 1998; Liu et al., 2000; Kim et al., 2001). The phenotypes of mutants or morphants of *Eya1* and *Six1* suggest that these genes are required both for the proliferation and survival of placodal neuronal progenitors (Zheng et al., 2003; Li et al., 2003; Bricaud and Collazo, 2006) as well as for the proper expression of neuronal determination and differentiation genes (Zou et al., 2004; Friedman et al., 2005; Bricaud and Collazo, 2006; Ikeda et al., 2007). However, it is unclear which target genes mediate the various effects of *Eya1* and *Six1*. Here we use gain- and loss-of-function approaches in *Xenopus* to address this question. We show that high levels of expression of *Eya1* and/or *Six1* promote proliferating neuronal progenitors via activation of *SoxB1* genes, while at lower levels they permit cell cycle exit and promote neuronal differentiation downstream of and/or parallel to *Ngnr1*. Our findings indicate that *Eya1* and *Six1* are required during multiple steps of placodal neurogenesis.

Materials and methods

Expression constructs

Six1, *Eya1*, *Sox3* and *GR–Sox3* mRNAs were made from pCS2$^+$-Eya1α (Ahrens and Schlosser, 2005), pDH105-Six1 (Pandur and Moody, 2000), pCS2-Sox3-V5His (Zhang et al., 2003) and pCS2-GR-Sox3-GFP (Zhang and Klymkowsky, 2007) plasmids. Myc-tagged *Eya1* mRNA (*myc-Eya*) was made from pCS2$^+$-myc-Eya1α generated by releasing the insert from pT-Adv-Eya1α (David et al., 2001) with EcoRI and subcloning it downstream of the myc-tag of pCS2$^+$-myc. The construct was verified in immunoblot analyses following in vitro transcription and translation (TNT-coupled reticulocyte lysate kit, Promega) using a mouse anti-c-myc antibody (9E10, Developmental Studies Hybridoma Bank). Myc-tagged or untagged versions of *Eya1* mRNA had identical effects on all markers analyzed.

To generate a hormone-inducible *Eya1* expression vector (pCS2$^+$-GR-myc-Eya1α), the ligand-binding domain of the human glucocorticoid receptor (GR) plus the myc-tag were released by digesting pCS2$^+$-GR-myc (Hutcheson and Vetter, 2001) with BamHI and NcoI. This fragment was inserted into the BamHI/NcoI site upstream of *Xenopus Eya1* replacing the myc-tag in the pCS2$^+$-myc-Eya1α plasmid. To generate a hormone-inducible *Six1* expression vector (pCS2$^+$-GR-myc-Six1), the full open-reading frame of *Xenopus Six1* was generated by PCR with primers containing XhoI sites and the pDH105-Six1 plasmid (Brugmann et al., 2004). The *Six1* fragment was inserted into the XhoI site

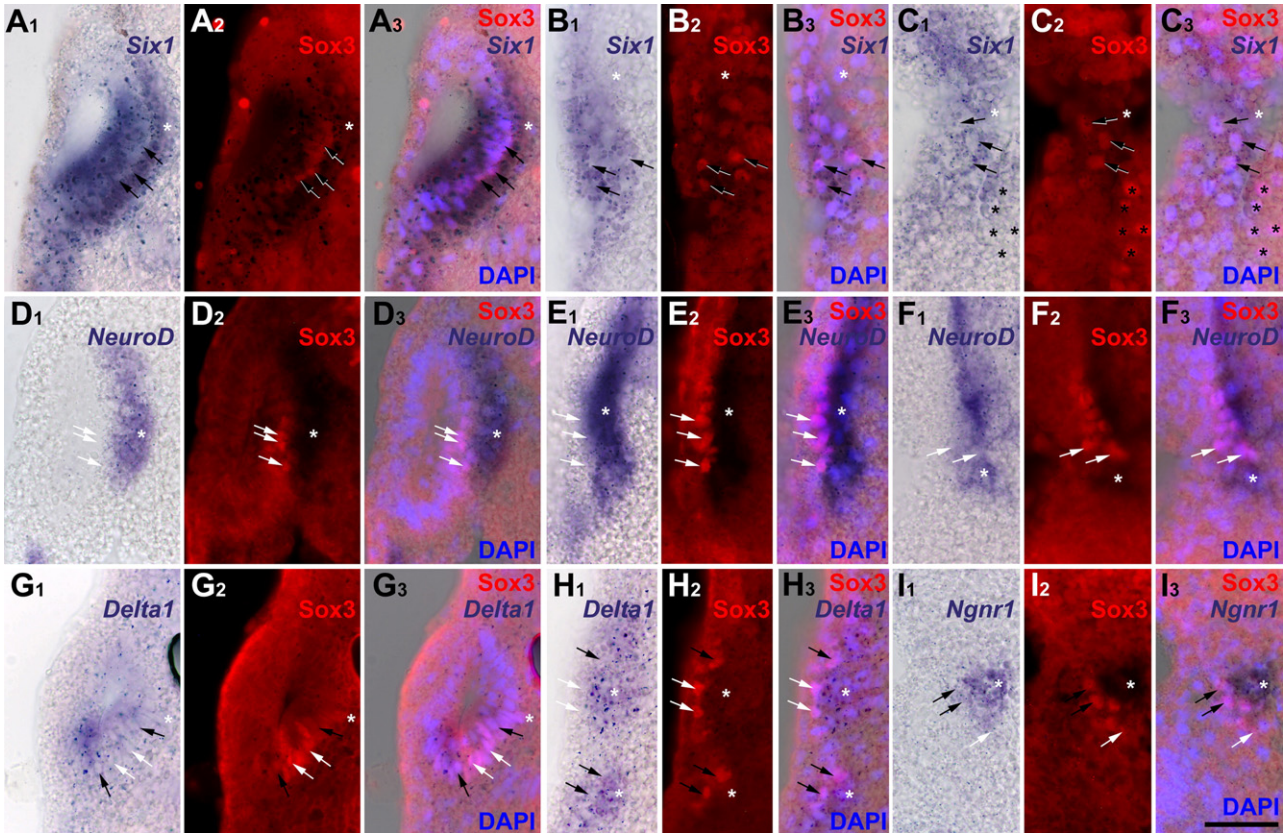

Fig. 1. Placodal distribution of Sox3-immunopositive cells in relation to *Six1*, *NeuroD*, *Delta1* and *Ngnr1* expression in transverse sections through stage 26 embryos. Each section is shown in bright field (A_1–I_1: gene expression), red fluorescence (A_2–I_2: Sox3 protein), and a superposition of these two with blue DAPI fluorescence (A_3–I_3). White asterisks indicate placodally derived cranial ganglia (main body of ganglia out of level of section in panels B, C and G). Black asterisks mark endodermal Sox3 nuclei. (A–C) *Six1* expression domains in otic vesicle (A), anterodorsal lateral line (B) and facial epibranchial (C) placodes encompass Sox3 immunopositive nuclei (arrows). (D–F) *NeuroD* is expressed in ganglia derived from otic vesicle (D), anterodorsal lateral line (E) and facial epibranchial placodes (F) immediately abutting Sox3-positive placodal cells (arrows). (G–I) *Delta1* expression in the otic vesicle (G), and the anterodorsal lateral line and glossopharyngeal epibranchial placodes and ganglia (H upper and lower part, respectively) and *Ngnr1* expression in the facial epibranchial placode (I) overlap with some (black arrows) but not all (white arrows) Sox3-cells. Bar in I: 50 μm (for all panels).

downstream of GR-myc in pCS2$^+$-GR-myc. Both plasmids were confirmed by sequencing and used as templates to generate mRNAs (*GR–Six1* and *GR–Eya1*).

To ensure that GR-fusion constructs function comparable to wild-type protein, injected embryos were cultured in dexamethasone (DEX) (10 μM) immediately after mRNA injection. For each construct these embryos displayed nearly the identical phenotype as those injected with the wild-type mRNAs (cf. Supplemental Table S1 to Table 2 and Brugmann et al., 2004). Embryos injected with GR-constructs and raised in the absence of DEX showed minimal effects (Supplemental Table S1) in accord with published accounts of this method (Hollenberg et al., 1993; Mattioni et al., 1994, Kolm and Sive, 1995; de Graaf et al., 1998). DEX treatment alone does not significantly alter gene expression (Supplemental Table S1).

Morpholinos

Morpholino antisense oligonucleotides (MO) against two different potential translational start sites in *Six1* (*Six1*MO1: 5′-GGAAGGCAGCATAGACATGGCTCAG-3′; *Six1*MO2: 5′-CGCACACGCAAACACATACACGGG-3′; both lissamine-tagged) were previously described and analyzed for specificity (Brugmann et al., 2004). Two different MOs against *Eya1* were generated (GeneTools): *Eya1*MO1 (5′-TACTATGTGGACTGGTTAGATCCTG-3′) targeted base pairs 10 to 34 of the *Eya1* coding region, whereas *Eya1*MO2 (5′-ATATTTGTTCTGTCAGTGGCAAGTC-3′) was directed against base pairs −7 to −31 in the 5′ UTR of *Eya1* (EST BJ066588, Genbank). The efficacy of Eya1MO1 was verified in western blots following in vitro transcription and translation (TNT-coupled reticulocyte lysate kit, Promega) of pCS2$^+$-Eya1α (1 μg/25 μl reaction) with and without MO (1 μg of MO/25 μl reaction) using guinea pig anti-*Xenopus*-Eya1 antibody as previously described (Ahrens and Schlosser, 2005). MOs directed against the Sox2 (5′-CTCCATCATGCTGTACATGCAGGCG-3′) and Sox3 (5′-AACATGCTATACATTTGGAGCTTCA-3′) UTR/coding regions (Supplemental Fig. S1) were synthesized (GeneTools). To evaluate MO efficacy and specificity, epitope-tagged 5′ UTR-Sox2 and Sox3 constructs (pCS2-utr-Sox3-V5 and pCS2-utr-Sox2-V5) that include the sequences targeted by the Sox2MO and Sox3MO, respectively, were generated by PCR. A plasmid encoding ΔNXTCF3 (Molenaar et al., 1996) was supplied by Oliver Destree. mRNAs synthesized from pCS2-

