Developmental Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Six1 and Eya1 both promote and arrest neuronal differentiation by activating multiple Notch pathway genes

Nick Riddiford[1], Gerhard Schlosser[*]

School of Natural Sciences and Regenerative Medicine Institute (REMEDI), National University of Ireland, Galway, Biosciences Research Building, Newcastle Road, Galway, Ireland

ARTICLE INFO

Keywords:
Hes8
Neurog1
Xenopus
Placodes
Neurogenesis

ABSTRACT

The transcription factor Six1 and its cofactor Eya1 are important regulators of neurogenesis in cranial placodes, activating genes promoting both a progenitor state, such as *hes8*, and neuronal differentiation, such as *neurog1*. Here, we use gain and loss of function studies in *Xenopus laevis* to elucidate how these genes function during placodal neurogenesis. We first establish that *hes8* is activated by Notch signaling and represses *neurog1* and neuronal differentiation, indicating that it mediates lateral inhibition. Using *hes8* knockdown we demonstrate that *hes8* is essential for limiting neuronal differentiation during normal placode development. We next show that Six1 and Eya1 cell autonomously activate both *hes8* and *neurog1* in a dose-dependent fashion, with increasing upregulation at higher doses, while neuronal differentiation is increasingly repressed. However, high doses of Six1 and Eya1 upregulate *neurog1* only transiently, whereas low doses of Six1 and Eya1 ultimately promote both *neurog1* expression and neuronal differentiation. Finally, we show that Six1 and Eya1 can activate *hes8* and arrest neuronal differentiation even when Notch signaling is blocked. Our findings indicate that Six1 and Eya1 can both promote and arrest neuronal differentiation by activating the Notch pathway genes *neurog1* and *hes8*, respectively, revealing a novel mechanism of Six1/Eya1 action during placodal neurogenesis.

1. Introduction

The gene *Six1* encodes a homeobox transcription factor, while *Eya1* encodes a protein known to act as one of the transcriptional coactivators of Six1 (Kumar, 2009b; Rebay, 2015; Tadjuidje and Hegde, 2013). Eya1 also has phosphatase activity and serves additional functions in the cytoplasm (Rebay, 2015; Tadjuidje and Hegde, 2013). Both genes were first described in *Drosophila*, where mutations of the genes encoding their insect homologues, *sine oculis* and *eyes absent*, lead to the absence of eyes (Kumar, 2009a; Silver and Rebay, 2005). In vertebrates, Six1 and Eya1 cooperate in the development of multiple tissues and organs such as trunk muscles, kidney and the cranial placodes (Moody and LaMantia, 2015; Schlosser, 2010; Wong et al., 2012), which are the focus of the present study.

Cranial placodes give rise to many of the cranial sense organs and ganglia of vertebrates. Apart from the adenohypophyseal and lens placode, all placodes (olfactory, otic, lateral line, profundal/trigeminal, epibranchial placodes) are neurogenic and give rise to neurons as one of their derivative cell types (Baker and Bronner-Fraser, 2001; Schlosser, 2010). All placodes arise from a common precursor, the pre-placodal ectoderm (PPE), which forms a crescent shaped region around the anterior neural plate and is defined by the expression of *Six1* and *Eya1* (Grocott et al., 2012; Pieper et al., 2011; Saint-Jeannet and Moody, 2014; Schlosser and Ahrens, 2004). The PPE subsequently breaks up into individual placodes, which maintain *Six1* and *Eya1* expression.

Loss of function studies in mice, zebrafish and *Xenopus* have shown that Six1 and Eya1 are essential regulators for several aspects of placodal development, most notably neuronal and sensory development. Mutants or morphants show decreased proliferation and increased apoptosis in placodally derived sense organs and ganglia (Chen et al., 2009; Li et al., 2003a; Ozaki et al., 2004; Schlosser et al., 2008; Zheng et al., 2003; Zou et al., 2008, 2006). In addition, there are deficiencies in sensory and neuronal differentiation, and expression of neuronal determination (proneural) genes such as *Neurog1*, *Neurog2* and *Atoh1* as well as the neuronal differentiation gene *NeuroD1* is reduced in olfactory, epibranchial and otic placodes (Bricaud and Collazo, 2006; Chen et al., 2009; Friedman et al., 2005; Ikeda et al., 2007; Schlosser et al., 2008; Zou et al., 2004). In humans, mutations in either *SIX1* or *EYA1* lead to branchio-oto-renal syndrome with congenital deafness (Kochhar et al., 2007).