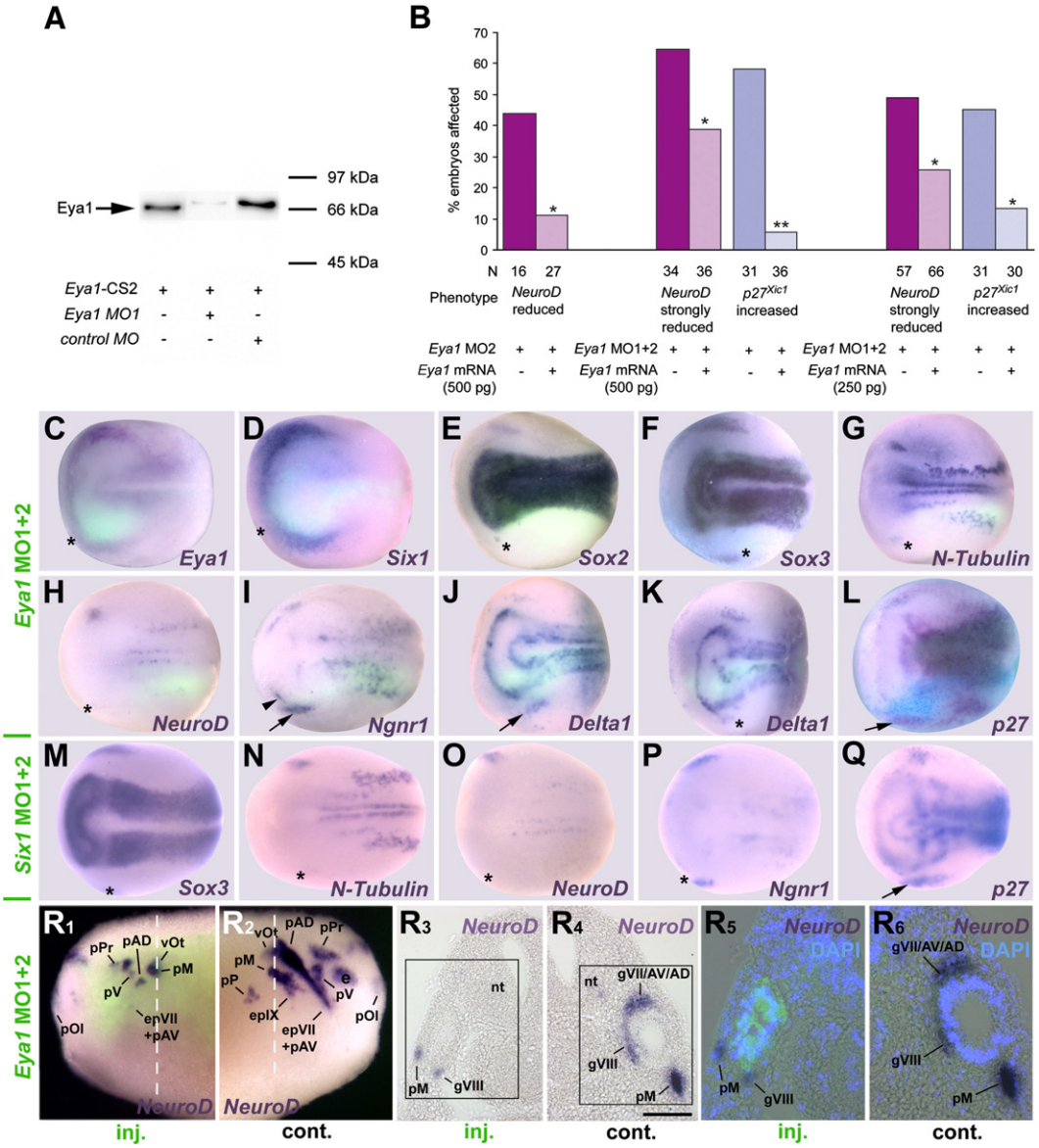

Fig. 2. Effects of *Eya1* and *Six1* knockdown on markers of neurogenesis and placodal ectoderm. (A) Immunoblot showing that *Eya1*MO1 but not control MO blocks synthesis of Eya1 protein. (B) Co-injecting *Eya1*MO2 or *Eya1*MO1+2 with myc-tagged *Eya1* mRNA significantly restores *NeuroD* and prevents ectopic *p27*^{Xic1} expression (X^2 test; *: $p<0.05$, **: $p<0.001$) in three independent rescue experiments. (C–Q) Neural plate stage embryos after unilateral injection (lower half) of *Eya1*MO1+2 (C–L) or *Six1*MO1+2 (M–Q). In all cases, where *myc-GFP* was co-injected as lineage tracer (C–K), embryos are shown superimposed with green fluorescent channel. Asterisks indicate reductions, arrows and arrowheads indicate increased marker gene expression. For *Delta1* after *Eya1*MO1+2 injections two different phenotypes are depicted (J, K). R: Tail bud stage embryo after unilateral injection (R_1, R_3, R_5: injected side; R_2, R_4, R_6: control side) of *Eya1*MO1+2 reveals reduction of *NeuroD* expression in all neurogenic placodes or derivative ganglia. R_3 and R_4 depict a section at the level indicated (white line) with boxed areas shown magnified in R_5 and R_6, respectively, superimposed with green (*myc-GFP* co-injected with *Eya1*MO1+2) and blue (DAPI) fluorescence. Residual *NeuroD* expression is confined to cells receiving little or no MO. Bar in R_4: 100 μm (for R_3, R_4). Abbreviations: e: eye; epVII+AV: facial epibranchial and anteroventral lateral line placode; epIX: glossopharyngeal epibranchial placode; gVII/AV/AD: ganglion of the facial, anteroventral and anterodorsal lateral line nerve; gVIII: vestibulocochlear ganglion; nt: neural tube; pAD: anterodorsal lateral line placode; pM: middle lateral line placode; pOl: olfactory placode; pP: posterior lateral line placode; pPr: profundal placode; pV: trigeminal placode; vOt: otic vesicle.

utr-Sox3-V5 and pCS2-utr-Sox2-V5 were injected into fertilized eggs (650 pg) together with Sox2 or Sox3 MOs (20 ng) and proteins were analyzed in western blots using anti-V5 and anti-XTCF3c antibodies as previously described (Zhang et al., 2003). A standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′) obtained from GeneTools was used in control injections.

Microinjections

Embryos of *Xenopus laevis* were staged according to Nieuwkoop and Faber (1967) and injected according to standard procedures (Sive et al., 2000). Capped mRNAs were synthesized with Message Machine Kit (Ambion) and injected into single blastomeres at the 2- to 16-cell stage that give rise to the dorsal ectoderm. Unless otherwise noted, the following amounts of mRNAs were injected: *Eya1* or *myc-Eya1*: 500–1000 pg; *Six1*: 250–500 pg; *GR–Eya1*: 400–500 pg; *GR–Six1*: 400–500 pg; *Sox3*: 500 pg; *GR–Sox3*: 500 pg. Morpholinos (see above) were injected singly or as a cocktail (20 ng each) into single blastomeres at the 2–16 cell stage. For rescue experiments, *Eya1MO2* alone or in combination with *Eya1MO1* (20 ng each) were co-injected with *myc-Eya1* (250–500 pg). *GR–Eya1+GR–Six1* (500 pg each) were co-injected with a combination of *Sox2MO* and *Sox3MO* (12–24 ng each). In injections of non-tagged mRNA or morpholinos, co-injection of *myc-GFP* (125–250 pg) (pCMTEGFP; kindly provided by Doris Wedlich) or *lacZ* (250 pg) identified the injected side. For activation of hormone-inducible constructs, embryos were incubated in DEX (10 μM; Sigma) from stages 16–18 onwards. To block protein synthesis, cycloheximide-treatment (50 μg/ml; Sigma) at stage 16–18 was followed after 30′ by incubation in cycloheximide supplemented with DEX (10 μM) for 3–4 h at room temperature.

Tissue grafts

Grafts were performed as previously described (Ahrens and Schlosser, 2005). The lateral placodal region of neural plate stage albino embryos injected unilaterally with *Eya1* (1000 pg) and *myc-GFP* (250 pg) at the 2-cell stage, was grafted orthotopically into pigmented host embryos of the same stage. Uninjected donor embryos were used in control experiments.

In situ hybridization and immunohistochemistry

Embryos injected with *myc-GFP* or with tagged morpholinos were sorted under a fluorescent stereomicroscope and then fixed according to standard procedures (Sive et al., 2000). *LacZ* injected embryos were fixed and then stained to reveal lacZ. Wholemount in situ hybridization was carried out as previously described (Schlosser and Ahrens, 2004) using digoxigenin-labeled antisense probes for *Eya1* (David et al., 2001), *Six1* (Pandur and Moody, 2000), *Sox3* (Penzel et al., 1997), *Sox2* (De Robertis et al., 1997), *N-Tubulin* (Oschwald et al., 1991), *NeuroD* (Lee et al., 1995), *Ngnr1* (Ma et al., 1996), *Delta1* (Chitnis et al., 1995), $p27^{Xic1}$ (Ohnuma et al., 1999), and *CyclinA1* (Carter et al., 2006).

After in situ hybridization, myc-tagged proteins were revealed immunohistochemically using mouse anti-c-myc antibody (9E10, Developmental Studies Hybridoma Bank) as previously described (Ahrens and Schlosser, 2005). Phosphohistone H3 (PH3) was identified with a rabbit anti-PH3 antibody (06-570, Upstate; 1:100) and revealed with either anti-rabbit-TRITC (T-6778, Sigma, 1:100) or anti-rabbit-IgG-HRP (111-035-003, Jackson ImmunoResearch, 1:200) followed by reaction in diaminobenzidine (Dent et al., 1989). PH3-immunopositive cells were counted in areas of defined size in the lateral placodal ectoderm of injected embryos. Uninjected sides of these embryos or *lacZ* injected embryos served as control.

Vibratome sections (30 μm) were cut after whole mount in situ hybridization. Immunohistochemistry on sections for myc-tagged proteins or Sox3 was performed as previously described (Schlosser and Ahrens, 2004). Sox3 was revealed using a polyclonal rabbit anti-Sox3 antibody (Zhang et al., 2003; 1:1000) and a TRITC-conjugated anti-rabbit-IgG antibody (T-6778, Sigma, 1:100). PCNA immunohistochemistry followed published protocols (Wullimann et al., 2005) except that anti-mouse-IgG2a-TXRD (1080-07, Southern Biotechnol., 1: 100) was used as secondary antibody. Secondary antibodies were sometimes coincubated with DAPI (100 ng/μl). Nonspecific binding of secondary antibodies was not observed when the primary antibody was omitted in control reactions.

Results

Placodal neurons differentiate adjacent to Eya1 and Six1 expression domains

As the pre-placodal ectoderm begins to separate into discrete placodes, it maintains expression of *Six1* and *Eya1*. Their expression weakens in delaminating ganglion cells, which express several genes regulating neurogenesis. (Pandur and Moody, 2000; Schlosser and Northcutt, 2000; David et al., 2001). To determine the progression of gene expression in placodal cells, we combined Sox3-immunostaining with in situ hybridization for *Eya1*, *Six1*, *NeuroD*, *Ngnr1* or *Delta1* (Fig. 1). Sox3-immunopositive nuclei were present in all neurogenic placodes except the profundal and trigeminal placodes and were always located fully within the expression domains of *Six1* (Figs. 1A–C) and *Eya1* (not shown). However, Sox3+ cells always constituted a subpopulation in these domains. Expression of neurogenesis markers was analyzed in otic, lateral line and epibranchial placodes. Each of these expresses *NeuroD* and *Delta1*, whereas *Ngnr1* is confined to epibranchial placodes. *Delta1* and *Ngnr1* were expressed in both the placodes and in incipient ganglion cells migrating away from the placodes. *Delta1* and *Ngnr1* expressing cells were both adjacent to Sox3+ cells and overlapping with them (Figs. 1G, H), suggesting that: 1) Sox3 expression is upstream of Delta1 and Ngnr1; and 2) cells that are down-regulating Sox3 expression initiate the expression of neuronal determination genes. In contrast, *NeuroD* expression was observed in incipient ganglion cells immediately adjacent to, but not overlapping with, Sox3+ nuclei (Figs. 1D–F). These results indicate that Sox3+ neuronal progenitors arise from the *Eya1* and *Six1*-positive pool, then initiate neuronal determination programs via *Ngnr1* and *Delta1*, but complete neuronal differentiation only after *Eya1*, *Six1* and *Sox3* expression declines.

Eya1 and Six1 are required for placodal neurogenesis

To test whether *Eya1* and/or *Six1* are required for placodal neurogenesis (Fig. 2, Tables 1, 2) we generated morpholino antisense oligonucleotides (MOs) that specifically block translation of *Six1* or *Eya1* mRNAs. The efficacy and specificity of the two *Six1* MOs were previously reported (Brugmann et al., 2004). The efficacy of *Eya1*MO1 was verified in vitro, where it strongly inhibited Eya1 protein synthesis (Fig. 2A). Both *Eya1*MO1 and *Eya1*MO2, when injected individually, affected

Table 1
Changes in marker gene expression in the placodal and non-neural ectoderm after injection of *Eya1* morpholinos

Injection	*Eya1* MO1[a]	*Eya1* MO2[a]
Phenotype	% (n)	% (n)
Eya1		
Reduced	40** (15)	75** (8)
Six1		
Reduced	41* (17)	52** (31)
Sox3		
Reduced	8 (26)	29* (45)
Increased/ectopic	15* (26)	11* (45)
N-Tubulin		
Reduced	58** (40)	73** (41)
Increased/ectopic	0 (40)	3 (41)
NeuroD		
Reduced	71** (38)	68** (79)
Ngnr1		
Reduced	18 (40)	36* (50)
Increased/ectopic	38** (40)	20* (50)
Delta1		
Reduced	42* (19)	50** (32)
Increased/ectopic	16* (19)	3 (32)

n: number of embryos analyzed at neural plate (stage 14–16) and tail bud (stage 21–26) stages.
[a] Significant differences (X^2 test; *: $p<0.05$, **: $p<0.001$) to control MO injections (see Table 2) are indicated.

Table 2
Changes in marker gene expression in the placodal and non-neural ectoderm after injection of various constructs

Injection	Control MO	*Eya1* MO1+2[a]	*Six1* MO1+2[a]	*Sox2*MO+ *Sox3*MO[a]	*Eya*1		*Six1*		*Eya1*+*Six1*		*Sox3*	*GR–Eya1*[b]	*GR–Six1*[b]	*GREya1*+ *GR-Six1*[b]	*GR–Sox3*[b]
Phenotype	% (n)	% (n)	% (n)	% (n)	% (n, np)	% (n, tb)	% (n, np)	% (n, tp)	% (n, np)	% (n, tb)	% (n)	% (n, tb)	% (n, np)	% (n, tb)	% (n, tb)
Eya1															
Reduced	4 (45)	54** (39)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Increased/ectopic	0 (45)	15* (39)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Six1															
Reduced	8 (38)	45** (81)	nd	nd	0 (62)	0 (77)	nd	nd	nd	nd	nd	nd	nd	nd	nd
Increased/ectopic	0 (38)	0 (81)	nd	nd	55 (62)[c]	44 (77)[c]	nd	nd	nd	nd	nd	nd	nd	nd	nd
Sox3															
Reduced	12 (60)	30* (156)	62** (153)	nd	29 (52)	53 (117)	52 (46)	59 (69)	85 (40)	90 (48)	nd	32 (34)	70 (125)	56 (135)	nd
Increased/ectopic	0 (60)	12* (156)	0 (153)	nd	29 (52)	22 (117)	4 (46)	0 (69)	35 (40)	38 (48)	nd	32 (34)	39 (137)	63 (135)	nd
Sox2															
Reduced	6 (32)	58** (51)	54** (26)	nd	30 (63)	50 (38)	86 (28)	71 (68)	42 (26)	36 (25)	68 (34)	35 (40)	58 (106)	46 (52)	nd
Increased/ectopic	0 (32)	26* (31)	0 (26)	nd	38 (63)	37 (38)	0 (28)	6 (33)	46 (26)	59 (32)	9 (34)	68 (40)	28 (114)	69 (52)	nd
N-Tubulin															
Reduced	9 (32)	90** (206)	66** (85)	80** (40)	60 (52)	48 (42)	88 (57)	86 (14)	79 (19)	88 (17)	91 (46)	41 (41)	41 (32)	43 (82)	42 (26)
Increased/ectopic	0 (32)	0 (206)	0 (85)	0 (40)	4 (52)	33 (42)	0 (57)	0 (14)	11 (19)	25 (20)	0 (46)	30 (41)	22 (32)	42 (82)	0 (26)
NeuroD															
Reduced	24 (89)	79** (238)	70** (182)	75** (60)	64 (63)	17 (201)	83 (78)	74 (87)	72 (39)	87 (37)	81 (67)	12 (42)	62 (141)	16 (195)	49 (78)
Increased/ectopic	0 (89)	1 (146)	0 (182)	0 (60)	3 (63)	45 (201)	0 (78)	0 (87)	5 (39)	87 (37)	0 (67)	52 (42)	29 (141)	71 (195)	0 (53)
Ngnr1															
Reduced	10 (41)	21 (116)	45** (75)	38* (37)	44 (50)	50 (117)	75 (68)	57 (62)	76 (45)	86 (21)	45 (51)	27 (33)	57 (136)	61 (79)	67 (30)
Increased/ectopic	0 (41)	34** (116)	36** (75)	38** (37)	14 (50)	9 (117)	4 (68)	14 (52)	4 (45)	0 (21)	18 (51)	24 (33)	5 (168)	18 (79)	0 (30)
Delta1															
Reduced	7 (30)	33* (75)	58** (79)	69** (36)	49 (33)	48 (58)	68 (40)	68 (66)	93 (27)	80 (25)	52 (60)	44 (27)	72 (127)	54 (41)	64 (36)
Increased/ectopic	0 (30)	22* (75)	18* (79)	0 (36)	27 (33)	4 (58)	48 (44)	0 (66)	0 (27)	0 (25)	32 (60)	0 (27)	2 (136)	10 (41)	0 (36)
p27^{Xic1}															
Reduced	17 (35)	22 (72)	29 (59)	42* (33)	43 (115)	76 (72)	75 (60)	82 (71)	80 (10)	nd	78 (55)	35 (161)	84 (83)	39 (209)	24 (46)
Increased/ectopic	0 (35)	51** (72)	34** (59)	49** (33)	48 (115)	20 (110)	15 (40)	0 (71)	0 (10)	nd	38 (55)	29 (161)	8 (49)	50 (209)	17 (46)
CyclinA1															
Reduced	nd	nd	nd	nd	nd	2 (46)	0 (27)	0 (38)	nd	nd	nd	0 (112)	0 (37)	0 (91)	nd
Increased/ectopic	nd	nd	nd	nd	nd	0 (46)	0 (27)	0 (38)	nd	nd	nd	0 (112)	0 (37)	1 (91)	nd

n: number of embryos analyzed at both neural plate (stage 14–16) and tail bud (stage 21–26) stage.
n, np: number of embryos analyzed at neural plate (stage 14–16) stage.
n, tb: number of embryos analyzed at tail bud (stage 21–26) stage.
nd: not determined.
[a] Significant differences (X^2 test; *: $p<0.05$, **: $p<0.001$) to control MO injections are indicated.
[b] Dexamethasone treatment from stages 16–18 on.
[c] Expression broader but weaker.

the expression of a wide range of markers in a similar fashion; co-injection of both MOs produced a higher frequency of stronger phenotypes (Tables 1, 2). Co-injection of a myc-tagged *Eya1* mRNA rescued the effects of *Eya1*MO2 or *Eya1*MO1+2 injection on *NeuroD* and *p27*^{Xic1} expression (Fig. 2B), demonstrating that these MOs specifically block *Eya1* synthesis. Unless otherwise noted, control MOs had no or significantly less frequent effects on the markers analyzed than injections of *Eya1*MO1+2 or *Six1*MO1+2 (Table 2).

Similar to findings after *Six1* loss of function (Brugmann et al., 2004), injection of *Eya1*MOs reduced expression of *Eya1* and *Six1* (Figs. 2C, D; Table 2), demonstrating its requirement for establishing the pre-placodal ectoderm. In addition, injection of either *Eya1*MOs or *Six1*-MOs resulted in significant reduction of placodal neurons as evidenced by reduced *N-Tubulin* and *NeuroD* expression in all neurogenic placodes (Figs. 2G, H, N, O, R), corroborating results in mutant mice that *Eya1* and *Six1* are required for neuronal differentiation in placodes (Zou et al., 2004, Ikeda et al., 2007). We further demonstrate that this requirement is cell-autonomous, since *NeuroD* is reduced in cells containing *Eya1*-MOs (Fig. 2R). We then investigated which neurogenesis genes are involved in the reduction of placodal neurons. Both *Eya1*MOs and *Six1*MOs generally reduced placodal expression of *Sox3* (Figs. 2F, M) and *Sox2* (Fig. 2E), although occasionally *Eya1*MOs led to an expansion

of the *Sox3* and *Sox2* expression domain (Table 2). This was manifested as a broader, but more faintly stained expression domain or scattered ectopic spots in the head. Loss of *Eya1* or *Six1* function often reduced *Ngnr1* (Fig. 2P) and *Delta1* (Fig. 2K) expression domains, likely because *Eya1* and *Six1* are required to establish the placodal ectoderm. However, in about a third of the embryos *Ngnr1* and *Delta1* domains were broadened (Figs. 2I, J), indicating that neuronal determination proceeds more effectively when *Eya1* or *Six1* levels are reduced. *Eya1* loss of function significantly reduced the number of mitotic (PH3-positive) cells (Fig. 9G), while reduction of either *Eya1* or *Six1* led to the expansion of the $p27^{Xic1}$ expression domain (Figs. 2L, Q), suggesting a role in blocking the cell cycle exit that is necessary for neuronal differentiation. These results demonstrate that loss of *Eya1* and *Six1* function interferes with proliferation, *SoxB1* expression and neuronal differentiation but allows neuronal determination to proceed. This suggests a dual requirement for *Eya1* and *Six1* during placodal neurogenesis upstream of *SoxB1* genes as well as downstream of and/or parallel to *Ngnr1* and $p27^{Xic1}$.

Eya1 and Six1 delay the onset of neuronal differentiation

To complement our loss of function studies, we overexpressed myc-tagged and untagged forms of *Eya1* and *Six1* either individually or in combination by mRNA injection (Figs. 3–5, Table 2). Injection of *Six1* and *Eya1+Six1* mRNAs expanded the pre-placodal ectoderm at the expense of neural crest and epidermal genes (Brugmann et al., 2004). Likewise, injection of *Eya1* mRNA led to broader and more diffuse expression of *Six1* in cranial ectoderm (Fig. 3A). However, injection of *Eya1, Six1* or *Eya1+Six1* mRNAs at levels that expand placodal ectoderm tended to repress the expression of genes required for neurogenesis at neural plate stages (Fig. 3, Table 2). Placodal *Sox2* and *Sox3* expression were frequently reduced, in particular after *Six1* mRNA injection (Figs. 3B, C, K, O) while *Eya1* mRNA injection also caused ectopic expression of low levels of *Sox2* and/or *Sox3* in both head and trunk ectoderm (Fig. 3D). *Eya1* and/or *Six1* overexpression reduced placodal expression of *Ngnr1*, *Delta1* and $p27^{Xic1}$ (Figs. 3G, H, I, N). For *Eya1*-injected embryos, *Ngnr1* and *Delta1* occasionally displayed a broader domain of expression, and ectopic $p27^{Xic1}$ expression was observed in head and trunk ectoderm in nearly half of the embryos (Fig. 3J). For *Six1*-injected embryos, *Ngnr1* and $p27^{Xic1}$ were rarely expanded, whereas this phenotype occurred frequently for *Delta1*. Finally, both *N-Tubulin* (Figs. 3E, L) and *NeuroD* (Figs. 3F, M) were strongly reduced by *Eya1, Six1* or *Eya1+Six1*.

To determine whether the effects of *Six1* or *Eya1* overexpression were transient, similarly injected embryos were analyzed at tail bud stages (Fig. 4, Table 2) when most placodes have separated and initiated neuronal differentiation. The effects of *Eya1* and/or *Six1* mRNA injection on *Sox3*, *Sox2*, and *Ngnr1* were similar to effects at neural plate stages (Figs. 4A, B, F, I, K, M). The frequencies at which *Delta1* and $p27^{Xic1}$ were expanded decreased significantly (Figs. 4G, H, L). While *NeuroD* and *N-Tubulin* continued to be frequently reduced, increased or ectopic expression was now also observed after injection of *Eya1* mRNA (Figs. 4C–E, J) in the vicinity of placodes and in spots throughout head ectoderm. After co-injection of *Eya1* and *Six1* mRNAs, ectopic spots of *NeuroD* expression in the cranial ectoderm occurred at high frequency (Figs. 4N, 10B). Often placodal expression of these genes was reduced throughout the region containing the exogenous *Eya1* or *Six1* mRNAs, with ectopic expression occurring at its border (Fig. 4D). These data suggest that increased levels of *Eya1* and *Six1* delay the expression of neuronal determination and differentiation genes, which ultimately results in larger numbers of placodal neurons. Moreover, *Eya1* and *Six1* affect neuronal differentiation synergistically and *Eya1* rather than *Six1* is limiting in vivo because ectopic neurons form only after *Eya1* but not after *Six1* overexpression.

To ensure that these phenotypes are not due to effects in the early embryo, prior to the induction of the pre-placodal ectoderm, we injected mRNAs encoding GR-fusion constructs of Eya1 and Six1 (*GR–Eya1*, *GR–Six1*; see Supplemental Table S1 for validation). These proteins were activated by adding DEX at neural fold stages (stage16–18), when pre-placodal ectoderm has become committed (Ahrens and Schlosser, 2005). Embryos were then analyzed at early tail bud stages. The phenotypes observed were very much as described for wild-type constructs analyzed at tail bud stages (Fig. 5; Table 2). However, injection of *GR–Eya1* or *GR–Six1* led to higher frequencies of ectopic expression of *Sox3* (Fig. 5I) and *Sox2* in head and trunk ectoderm; this was particularly extensive after co-injection of both constructs. Ectopic expression of *Sox2* and *Sox3* were seen only with *GR–Six1* but not wild-type *Six1*. Similarly, ectopic expression of *N-Tubulin* and *NeuroD* was only seen after *GR–Six1* but not after wild-type *Six1* injection.