* Corresponding author.
E-mail address: Gerhard.schlosser@nuigalway.ie (G. Schlosser).
[1] Current address: Institut Curie, CNRS UMR 3215, INSERM U934, Stem Cells and Tissue Homeostasis Group, FP75005 Paris, France.

http://dx.doi.org/10.1016/j.ydbio.2017.09.027
Received 12 June 2017; Received in revised form 19 September 2017; Accepted 19 September 2017

Taken together, these previous studies suggest that Six1 and Eya1 play a dual role in placodal neurogenesis, promoting both the expansion of proliferative progenitors as well as sensory or neuronal differentiation. We have previously shown in *Xenopus* that these effects of Six1 and Eya1 are strongly dose-dependent: High doses of Six1 and Eya1 promote upregulation of progenitor genes *sox2* and *sox3*, and massive proliferation and repression of neuronal differentiation whereas lower doses lead to increased and partly ectopic expression of the neuronal differentiation gene *neurod1* and $p27^{Xic1}$, an inhibitor of cyclin dependent kinases (Schlosser et al., 2008).

To further elucidate how Six1 and Eya1 regulate neurogenesis, we recently completed an RNA-Seq screen for their direct target genes in the developing placodes using overexpression of hormone-inducible constructs of Six1 and Eya1 in the presence of protein synthesis inhibitors (Riddiford and Schlosser, 2016). As well as recovering *atoh1*, which was previously shown to be directly regulated by Six1/Eya1 (Ahmed et al., 2012a), this screen identified hundreds of novel putatively direct targets of Six1 or Eya1, including many transcriptional regulators of both progenitor fates (e.g. *sox2*, *sox3*, *hes8, hes9.1*) and neuronal or sensory differentiation (e.g. *neurog1*, *pou4f1.2*, *islet2*, *tlx1*, *gfi1*). Subsequent loss of function studies showed that Six1 and Eya1 are required for placodal expression of all of these genes (Riddiford and Schlosser, 2016).

We now present gain and loss of function studies in *Xenopus laevis* that address how two of the target genes identified in this screen, *hes8* and *neurog1* mediate the function of Six1 and Eya1 during placodal neurogenesis. We show that *hes8* belongs to the *Hes5* family of transcriptional repressors, which have previously been shown to mediate repression of proneural genes and neuronal differentiation in response to Notch signaling (Hatakeyama et al., 2004; Holmberg et al., 2008; Kobayashi and Kageyama, 2014; Ma et al., 1998; Ohtsuka et al., 1999; Pierfelice et al., 2011). We establish that *hes8* is activated by Notch signaling and represses *neurog1* and neuronal differentiation. *hes8* knockdown shows that *hes8* is essential for limiting neuronal differentiation during normal placode development. We also demonstrate that both *hes8* and *neurog1* are cell autonomously activated by Six1 and Eya1 in a dose-dependent fashion, whereby higher doses promote upregulation of *hes8* and *neurog1* and repression of neuronal differentiation. For *neurog1* activation, Six1 and Eya1 synergize with Sox3, however, upregulation of *neurog1* at high doses of Six1 and Eya1 is observed only transiently, while low doses of Six1 and Eya1 ultimately promote both *neurog1* expression and neuronal differentiation. We finally show that Six1 and Eya1 can activate *hes8* and arrest neuronal differentiation even when Notch signaling is blocked. Taken together, our findings indicate that Six1 and Eya1 can both promote and arrest neuronal differentiation by activating the Notch pathway genes *neurog1* and *hes8*, respectively, revealing a novel mechanism of Six1/Eya1 action during placodal neurogenesis.

2. Materials and methods

2.1. Phylogenetic reconstruction

Amino acid residues of vertebrate *Hes* genes (see Table S1 for sequence accession numbers) were aligned across the conserved basic helix-loop-helix (bHLH) and Orange domains. Positions with alignment gaps in a majority of the sequences were removed and the regions concatenated, resulting in an alignment (Fig. S1) of 76 residues shown here as a 50% consensus with invariant sites underlined 5′ - RKP XVEKXRRXRINXSLEQLKXLLXXXXXXXX-XXSKLEKADILEMTVXX LGYSXCLXEXXXFLXXXXXXXLLXHL - 3′. Phylogenetic relationships of the genes were estimated via Bayesian inference (Ronquist and Huelsenbeck, 2003) using the WAG (Whelan and Goldman, 2001) amino acid substitution model including gamma among site rate variation. Analyses were run for 1,100,000 generations, with samples saved every 200 generations, and the first 50,000 generations eliminated as 'burn-in' prior to forming a consensus tree using the sumt command. In addition to the Bayesian approach, a Maximum Likelihood (ML) tree was also generated using RaxML (Stamatakis et al., 2005) to verify topology, and is shown in Fig. S2.