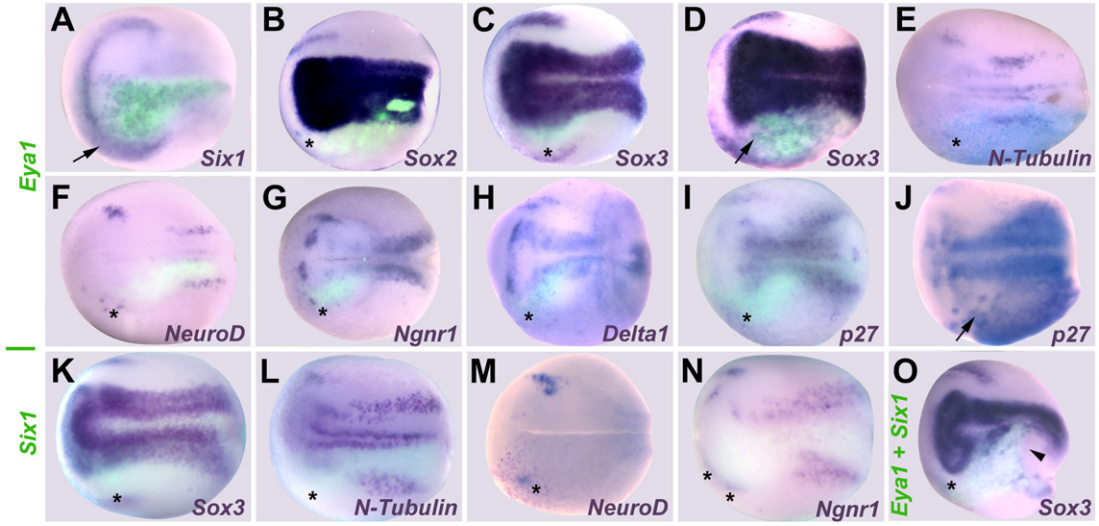

Fig. 3. Effects of *Eya1* and *Six1* overexpression on markers of neurogenesis and placodal ectoderm at neural plate stages. Embryos after unilateral injection (lower half) of *Eya1* (A–J), *Six1* (K–N) or *Eya1+Six1* (O) mRNA. In all cases, where *myc-GFP* was co-injected as lineage tracer (A–D, F–I, K, L, N, O), embryos are shown superimposed with green fluorescent channel. Black asterisks indicate reductions in the placodal or non-neural ectoderm. Arrows mark increased or ectopic marker gene expression in the placodal or non-neural ectoderm; black arrowhead identifies deformed blastopore due to gastrulation defects (O). For *Sox3* (C, D) and $p27^{Xic1}$ (I, J) after *Eya1* injections two different phenotypes are depicted.

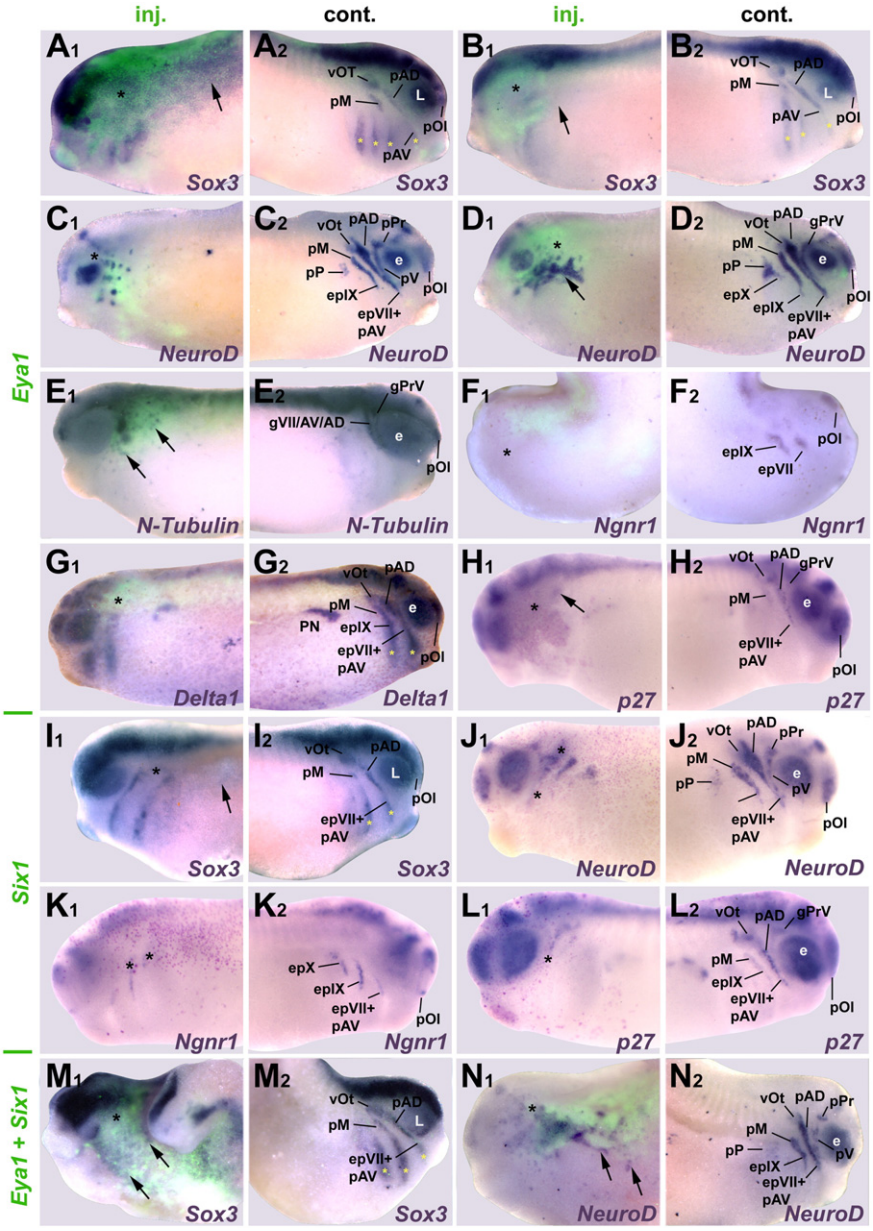

Fig. 4. Effects of *Eya1*, *Six1* and *Eya1+Six1* overexpression on various neurogenesis markers at tail bud stages (stage 26). Injected (A_1–N_1) and control (A_2–N_2) sides of tail bud stage embryos after unilateral injection of *Eya1* (A–H), *Six1* (I–L) or *Eya1+Six1* (M, N) mRNAs. In all cases, where *myc-GFP* was co-injected as lineage tracer (A–G, M–N), embryos are shown superimposed with green fluorescent channel. Black asterisks indicate reductions, while black arrows mark increased or ectopic marker gene expression in the placodal or non-neural ectoderm. Yellow asterisks identify expression in pharyngeal pouches. For *Sox3* (A, B) and *NeuroD* (C, D) after *Eya1* injections two different phenotypes are depicted. Abbreviations as in Fig. 2. gPrV: profundal-trigeminal ganglionic complex; L: lens placode; PN: pronephros.

High levels of Eya1 and Six1 inhibit neuronal differentiation but expand the pool of proliferative SoxB1-positive neuronal progenitors

One explanation for the diverse phenotypes observed involves a threshold effect; at protein levels above the threshold level these proteins act to maintain cells in a *SoxB1*-positive proliferative neuronal progenitor state, whereas below this level ectopic neurons can differentiate from the expanded pool of progenitors. This model was supported by injecting increasing doses of *GR–Eya1+GR–Six1* followed by DEX activation at stage 16–18 (Fig. 6). The frequency of embryos with ectopic *Sox3* expression as well as the extent of ectopic *Sox3* expression increased with increasing doses of injected *Eya1* and *Six1* mRNA (although *Sox3* expression in the placodes was often reduced) (Fig. 6A). In contrast, the frequency and extent of increased and/or ectopic expression of *Ngnr1* or *NeuroD* declined and the frequency and extent of reductions in $p27^{Xic1}$, *Ngnr1* or *NeuroD* expression increased (Fig. 6B–D). This suggests that increasing concentrations of *Eya1* and *Six1* increasingly delay cell cycle exit. In contrast to *Ngnr1* and *NeuroD*, frequency and extent of increased and/or ectopic expression of $p27^{Xic1}$ also increased with increasing doses of injected *Eya1* and *Six1* mRNA (although $p27^{Xic1}$ expression in the placodes was often reduced in the same embryos) (Fig. 6B), suggesting that high levels of *Eya1* and *Six1* prevent onset of neuronal differentiation even in cells that have left the cell cycle.

In addition, analysis of the expression domains of neurogenesis genes in relation to injected *Eya1* or *Six1* in tissue sections through the placodal region of tail bud embryos showed that in ectoderm containing exogenous *Eya1*, identified by myc-immunostaining, *N-Tubulin*, *NeuroD*, *Ngnr1*, $p27^{Xic1}$ and *Delta1* were repressed, whereas residual and ectopic expression were located in adjacent areas

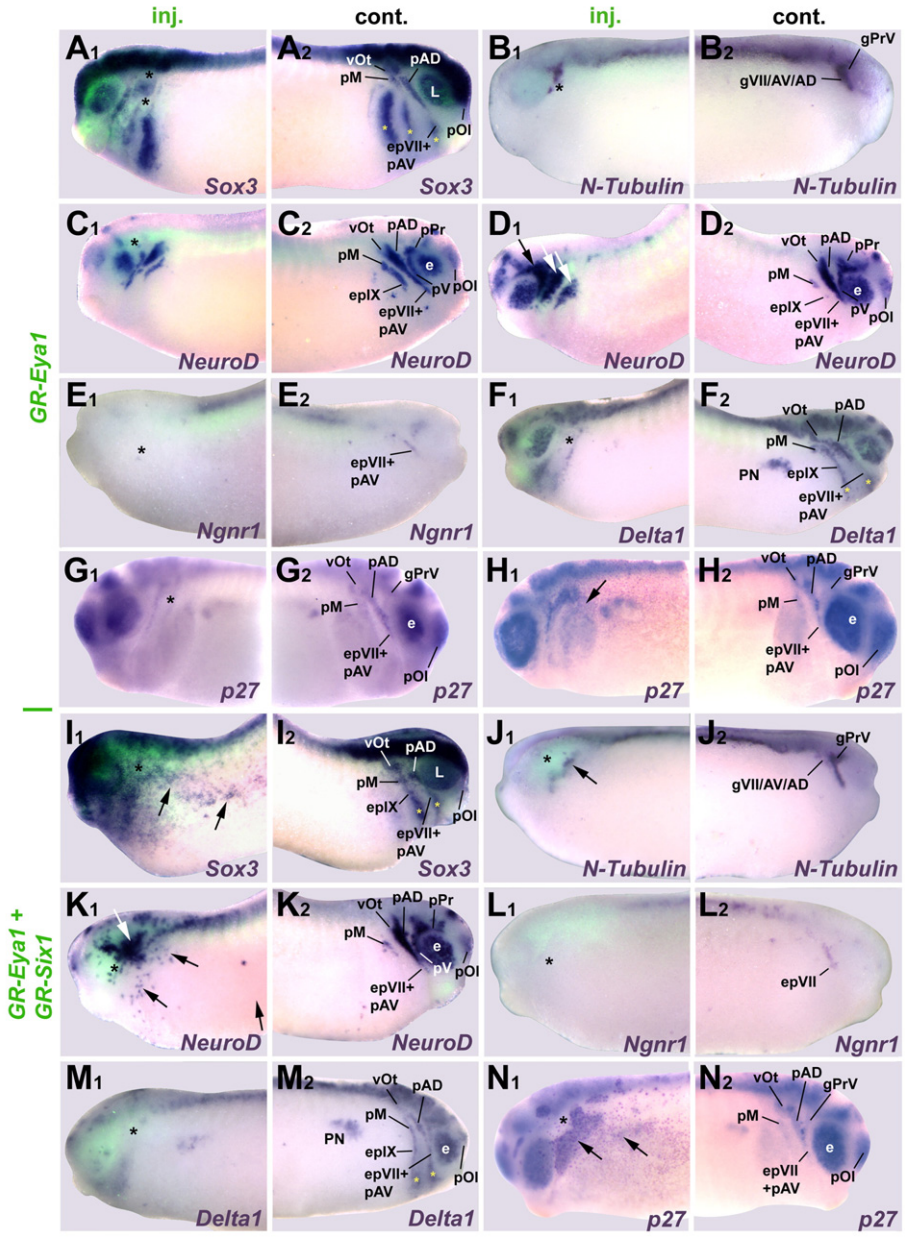

Fig. 5. Effects of overexpression of *GR–Eya1* or *GR–Eya1+GR–Six1* on various neurogenesis markers at tail bud stages (stage 26). Injected (A_1–N_1) and control (A_2–N_2) sides of tail bud stage embryos after unilateral injection of *GR–Eya1* (A–H) or *GR–Eya1+GR–Six1* (I–N) and DEX activation at stage 16–18. In all cases, where *myc-GFP* was co-injected as lineage tracer (A–F, I–M), embryos are shown superimposed with green fluorescent channel. Black asterisks indicate reductions, while black or white arrows mark increased or ectopic marker gene expression in the placodal or non-neural ectoderm. Yellow asterisks identify expression in pharyngeal pouches. For *NeuroD* (C, D) and *p27^{Xic1}* (G, H) after *GR–Eya1* injection two different phenotypes are depicted. Abbreviations as in Figs. 2 and 4.

with weak *Eya1* expression (Fig. 7). Similar results were found with *GR–Eya1* and co-expression with *Six1* or *GR–Six1*.

To test whether this distribution of neuronal differentiation is due to non-cell autonomous action of *Eya1*, we orthotopically grafted placodal ectoderm from *Eya1* mRNA injected embryos or uninjected control embryos into uninjected hosts (Fig. 8). While some reductions of placodal *NeuroD* expression were observed in grafts from both control (2/5) and *Eya1* injected (10/19) embryos, an ectopic increase of *NeuroD* expression was only observed in grafts from *Eya1* injected donors (5/19) and was confined to the graft. This indicates that *Eya1* promotes neuronal differentiation in a cell-autonomous fashion.

High levels of *Eya1* appear to be incompatible with high levels of *Sox3* expression since native *Sox3* expression domains in the placodes are repressed in areas of intense exogenous *Eya* expression. At the same time, there is ectopic low level expression of *Sox3* throughout these regions, and the ectoderm is dramatically thickened and contains multiple layers of cells compared to the thin bilayered ectoderm on the control side (Fig. 9A–C). *Sox3*-expressing cells within these ectodermal thickenings are immunopositive for proliferating cell nuclear antigen (PCNA), indicating that the thickenings result from increased proliferation (Figs. 9A, B).

To quantify the effects on cell proliferation we counted nuclei immunopositive for PH3, a marker for cells in mitosis (Saka and Smith, 2001), in the placodal region after injection of mRNAs encoding wild-type *Eya1* and/or *Six1*. Each significantly increased the proportion of PH3-positive cells at neural plate stages and co-injection augmented this effect (Figs. 9D–G). By neural tube stages the

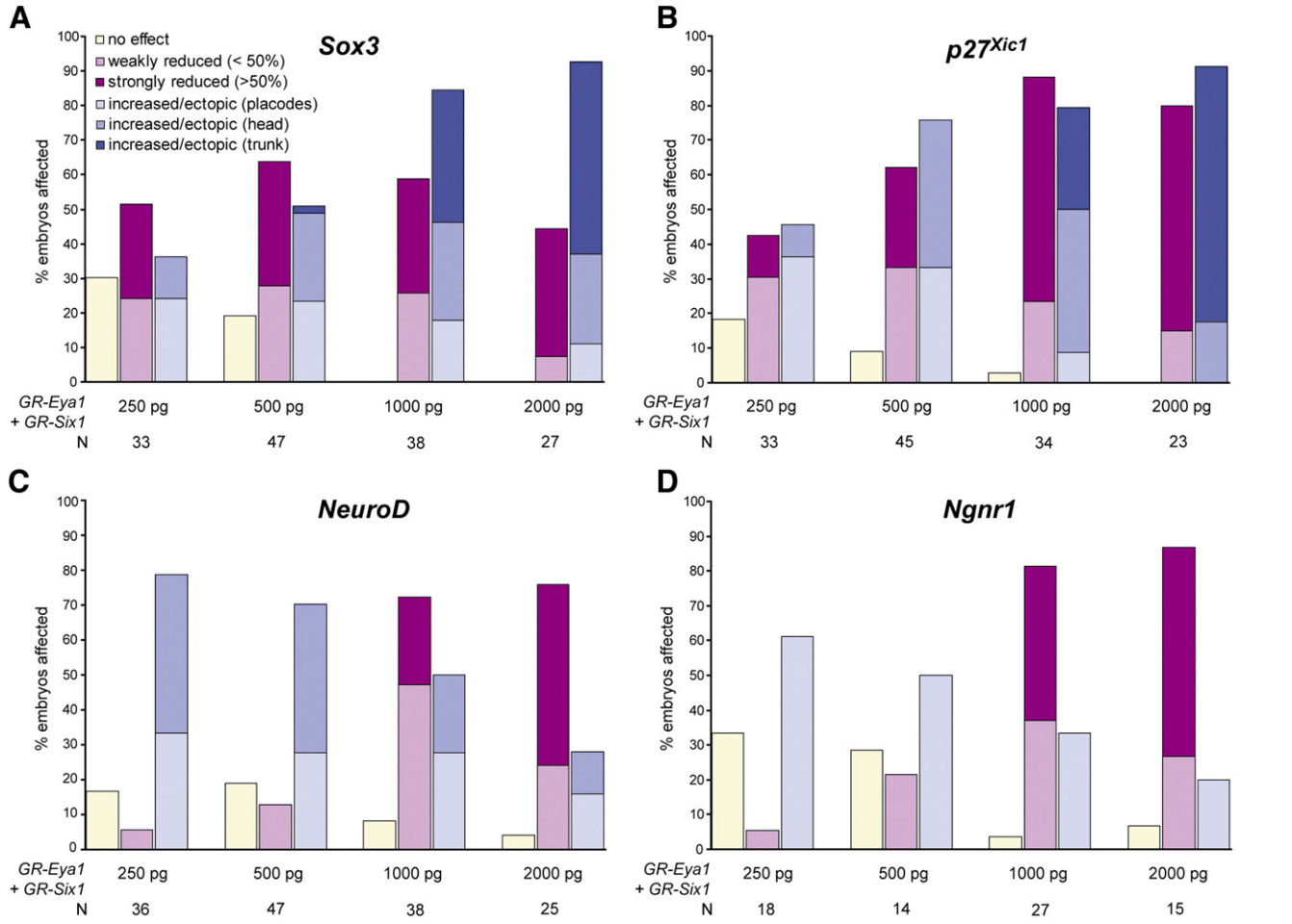

Fig. 6. Dose-dependent effects of *Eya1* on neurogenesis markers. Changes in placodal expression of *Sox3* (A), *p27*^{Xic1} (B), *NeuroD* (C), and *Ngnr1* (D) at stages 21–26 after injecting one blastomere at the two cell stage with various doses of *GR–Eya1+GR–Six1* mRNA followed by DEX activation at stage 16–18. For all markers analyzed, reductions of marker gene expression was categorized as weak (less than 50% reduced) or strong (more than 50% reduced), while increased or ectopic marker gene expression was categorized as being confined to the vicinity of placodes (placodes), extending to other parts of head ectoderm (head) or extending into trunk ectoderm (trunk). Note that reductions in placodal marker gene expression were sometimes associated with increased and/or ectopic marker gene expression elsewhere in the same embryo.

mitotic index returned to control levels indicating that this is a transient response. After injections of mRNA encoding *GR–Eya1* and/or *GR–Six1* and DEX activation at neural fold stages, a similar significant increase of the mitotic index was observed (Fig. 9G). These results are compatible with the observation that both wild-type and inducible forms of *Eya1* and *Six1* reduced placodal expression of *p27*^{Xic1} (Figs. 3I, 4H, L, 5G, N), indicating that they delay cell cycle exit. However, neither *Eya1* nor *Six1* affected *CyclinA1* expression in the developing placodal ectoderm (Table 2), indicating that neuronal progenitor pool expansion is mediated by other target genes of *Eya1* and *Six1*.

Taken together these findings substantiate the proposal that high levels of *Eya1* and *Six1* synergistically delay cell cycle exit and prevent neuronal differentiation but promote the proliferation of *SoxB1*-positive neuronal progenitor cells. To confirm that the ectopic neurons observed in areas of low exogenous *Eya1* and *Six1* expression originate from Sox3+ neuronal progenitors, we examined *NeuroD* expression in relation to *Sox3*-immunostaining (Fig. 10). While *Sox3*-immunostaining and *NeuroD* expression are mutually exclusive, ectopic *NeuroD* expressing cells are found adjacent to Sox3+ cells and some of the latter are localized within ectoderm expressing high levels of exogenous *Eya1* (Fig. 10A) or *Eya +Six1* (Fig. 10B). This spatial relationship suggests that Sox3+ cells differentiate as neurons once *Eya1* and *Six1* levels decline below a certain threshold.

Eya1 and Six1 control placodal neurogenesis in a SoxB1-dependent fashion

Our model implies that *Eya1* and *Six1* modulate placodal neurogenesis in a *SoxB1*-dependent fashion. To test this hypothesis, we injected either *SoxB1* morpholinos or mRNAs into one blastomere at the two cell stage and analyzed neurogenesis marker expression at neural plate and tail bud stages (Fig. 11, Table 2). To test efficacy and specificity of MOs designed to block the translation of *Sox2* and *Sox3* mRNAs (Supplemental Fig. S1), we co-injected them together with *utr-Sox2* and *utr-Sox3* mRNA into fertilized eggs. The *Sox2*MO inhibited protein synthesis from the *utr-Sox2* mRNA but not from the *utr-Sox3* mRNA and vice versa (Fig. 11A). Neither MO affected TCF3 synthesis, an internal control.