2.2. Expression constructs

The *pCS2*$^{+}$*-mt-eya1α* (Schlosser et al., 2008) and *pCS2*$^{+}$*-mt-six1* (Brugmann et al., 2004) plasmids injected to test for cell-autonomous effects were previously described. *Six1*, *eya1, gr-six1, gr-eya1, gr-sox3, hes8, notch-ICD* and *su(h)-DBM* mRNAs were made from *pDH105-six1* (Pandur and Moody, 2000), *pCS2*$^{+}$*-eya1α* (Ahrens and Schlosser, 2005), *pCS2*$^{+}$*-gr-mt-six1* (Schlosser et al., 2008), *pCS2*$^{+}$*-gr-mt-eya1α* (Schlosser et al., 2008), *pCS2-gr-sox3-gfp* (Zhang and Klymkowsky, 2007), *pCS2*$^{+}$*-hes8* (Riddiford and Schlosser, 2016), *pCS2*$^{+}$*-notch-ICD* (Wettstein et al., 1997) and *pCS2*$^{+}$*-su(h)-DBM* (Wettstein et al., 1997) plasmids. *pCS2*$^{+}$*-hes8* contains only the coding sequence of *hes8* and lacks the 5′UTR essential for binding of our translation blocking morpholino antisense oligonucleotide (MO) against *hes8* (see below). To verify efficiency of MO binding in western blots, we therefore generated a *hes8-gfp* fusion construct with a partially restored 5′UTR containing the missing *hes8* MO binding sequence. To construct *pCS2*$^{+}$*-hes8-gfp*, we PCR amplified the insert from *pCS2*$^{+}$*-hes8* with forward primers containing the MO binding sequence and a *Cla*I restriction site and reverse primers with an *Eco*RI restriction site (forward: 5′-CCCATCGATGGGCACAGACAGAGTGCAATAAATGGCTTCTACCCACG-TT-3′; reverse: 5′-CCGGAATTCCGGTTACCACGGCCTCCAGATT-3′) and after restriction digestion subcloned the PCR fragment upstream of *gfp* into *pCMTEGFP* (kindly provided by Doris Wedlich), replacing the myc tag (*mt*) of this plasmid. A stop codon and frameshift erroneously introduced downstream of *hes8* during this procedure were then corrected by PCR site directed mutagenesis (forward primer: 5′-CTGGAGGCCGTGGTACAGGAATTCTATGGTGAGCAAGGGC-3′; reverse primer: 5′-GCCCTTGCTCACCATAGAATTCCTGTACCACGGCC TCCAG-3′). The sequence was confirmed by sequencing.

2.3. Morpholinos

Translation blocking MOs against *six1* (*six1* MO1: 5′-GGAAGGCAGCATAGACATGGCTCAG-3′; *six1* MO2: 5′-CGCACAC GCAAACACATACACGGG-3′; (Brugmann et al., 2004), and *eya1* (*eya1* MO1: 5′-TACTATGTGGACTGGTTAGATCCTG-3′; *eya1* MO2: 5′-ATATTTGTTCTGTCAGTGGCAAGTC-3′; (Schlosser et al., 2008)) were previously described and analyzed for specificity. A new translation blocking MO was designed for *hes8* (5′-AGAAGCCATTTA TTGCACTCTGTCT-3′) targeting positions −16 to +9 around the *hes8* start codon. The specificity of *hes8* MO was verified in western blots following in vitro transcription and translation (TNT-coupled reticulocyte lysate kit, Promega) of *pCS2*$^{+}$*-hes8-gfp* (1 μg /25 μl reaction) with and without MO (25 μM) visualized by rabbit anti-GFP antibody (Santa Cruz, sc8334; 1:1000) as previously described (Ahrens and Schlosser, 2005). A standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′) and an *eya1* 5 mismatch MO (5′-ATtTTaGTTCTGaCAGTGGgAAcTC-3′) obtained from GeneTools were used in control injections.

2.4. Microinjections

Embryos of *Xenopus laevis* were obtained by hormone induced egg laying followed by in vitro fertilization or natural matings, 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 4-cell stage that give rise to the dorsal ectoderm. 500 pg of *pCS2*$^{+}$*-mt-eya1α* and *pCS2*$^{+}$*-mt-six1* plasmids were injected. The following amounts of mRNAs were injected: *six1*: 500 pg; *eya1*: 500 pg; *gr-six1*: 250–2000 pg; *gr-eya1*: 250–2000 pg;

gr-sox3: 500 pg*; hes8*: 62.5–500 pg*; notch-ICD*: 500 pg; and *su(h)-DBM*: 500 pg. For dosage experiments 250–2000 pg *gr-six1* were coinjected with equal amounts of *gr-eya1*. Morpholinos (see above) were injected singly or as a cocktail (1–2 ng each) into single blastomeres at the 2–16 cell stage. Co-injection of *mt-gfp* (125 pg) or *lacZ* (250 pg) identified the injected side. For activation of hormone-inducible constructs, embryos were incubated in dexamethasone (10 μM; Sigma) from stages 11–13 onwards. To block protein synthesis, cycloheximide-treatment (50 μg/ml; Sigma) at stage 11–13 was followed after 30′ by incubation in cycloheximide supplemented with dexamethasone (10 μM) for 3–4 h at room temperature.