In embryos co-injected with *Sox2* and *Sox3* morpholinos, *N-Tubulin* (Fig. 11B) and *NeuroD* expression (Fig. 11C) were strongly repressed in all neurogenic placodes including the profundal and trigeminal placodes. *Delta1* expression was also strongly repressed (not shown) suggesting that reduced neuronal differentiation was not due to an increase in lateral inhibition. On the other hand, the expression of *p27*^{Xic1} and *Ngnr1* was sometimes reduced but often enhanced in the placodal region (Fig. 11D, E). Thus, *Sox2* and *Sox3* appear essential for maintaining cells in the cell cycle and to promote neuronal differentiation after cell cycle exit, since elevation of *p27*^{Xic1} and *Ngnr1* after

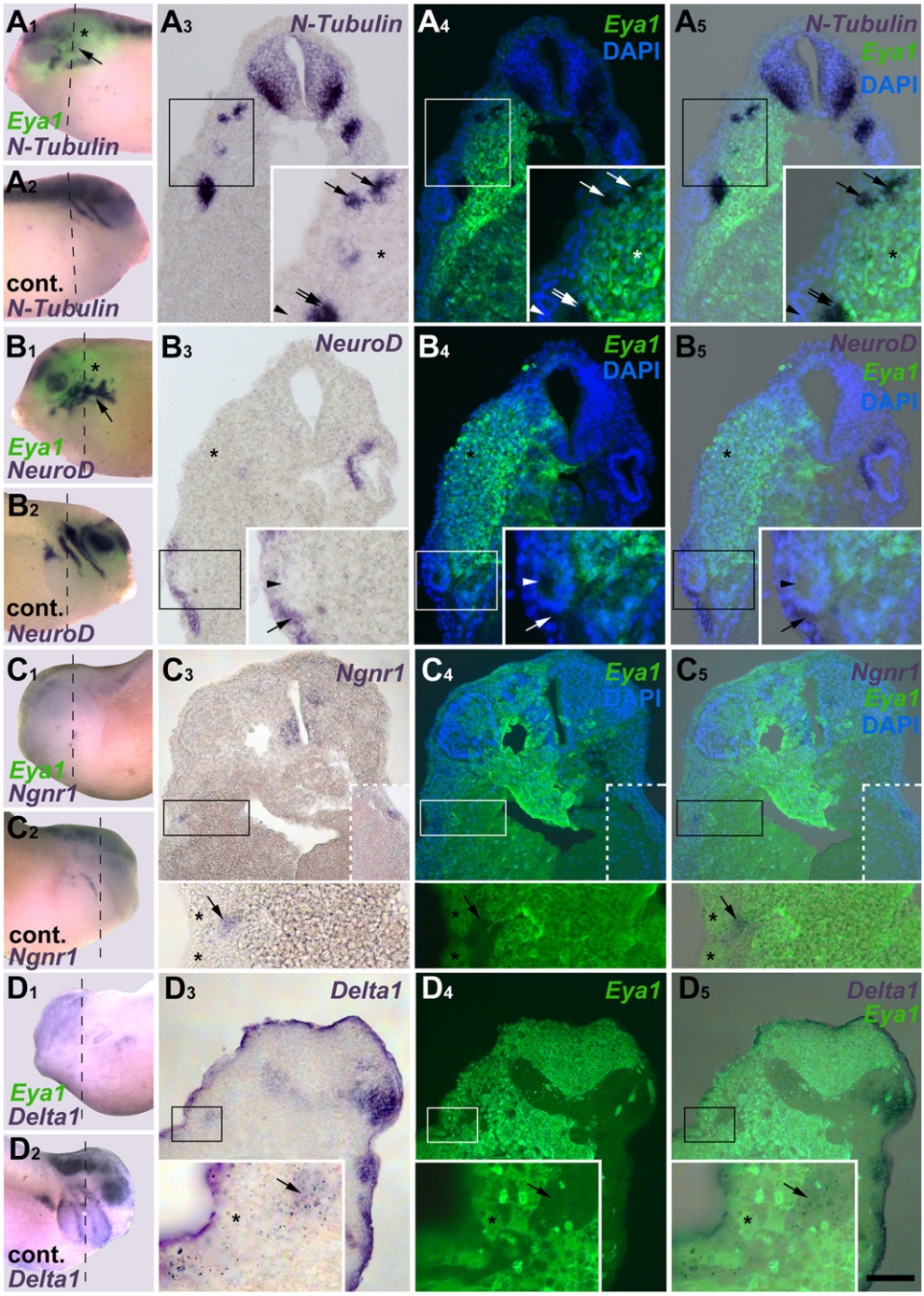

Fig. 7. Dose-dependent effects of *Eya1* on neurogenesis markers as revealed in tissue sections. Distribution of *N-Tubulin* (A), *NeuroD* (B), *Ngnr1* (C) and *Delta1* (D) in stage 26 embryos after injection of *myc-Eya1* or co-injection of *Eya1* with *myc-GFP* mRNA. Embryos are shown as wholemounts (injected side: A_1–D_1; control side: A_2–D_2) and in transverse sections (at level indicated by black line) shown in bright field (A_3–D_3), superimposition of green (myc-immunostaining) and blue (DAPI) fluorescence (A_4–D_4, no DAPI image available in panel D), and as a merged image (A_5–D_5). Boxed areas are magnified in inserts. The DAPI channel is not shown in the inserts of panel C to make *Ngnr1* staining more clearly visible. Hatched boxed area in panels C_3–C_5 shows *Ngnr1* positive epibranchial placode on control side in an adjacent section. Asterisks indicate reductions, while arrows mark ectopic marker gene expression in the placodal or non-neural ectoderm. Double arrows identify areas of residual expression. Arrowheads mark displaced otic vesicle on injected side. Bar in panel D_5: 100 μm (for A_{3-5} – D_{3-5}).

SoxB1 knockdown is not accompanied by increased neuronal differentiation.

Overexpression of *Sox3*, however, reveals that high levels of SoxB1 proteins are incompatible with neuronal differentiation. Injections of *Sox3* or of *GR–Sox3* followed by DEX activation at stages 16–18 gave similar results. *N-Tubulin* (Fig. 11F, J) and *NeuroD* (Fig. 11G, K) were strongly repressed in all neurogenic placodes and the *Sox3*-over-expressing regions of the ectoderm were thickened and PCNA-immunopositive (not shown). However, unlike the results with *Eya1* or *Six1*, we never found ectopic expression of *N-Tubulin* or *NeuroD*. $p27^{Xic1}$ (Fig. 11I), *Ngnr1* (Fig. 11H, L) and *Delta1* (not shown) were mostly reduced by increasing *Sox3* or *GR–Sox3* levels although increased or ectopic expression was sometimes observed, indicating that *Sox3* overexpression delays cell cycle exit and onset of neuronal differentiation. Increasing *Sox3* expression, therefore, mimics many of the effects of *Eya1* and *Six1* on placodal neurogenesis suggesting that

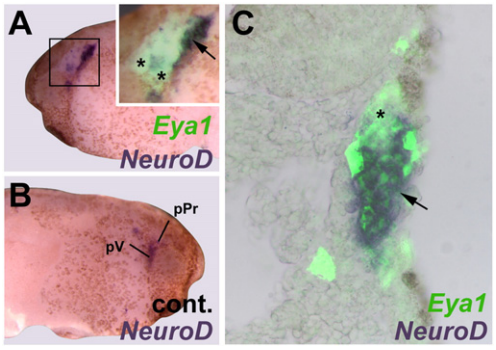

Fig. 8. Cell-autonomous effects of *Eya1* on placodal neurogenesis. Panels show distribution of *NeuroD* in grafts of placodal ectoderm from embryos injected with *Eya1* and *myc-GFP* mRNA to uninjected hosts. Boxed area is magnified in insert of panel A. Insert of panels A and C are shown superimposed with green fluorescent channel to reveal myc-immunostaining. While *NeuroD* expression in profundal (pPr) and trigeminal (pV) placodes is reduced in the graft (asterisk, A, C) compared to control side (B), *NeuroD* is expressed ectopically (arrow) at graft border but not in host ectoderm (A, C).

these effects are *SoxB1*-mediated. However, in contrast to *Eya1* or *Six1* injection, *Sox3* injection never led to ectopic neuronal differentiation indicating that *Eya1* and *Six1* probably promote neuronal differentiation by additional and *Sox3*-independent mechanisms.

To directly test whether *Eya1* and *Six1* promote placodal neurogenesis in a *SoxB1* dependent fashion, we injected *GR–Eya1+GR–Six1* together with *Sox2MO* and *Sox3MO* followed by DEX activation at stage 16–18. Co-injection of *SoxB1* MOs significantly compromised the ability of *GR–Eya1+GR–Six1* to generate ectopic *NeuroD* expression (Figs. 11M, N). To determine whether *SoxB1* genes are direct target genes of *Eya1* and *Six1* we treated *GR–Eya1+GR–Six1* injected embryos with the protein synthesis inhibitor cycloheximide (CHX) prior to and during DEX activation at stage 16–18. *Eya1* and *Six1* retained the ability to ectopically induce *Sox2* (Figs. 11P, R) and *Sox3* (Figs. 11O, R) under these conditions, indicating that the latter are likely direct targets of transcriptional regulation by *Eya1* and *Six1*. Ectopic induction of *Sox2* and *Sox3* was stronger and more frequent with CHX treatment suggesting that there also is an indirect inhibitory effect on *Sox2* and *Sox3* expression that is relieved when protein synthesis is blocked. Ectopic *NeuroD* and $p27^{Xic1}$ expression were no longer observed after CHX treatment (Figs. 11Q, R) indicating that these genes are likely to be indirect targets of regulation by *Eya1* and *Six1*.

Discussion

Eya1 and Six1 regulate several steps in placode development

Eya1 and *Six1* are required for establishing the pre-placodal ectoderm in *Xenopus* and distinguishing it from the adjacent neural crest and non-neural epidermis (Brugmann et al., 2004). In addition, members of the *Eya* and *Six1/2* gene families have important roles in the proliferation and survival of neuronal and sensory progenitors (Zheng et al., 2003; Li et al., 2003; Bricaud and Collazo, 2006; Zou et al., 2006; Kriebel et al., 2007) as well as for the proper expression of neuronal determination and differentiation genes in the olfactory, otic and epibranchial placodes (Zou et al., 2004; Friedman et al., 2005; Bricaud and Collazo, 2006; Ikeda et al., 2007). We have extended these observations to demonstrate that both *Eya1* and *Six1* are required for neuronal differentiation in all neurogenic placodes, substantiating the suggestion that *Eya1* and *Six1* have pan-placodal functions (Schlosser, 2006). In addition, we show that they are important in multiple steps during placodal neurogenesis, that their specific roles are determined by their levels of expression, and many of their effects are mediated by *SoxB1* genes (Fig. 12).

After establishing the pre-placodal ectoderm, *Eya1* and *Six1* continue to be expressed at high levels in the developing placodes (Pandur and Moody, 2000; Schlosser and Northcutt, 2000; David et al., 2001). We show that a subset of these cells express *Sox2* and *Sox3*, and that *Eya1* and *Six1* are required for *Sox* expression. Our expression analysis suggests that incipient placodal neurons are mostly *Eya1*, *Six1* and *Sox3*-negative but originate from Sox3+ cells. This extends observations in the zebrafish and chick, where neurons in the otic and epibranchial placodes were found to differentiate from Sox3+ cells (Abu-Elmagd et al., 2001; Neves et al., 2007; Sun et al., 2007; Nikaido et al., 2007). It appears that it is the reduction of *Eya1* and *Six1* protein levels that permits expression of neuronal determination and differentiation markers. Thus, levels of expression of *Eya1* and *Six1* regulate the progression from proliferating placodal progenitor to differentiated placodal neuron (Fig. 12).

High levels of Eya1 and/or Six1 maintain cells in a proliferative neuronal progenitor state

Our gain-of-function experiments suggest that at high levels of expression *Eya1* and *Six1* keep cells in a proliferative neuronal progenitor state and block onset of neuronal determination. Although *Six1* is able to directly activate cell cycle regulators such as *CyclinA1* and *c-Myc* (Li et al., 2003; Coletta et al., 2004), neither *Eya1* nor *Six1* affected *CyclinA1* expression in our experiments. This suggests that other target genes of *Eya1* and *Six1* are involved in progenitor pool expansion. Our finding that *Sox2* and *Sox3* are likely direct targets of *Eya1* and *Six1* suggests that *SoxB1* genes may be involved in this process.

SoxB1 genes are well known to be required for neuronal differentiation in the central nervous system and to bias lineage choice towards neural or neuronal fates (Pevny et al., 1998; Kishi et al., 2000; Zhao et al., 2004; Kan et al., 2004, 2007; Wang et al., 2006). However, overexpression of *SoxB1* genes interferes with neuronal differentiation and often reduces *Ngnr1* and $p27^{Xic1}$ expression (Bylund et al., 2003; Graham et al., 2003; Hardcastle and Papalopulu, 2000), while loss of *SoxB1* function increases their expression, indicating that SoxB1 proteins maintain a proliferative neural or neuronal progenitor cell. Our loss of function experiments show that neuronal differentiation in placodes is blocked even though $p27^{Xic1}$ and *Ngnr1* are frequently increased indicating that *SoxB1* genes are also required for neuronal differentiation in placodes, acting either downstream of and/or parallel to $p27^{Xic1}$ and *Ngnr1*. However, we also observed that increasing *Sox3* expression in the placodal ectoderm reduced the expression of both neuronal determination and differentiation genes, while knockdown could expand *Ngnr1* and $p27^{Xic1}$. These findings suggest that *SoxB1* genes play a dual role in the neurogenic placodes as they do in the CNS: they are (1) required for neuronal differentiation, but (2) maintain cells in a proliferative neuronal progenitor state.