2.5. DAPT treatment

Embryos were treated with N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylester (DAPT) from stage 11 on. A stock solution of 10 mM DAPT in DMSO was diluted 1:100 for treatments. Control embryos were treated with 1:100 DMSO.

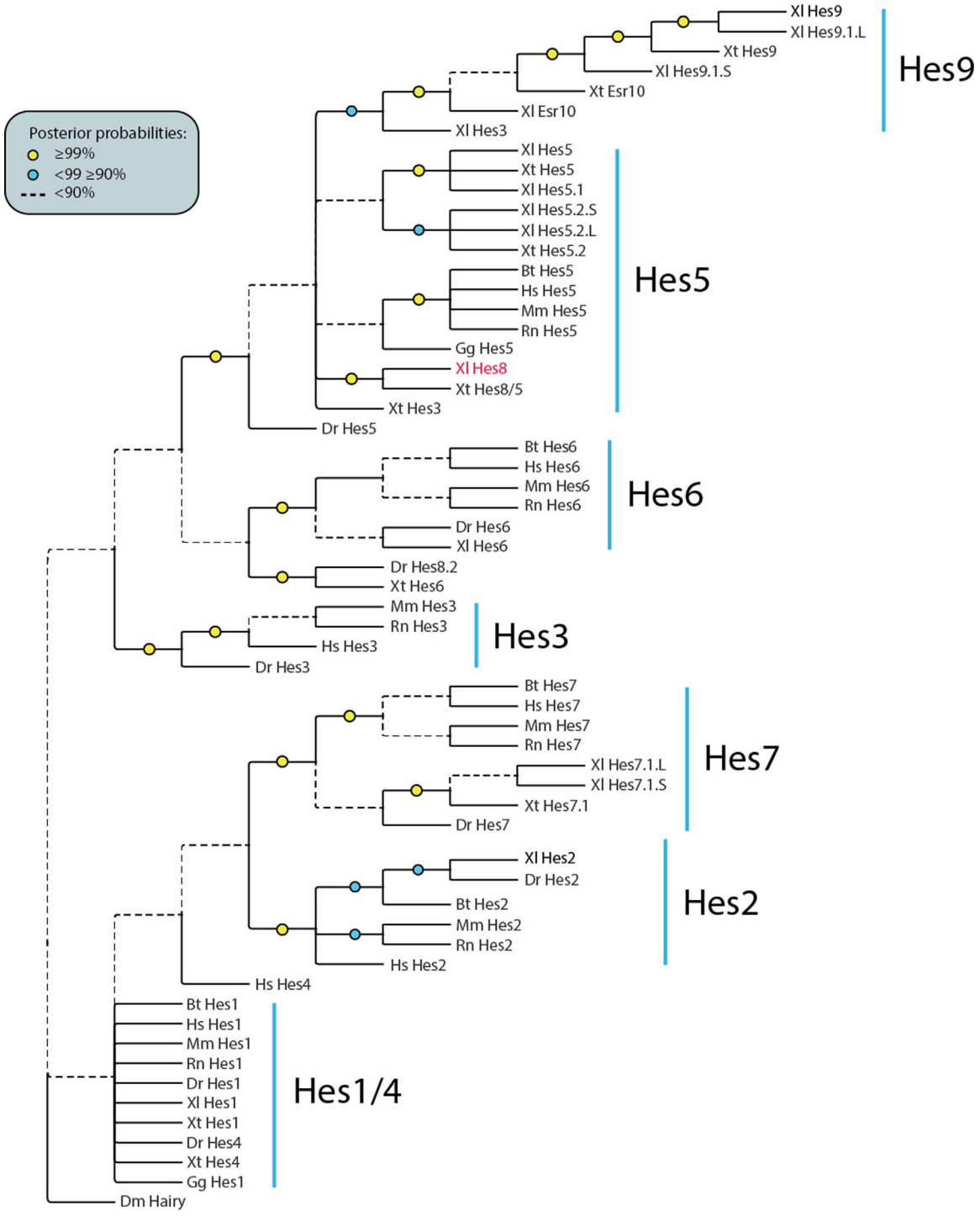

Fig. 1. Phylogeny of Hes proteins. Bayesian inference analysis of vertebrate Hes proteins based on a concatenated alignment of 76 amino acid residues encompassing the conserved bHLH and Orange domains (Fig. S1). *X. laevis* Hes8 shown in red. Nodal support shown as posterior probabilities where ≥ 90 is shown as solid lines and support below 90% shown as dashed lines. Accession numbers can be found in Table S1. Abbreviations: Dr: *Danio rerio*, Xl: *Xenopus laevis*, Xt: *Xenopus tropicalis*, Gg: *Gallus gallus*, Bt: *Bos taurus*, Hs: *Homo sapiens*, Rn: *Rattus norvegicus*, Mm: *Mus musculus*, Dm: *Drosophila melanogaster* (outgroup).

2.6. In situ hybridization and immunohistochemistry

Embryos injected with *mt-gfp* were sorted under a fluorescent stereomicroscope and were 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 under high stringency conditions at 60 °C as previously described (Schlosser and Ahrens, 2004) using digoxigenin-labeled antisense probes against *hes8* (Riddiford and Schlosser, 2016), *neurog1* (Nieber et al., 2009), *neurog2* (= *Ngnr:* (Ma et al., 1996), *delta1* (Chitnis, 1995), *neurod1* (Lee et al., 1995), *tubb2b* (= *N-Tubulin:* (Oschwald et al., 1991)), *sox3* (Penzel et al., 1997), *pou4f1.2* (Hutcheson and Vetter, 2001). 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). Since embryos sometimes displayed complex phenotypes with reduced marker expression in some placodes but increased marker expression in other placodes or adjacent non-neural ectoderm, we treated reductions and increases as independent and not mutually exclusive categories. All embryos were scored first for evidence of reduced marker expression in any of the placodes and second for evidence of increased marker expression in any of the placodes or adjacent non-neural ectoderm.

3. Results

Xenopus laevis hes8 was recently identified together with other *Hes* genes (*hes2*, *hes9.1*) in a screen for direct target genes of *six1* and *eya1* in the cranial placodes (Riddiford and Schlosser, 2016). To elucidate the role of *hes8* in Six1/Eya1 mediated neurogenesis we first investigated its phylogenetic relationship to other *Hes* genes.

3.1. Phylogenetic relationship of hes8

Hes8 was annotated as a predicted *X. tropicalis* gene that, when further scrutinized, appeared to bear close sequence similarity to other vertebrate Hes5 orthologs. In order to correctly classify *hes8*, amino acid sequences of vertebrate *Hes* genes (see Table S1 for accession numbers) were aligned across the conserved basic helix-loop-helix (bHLH) and Orange domains (Fig. S1), and analyzed using both Bayesian and Maximum Likelihood methods. The Bayesian tree clearly resolves most Hes subfamilies, showing strong support (posterior probability ≥ 99%) for Hes2, Hes3, Hes7, and good support (posterior probability ≥ 90%) for Hes9, as shown in Fig. 1. Hes1 and Hes4 remain unresolved as a polytomy. The Hes5/Hes9 subfamily is strongly supported as a group, consistent with a previous report that the *Hes9* gene family arose either from a *Hes5* duplication or a divergent *Hes5* pseudoallele (Li et al., 2003b). Hes8 clusters within the Hes5/Hes9