Surprisingly, we find *SoxB1* genes to be required for neuronal differentiation even in the profundal and trigeminal placodes, which do not express *Sox2* or *Sox3* from neural plate stages on (Schlosser and Ahrens, 2004). These are the earliest placodal neurons to be born and to differentiate (Lamborghini, 1980; Schlosser and Northcutt, 2000), and therefore may be influenced by the more widely expressed *Sox2*/*Sox3* at gastrula stages (Penzel et al., 1997; Mizuseki et al., 1998).

Our experiments demonstrate that *Sox2/Sox3* are regulated in the placodes by *Eya1* and *Six1*. Loss- and gain-of-function of either *Eya1* or *Six1* affect neuronal determination and differentiation genes in a similar manner to the loss- and gain-of function of *Sox2/Sox3*, suggesting that these two sets of transcription factors act in the same pathway. Furthermore, the endogenous expression of *Sox2* and *Sox3* in the placodes is confined to domains of *Eya1* and *Six1* expression, and requires both of these genes. These data suggest that the ability of *Eya1* and *Six1* to promote proliferative neuronal progenitors, from which

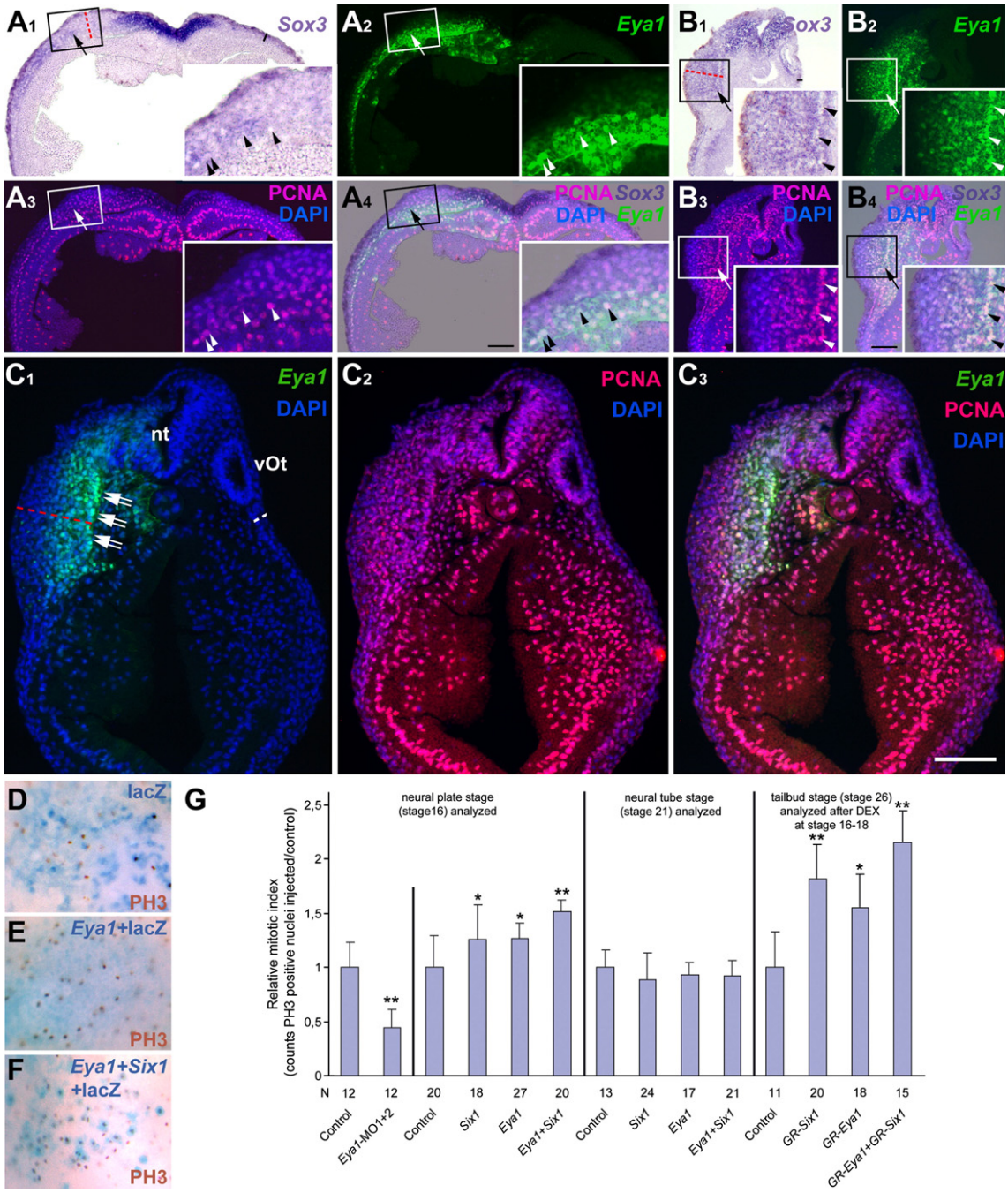

Fig. 9. *Eya1* and *Six1* promote proliferative *SoxB1*-positive neuronal progenitors. (A–C) Transverse sections through neural plate (A) or tail bud stage (B, C) embryos that received unilateral *myc-Eya1* mRNA injection at the two cell stage. (A, B) Panels show *Sox3* expression in bright field (A_1–B_1), distribution of injected myc-Eya1 protein (A_2–B_2), PCNA together with DAPI staining (A_3–B_3) and a merged image (A_4–B_4). (C) Panels show distribution of injected myc-Eya1 protein (C_1), PCNA (C_2) and a merged image (C_3) together with DAPI staining. Ectoderm that received high levels of *Eya1* expresses *Sox3* (A_1–B_1, arrows), is drastically thickened (A_1–C_1, red lines) compared to control side (black or white lines) and consists of multiple layers of PCNA positive cells (A_3, B_3, C_2). The otic vesicle has failed to form on the injected side of embryos in panels B and C. Arrowheads identify cells coexpressing myc-Eya1 and PCNA. Double arrows in panel C identify the basal lamina of the ectoderm on the injected side. Abbreviations: nt: neural tube. vOt: otic vesicle. All bars: 100 μm. (D–G) Effects of *Eya1* and *Six1* gain and loss of function on phosphohistone H3 (PH3). (D–F) PH3 immunopositive nuclei in placodal ectoderm of neural plate stage embryos after injection of *lacZ* (D) *Eya1+lacZ* (E) or *Eya1+Six1+ lacZ* mRNAs (F). (G) Relative mitotic index after injections of *Six1* or *Eya1 mRNAs* or morpholinos. Standard deviations and significant increases are indicated (*t*-test; *: $p<0.05$, **: $p<0.001$).

ectopic neurons later differentiate, is mediated by their capacity to activate *SoxB1* expression (Fig. 12). We confirmed this by showing that *SoxB1* knockdown interferes with the ability of *Eya1* and *Six1* to induce ectopic neurons. Our gain-of-function experiments further suggest that when expressed at high levels *Eya1* and *Six1* promote a proliferative SoxB1+ neuronal progenitor state. As levels of expression of both sets of genes begin to decrease, the SoxB1+ cells transition to a state in which cell cycle exit is permitted, the neuronal fate is determined, and neurons differentiate from an expanded pool of neuronal progenitors.

Low levels of Eya1 and/or Six1 promote neuronal differentiation

Our observation that ectopic neuronal differentiation occurs only after *Eya1* or *Six1* but not after *Sox3* overexpression, indicates that low levels of *Eya1* and *Six1* do not merely play a permissive role for the

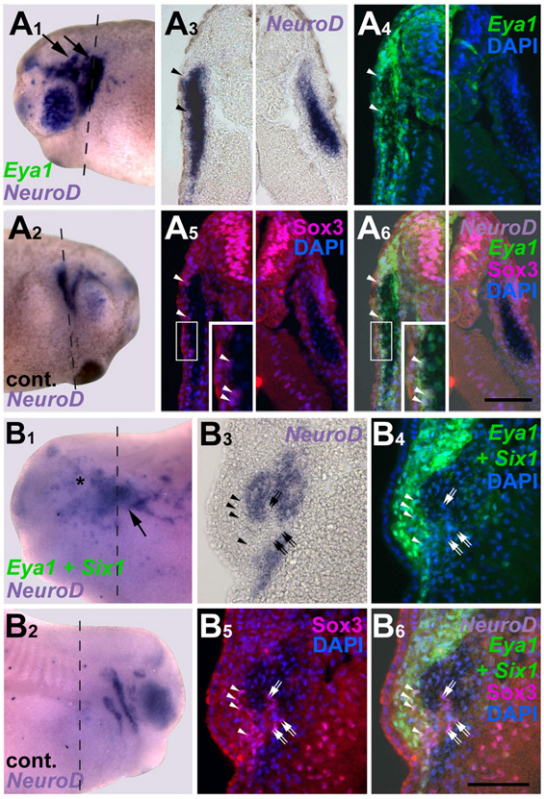

Fig. 10. Ectopic neurons in embryos overexpressing *Eya1* or *Eya1+Six1* originate from Sox3 immunopositive neuronal progenitors. Panels show distribution of *NeuroD* expressing cells in relation to Sox3-immunopositive cells in tail bud stage embryos after injection of *myc-Eya1* mRNA alone (A) or together with *Six1* mRNA (B). Embryos are shown as wholemounts (injected side: A_1–B_1; control side: A_2–B_2) and in transverse sections (at level indicated by black line). Panels show bright field images (A_3–B_3), myc-immunostaining together with DAPI (A_4–B_4), Sox3-immunostaining together with DAPI (A_5–B_5), and merged images (A_6–B_6). Asterisks indicate reductions, while arrows mark ectopic *NeuroD* expression in the non-neural ectoderm. Ectopic *NeuroD* expression is found next to Sox3 immunopositive cells on the fringes of ectodermal regions that received high levels of myc-Eya1. Sox3 cells reside in ectoderm that received high levels of myc-Eya1 (arrowheads) as well as in areas of lower myc-Eya1 levels immediately abutting *NeuroD* expressing cells (double arrows). Boxed areas are shown at higher magnification in inserts. All bars: 100 μm

onset of neuronal determination but additionally promote neuronal differentiation downstream of and/or in parallel to *Sox3* (Fig. 12). Furthermore, we do not observe ectopic neuronal differentiation in *Eya1* or *Six1* loss of function experiments even though *Ngnr1* and $p27^{Xic1}$ are often increased. This suggests that *Eya1* and *Six1* are both required for neuronal differentiation to proceed beyond expression of proneural genes.

Neurogenesis in different placodes is known to rely on different proneural genes with interspecific differences between vertebrates. In mammals, neuronal determination depends on *Ngn1* in the trigeminal and otic placodes, on *Ngn2* in the epibranchial placodes and on *Ash1* in the olfactory placode (Fode et al., 1998, Ma et al., 1998; Cau et al., 2002), while in the zebrafish neuronal determination in all (except possibly the olfactory placodes) depends on *ngn1* (Andermann et al., 2002). Although two different *Ngn* genes are also present in *Xenopus* (Furlong and Graham, 2005), only the expression of the *Ngn2* homologue *Ngnr1* has been described and it is confined to the olfactory, profundal/trigeminal and epibranchial placodes (Schlosser and Northcutt, 2000). This suggests that in *Xenopus* similar to amniotes, neuronal determination in different placodes relies on different neuronal determination genes and that possibly the *Xenopus Ngn1* homologue may play this role in placodes that do not express *Ngnr1* (otic and lateral line placodes). Because knockdown of *Eya1* or *Six1* blocks neuronal differentiation in placodes that express *Ngnr1* as well as in those that do not express it, we propose that these genes promote neuronal differentiation both downstream of and/or in parallel to *Ngnr1* and via other pathways (possibly downstream of and/or in parallel to other neuronal determination genes such as *Ngn1*).