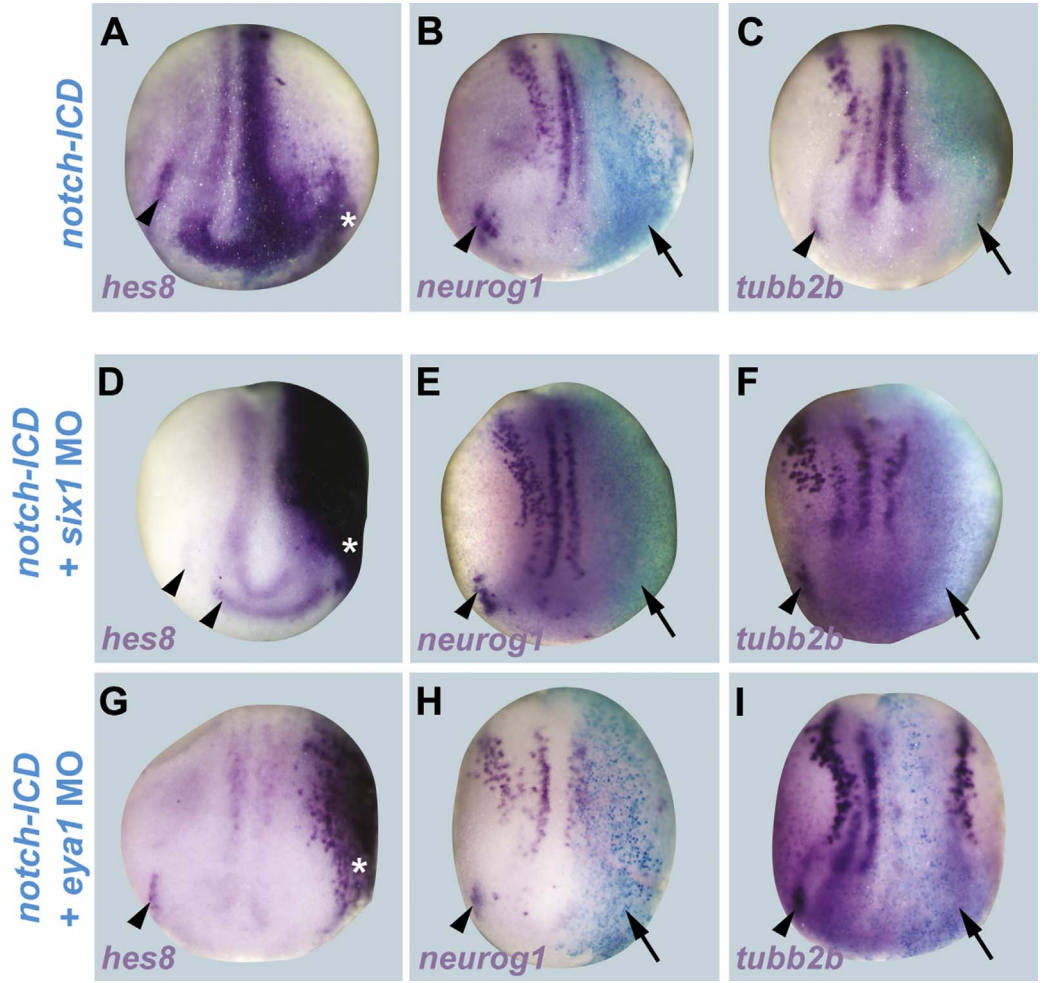

Fig. 2. *Hes8* is activated by Notch signaling. Expression of *hes8*, *neurog1* and *tubb2b* in neural plate stage *Xenopus* embryos after injection of *notch-ICD* either alone (**A-C**) or together with *six1* MO (**D-F**) or *eya1* MO (**G-I**). Control side is shown on the left and injected side on the right (as indicated by blue lacZ staining). In each case, injection of *notch-ICD* leads to upregulation of *hes8* (**A, D, G**) and repression of *neurog1* (**B, E, H**) and *tubb2b* (**C, F, I**). Arrowheads indicate expression domains in developing placodes. Arrows indicate reductions in placodal expression while asterisks indicate increased expression in placodes or adjacent non-neural ectoderm.

clade, but is outside the Hes9 clade.

As well as successfully resolving the classification of *Xenopus Hes* genes, this phylogeny represents the most complete survey of *Hes* genes in vertebrates to date, and would additionally recommend the reclassification of the *X. laevis* gene *hes3* (accession NP_001082163), which clusters with strong support within the *Hes9* clade, as a *Hes9* gene, as well as the *D. rario* gene *hes8.2* (accession NP_001159638) as a *Hes6* gene. The Maximum Likelihood tree resolves gene families almost identically to the Bayesian analysis, showing agreement among all subfamilies (Fig. S2).

3.2. Hes8 represses neurog1 and neuronal differentiation as consequence of lateral inhibition

We next tested whether *hes8,* like *hes5,* can act as a repressor of neuronal differentiation in response to Notch signaling. Activation of the Notch pathway by injection of *notch-ICD* led to broadening of *hes8* expression and concomitant reduction of *neurog1* and *tubb2b* in placodes and neural plate (Fig. 2A-C, Table 1). This suggests that *hes8* is upregulated in response to Notch signaling and is compatible with a potential role of *hes8* in mediating repression of neuronal differentiation downstream of Notch (lateral inhibition). To test this, we injected four different doses (62.5 pg, 125 pg, 250 pg, 500 pg) of *hes8* mRNA into one prospective dorsal blastomere at the four cell stage. Injection of all but the highest dose had similar phenotypic effects resulting in the downregulation of neuronal determination (*neurog1*, *neurog2*) and differentiation genes (*neurod1*), as well as *delta1* (a Notch ligand and neurogenin target gene) and the terminal neuronal differentiation marker *tubb2b* in placodes and the neural plate (Fig. 3A-C, E-G; Table 2). Placodal *sox3* expression was likewise reduced (Fig. 3D; Table 2). Overexpression of very high doses (500 pg) of *hes8* instead led to strong gastrulation defects with expansion of neural and neuronal markers on the injected side possibly as a result of compromised convergent extension (not shown). Thus, *hes8* is sufficient to repress neuronal differentiation upstream of, and possibly also in parallel to, *neurog1/neurog2*. Taken together, this suggests that *hes8*, like *hes5*, mediates lateral inhibition by repressing neuronal differentiation in response to Notch signaling.

Since we previously identified *hes8* as a putative direct target of Six1 and Eya1 (Riddiford and Schlosser, 2016), we next co-injected *six1* or *eya1* MOs with *notch-ICD* to test whether Six1 or Eya1 are required for Notch-mediated *hes8* activation. *Hes8* was strongly upregulated on the injected side even when Six1 or Eya1 were knocked down (Fig. 2D-I, Table 1) suggesting that *hes8* can be activated by Notch signaling even in the absence of Six1 or Eya1.

3.3. Hes8 is required for limiting neuronal differentiation during normal placodal development

To elucidate whether *hes8* is required for normal placodal neurogenesis, we designed a translation blocking MO that specifically and effectively knocks down Hes8 (Fig. 3H, I). Injection of 1–2 ng of *hes8* MO into one blastomere at the 2- or 4-cell stage resulted in complex phenotypes, with most marker genes showing both reductions and increases in expression (Fig. 3, I-R, Table 2). *hes8* expression itself was typically broadened and increased after *hes8* MO injection suggesting that Hes8 represses its own transcription (Fig. 3K, Table 2). The number of embryos with broadened *neurog1*, *neurog2*, *delta1* and *pou4f1.2* expression in the developing placodes was also significantly increased, indicating that Hes8 limits the expression of proneural genes and some of their target genes during normal placodal development (Fig. 3L-N, P). Coinjection of *hes8* mRNA significantly reduces the frequency of embryos with increased placodal *neurog1* expression (rescue) indicating that *hes8* MOs act by specifically depleting Hes8 (Fig. 3J). Reductions of placodal expression of *hes8*, *neurog1*, *neurog2*, *delta1* and *pou4f1.2* were also occasionally observed after *hes8* MO

Table 1
Changes in marker gene expression in the placodal and non-neural ectoderm after injection of various constructs.

Injection	eya1 5 mm MO		eya1 MO1+2[1]		six1 MO1+2[1]		eya1+DMSO		six1+DMSO		notch-ICD		eya1 MO1+2+notch-ICD		six1 MO1+2+notch-ICD		eya1+notch-ICD		six1+notch-ICD		su(h)-DBM		eya1+su(h)-DBM		six1+su(h)-DBM		eya1+DAPT		six1+DAPT	
Phenotype	%		%		%		%		%		%		%		%		%		%		%		%		%		%	
	(n)		(n)		(n)		(n)		(n)		(n)		(n)		(n)		(n)		(n)		(n)		(n)		(n)		(n)	
hes8																												
Reduced	11		83**		74**		61		73		0		0		0		0		0		95		55		73		65		59	
	(27)		(35)		(35)		(36)		(22)		(33)		(17)		(38)		(36)		(41)		(19)		(62)		(22)		(34)		(27)	
Increased/ ectopic	0		0		0		32		27		100		100		74		100		100		0		69		50		29		37	
	(27)		(35)		(35)		(37)		(22)		(33)		(17)		(38)		(36)		(41)		(19)		(62)		(22)		(34)		(27)	
neurog1																												
Reduced	10		49**		65**		51		77		96		69		87		79		83		11		14		35		44		86	
	(39)		(43)		(51)		(49)		(47)		(25)		(13)		(15)		(19)		(18)		(27)		(56)		(23)		(52)		(49)	
Increased/ ectopic	3		16		0		27		2		0		0		7		0		0		81		71		57		27		2	
	(39)		(43)		(51)		(49)		(47)		(25)		(13)		(15)		(19)		(18)		(27)		(56)		(23)		(52)		(49)	
tubb2b																												
Reduced	33		74*		67*		94		93		87		93		100		96		100		18		30		48		95		81	
	(27)		(24)		(94)		(17)		(30)		(39)		(14)		(18)		(23)		(14)		(28)		(40)		(23)		(19)		(26)	
Increased/ ectopic	0		0		0		0		3		0		0		0		0		0		46		52		44		0		0	
	(21)		(24)		(94)		(17)		(30)		(39)		(14)		(18)		(23)		(14)		(28)		(40)		(23)		(19)		(26)	

n: number of embryos analyzed at neural plate (stage 14–16); some embryos could only be checked for reduced or for increased expression and numbers analyzed for reduced expression are, thus, not always identical to numbers analyzed for increased expression.

[1] Significant differences (Fisher's exact test; *: $p < 0.05$, **: $p < 0.001$) to *eya1* 5 mm MO injections are indicated; data for *hes8* and *neurog1* from Riddiford and Schlosser (2016).