Increased levels of *Eya1* or *Six1* also reduce *Delta1*, suggesting that relief of Notch-mediated lateral inhibition (Chitnis et al., 1995) allows *Eya1* and *Six1* to promote ectopic neuronal differentiation. However, several observations indicate that this is unlikely to be a major contributor. First, repression of *Delta1* is confined to regions of high levels of *Eya1* and *Six1*, where cells maintain a proliferative progenitor state, whereas ectopic neuronal differentiation occurs in adjacent regions, which maintain *Delta1* expression. Second, *Ngnr1* as well as *NeuroD* is expected to be increased after relief of lateral inhibition (Ma et al., 1996), which we did not observe. Third, increases in *Delta1* after *Six1* or *Eya1* loss-of-function are observed much too rarely to account for the frequent reductions in neuronal differentiation.

Are other factors interacting with Eya1 and/or Six1 in placodal neurogenesis?

In the experiments presented herein, *Eya1* and *Six1* have similar effects on the expression of most neurogenesis markers and the phenotypes were stronger after co-expression of both. These results support their known ability to directly interact to affect transcription (Ohto et al., 1999; Ikeda et al., 2002). However, other *Eya* genes and genes of the *Six1/2* and *Six4/5* subfamilies are also expressed in multiple placodes in different vertebrates with some interspecific differences in expression patterns (Brugmann and Moody, 2005; Schlosser, 2006). This suggests that some of these other genes may also be involved in the regulation of placodal neurogenesis perhaps with redundant functions. Indeed, in mice trigeminal neurogenesis is affected only in *Six1/Six4* double knockouts but not in mutants of either gene alone (Konishi et al., 2006). In *Xenopus*, *Six2* and *Six4* are expressed in most placodes similar to *Six1* (Ghanbari et al., 2001). *Xenopus Eya3* is not strongly expressed in the pre-placodal ectoderm, in contrast to *Eya1*, but subsequently is expressed in the lens and otic placodes, while the expression of other *Xenopus Eya* genes has not yet been reported (Kriebel et al., 2007). Taken together, this suggests that *Six2*, *Six4, Eya3* and possibly other uncharacterized family members (*Six5*, *Eya2*, *Eya4*) as well may cooperate with *Six1* and *Eya1* in regulating neurogenesis in at least some placodes in *Xenopus*.

From our experiments, we noted that *Eya1* and *Six1* effects were not always identical. *Eya1*MOs caused expansion of *Sox2* and *Sox3* whereas *Six1*MOs did not. Injection of *Eya1* mRNA expanded *Ngnr1* and $p27^{Xic1}$ domains more frequently and reduced *SoxB1* expression less frequently than did injection of *Six1* mRNA. Finally, *Eya1* knockdown had a much smaller effect on *Sox3* compared to *Six1* knockdown. These results suggest that both *Eya1* and *Six1* may synergize with some other factors during placodal neurogenesis.

In conclusion, we have identified multiple previously unrecognized roles of *Eya1* and *Six1* in the regulation of placodal neurogenesis. Based on the data presented herein, we propose a model of the gene cascade regulating this important developmental process (Fig. 12). High levels of *Eya1* and *Six1* expression in the neurogenic placodes promote a *SoxB1*-dependent proliferative neuronal progenitor state and block onset of neuronal determination. As *Eya1* and *Six1* are downregulated, *SoxB1* expression subsides and cell cycle inhibitors ($p27^{Xic1}$) and proneural genes (*Ngnr1* or other unidentified genes) are de-repressed, thereby permitting neuronal determination and differentiation to proceed. Low *Eya1*, *Six1* and *SoxB1* expression levels then promote neuronal differentiation downstream of and/or in parallel to *Ngnr1* and possibly other proneural genes. Because *Eya1* and *Six1* only promote neuronal differentiation in cranial ectoderm, additional regionalized and yet unidentified factors must contribute to the

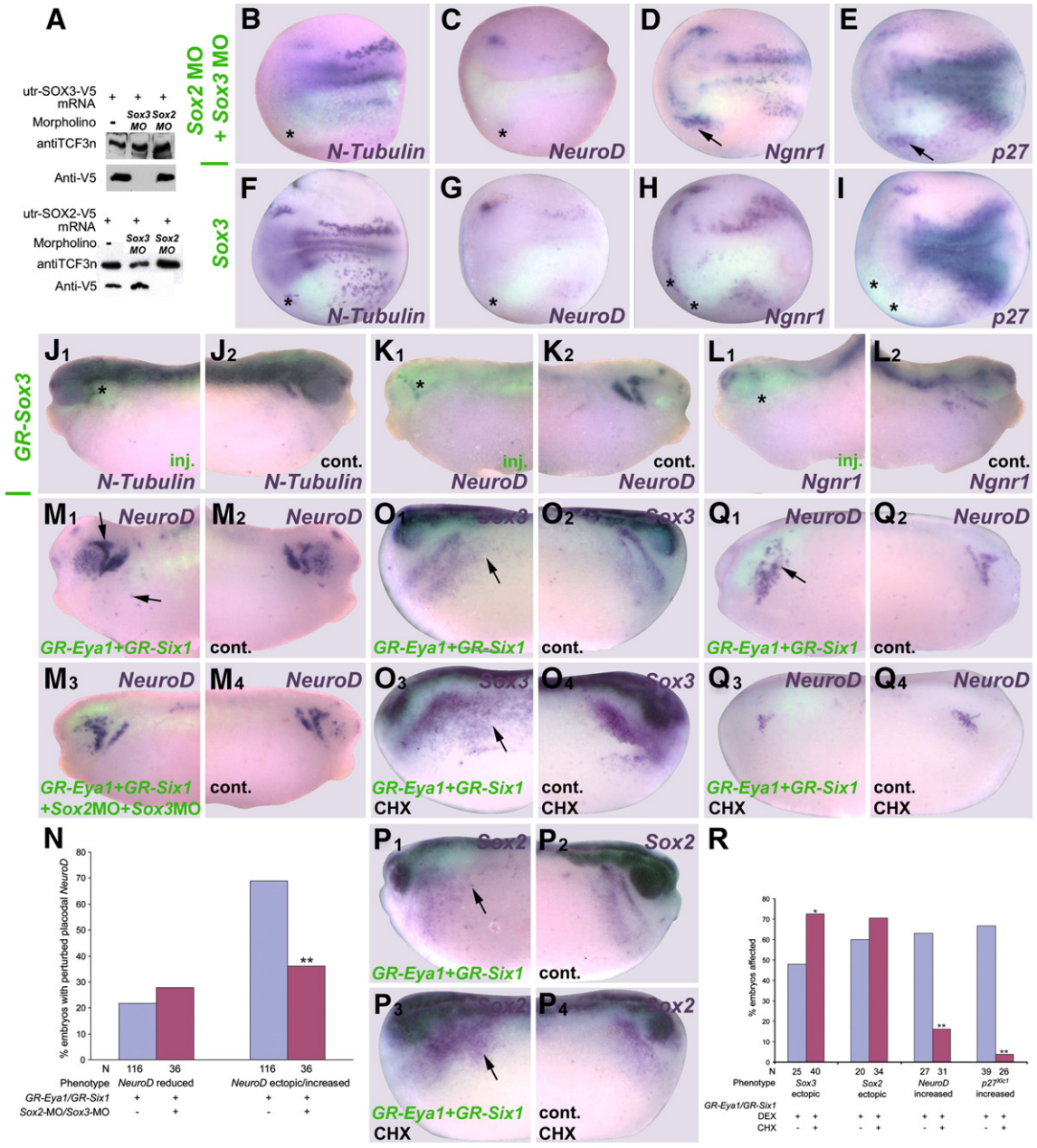

Fig. 11. Role of *SoxB1* genes in placodal neurogenesis. (A) Immunoblot showing that *Sox2*MO and *Sox3*MO specifically and efficiently block protein synthesis from co-injected *utr-Sox2* and *utr-Sox3* mRNA, respectively. (B–E, F–I) Effects of unilateral injection (lower half) of *Sox2+Sox3*MO (B–E), or *Sox3* mRNA (F–I) on various neurogenic markers at neural plate stage. Embryos are shown superimposed with green fluorescent channel to reveal distribution of myc-GFP co-injected as lineage tracer. Asterisks indicate reductions, while arrows mark increased or ectopic marker gene expression in the placodal or non-neural ectoderm. (J–L) Effects of unilateral injection of *GR–Sox3* and DEX activation at stage 16–18 on neurogenic markers at tail bud stages (J_1–L_1: injected side; J_2–L_2: control side). Asterisks indicate reductions in the placodal or non-neural ectoderm. (M, N) Ectopic neuronal differentiation (arrows) after injection of *GR–Eya1+GR–Six1* mRNAs (M_1: inj. side; M_2: control side) and DEX activation at stage 16–18 is significantly reduced after co-injection of *Sox2/Sox3* MO (M_3: inj. side; M_4: control side; N: quantitation, X^2 test; **: $p<0.001$). (O–R) Ectopic expression (arrows) of *Sox3* (O_1, O_3: inj. side; O_2, O_4: control side) and *Sox2* (P_1, P_3: inj. side; P_2, P_4: control side) but not of *NeuroD* (Q_1, Q_3: inj. side; Q_2, Q_4: control side) and $p27^{Xic1}$ after injection of *GR–Eya1+GR–Six1* mRNAs and DEX activation at stage 16–18 persists and is more extensive with CHX treatment (R: quantitation; X^2 test; *: $p<0.05$, **: $p<0.001$).

regulation of placodal neurogenesis. Our data demonstrate that *Eya1* and *Six1* play a central role in multiple steps of placodal neurogenesis: they regulate neuronal progenitor pool size, mediated via the *SoxB1*-genes, control the onset of neuronal determination and differentiation, and promote neuronal differentiation.

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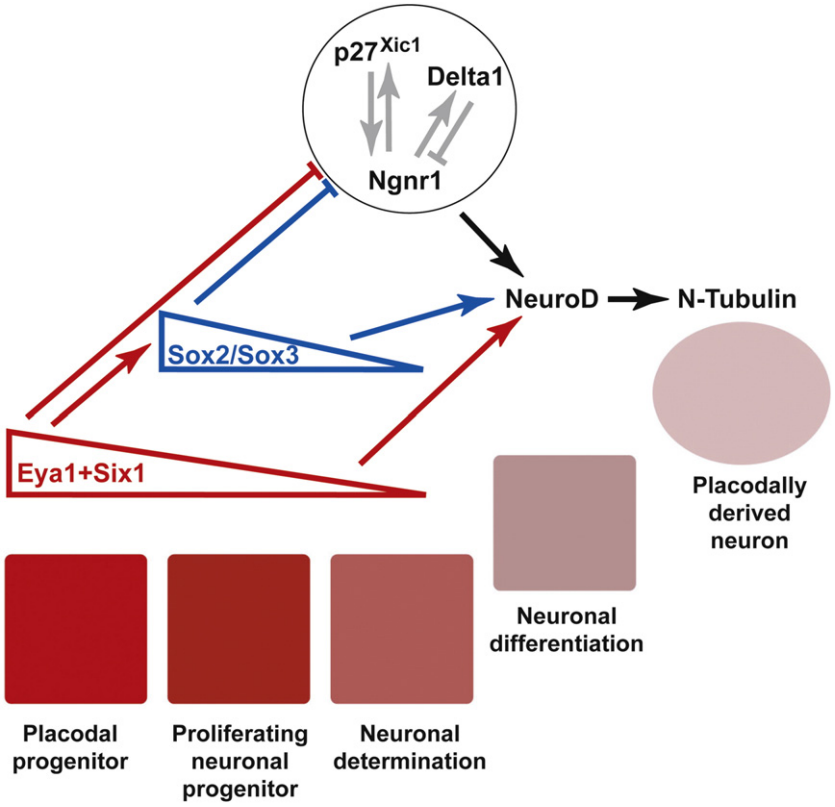

Fig. 12. Model of regulatory interactions between *Six1*, *Eya1* and *SoxB1* genes in placodal ectoderm. Blue and red arrows and bars indicate positive and negative regulatory interactions, respectively, of Eya1+Six1 (red) and SoxB1 (blue) proteins supported by present study. The circle encloses a network of proteins known to cross-regulate during early stages of neuronal determination. High levels of Eya1 and Six1 in the placodes (red squares) maintain proliferative neuronal progenitors. These levels decrease as cells leave the placodes (pink squares), permitting cell cycle exit and onset of neuronal determination and promoting neuronal differentiation. See text for details.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.05.523.

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