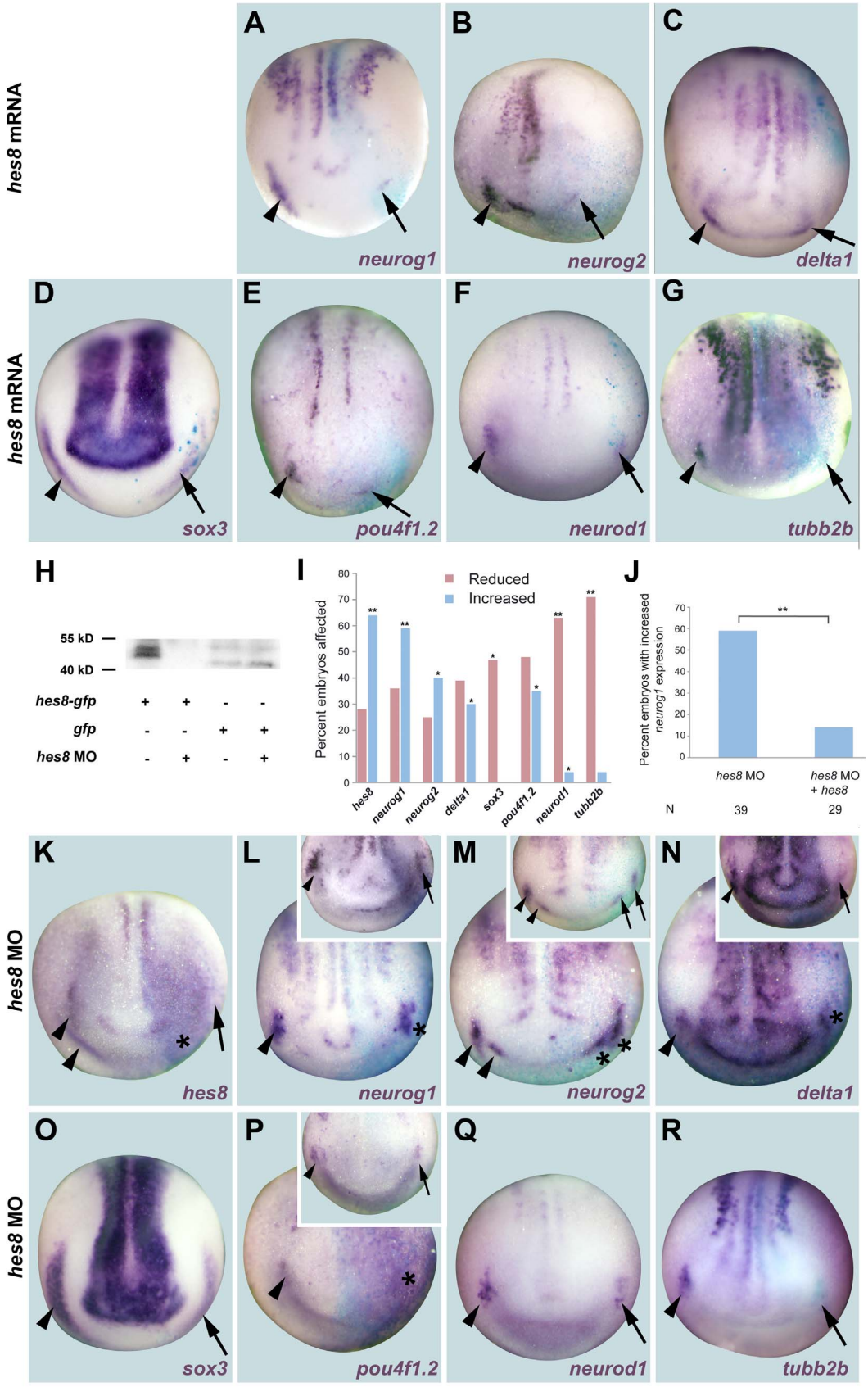

Fig. 3. *Hes8* represses neuronal determination genes. A-G, K-R: Expression of various neurogenesis genes in neural plate stage *Xenopus* embryos after injection of *hes8* mRNA (**A-G**) or *hes8* MO (**K-R**). Control side is shown on the left and injected side on the right (as indicated by blue LacZ staining). Arrowheads indicate expression domains in developing placodes. Arrows

indicate reductions in placodal expression while asterisks indicate increased expression in placodes or adjacent non-neural ectoderm. Hes8 overexpression leads to repression of all markers analyzed, while Hes8 knockdown promotes expansion of *hes8*, *neurog1*, *neurog2*, *delta1* and *pou4f1.2* but reduction of *sox3*, *neurod1* and *tubb2b* expression. **H**: Western blot after in vitro transcription and translation of *hes8* in the presence or absence of *hes8* MO demonstrates efficacy of *hes8* MO. **I**: Frequency of embryos with reductions of neurogenesis genes in PPR/placodes (red) or increases of neurogenesis genes in PPR/placodes or adjacent non-neural ectoderm (blue) after *hes8* MO injections (see Table 3 for numbers). Significant changes to control MO are indicated (Fisher's exact test; *: $p < 0.05$, **: $p < 0.001$). **J**: Increased placodal *neurog1* expression after injection of *hes8* MO is rescued by coinjection of *hes8* mRNA.

injection but were not significantly more frequent than after injection of control MOs. In contrast, embryos with reduced placodal expression of *sox3*, *neurod1* and *tubb2b* were significantly more frequent after *hes8* MO knockdown and these markers were almost never increased (Fig. 3O, Q, R). This suggests that Hes8 is required to promote *sox3* expression as well as *neurod1*-mediated neuronal differentiation downstream of the neurogenins during normal placodal development, possibly by repressing other repressors.

Table 2
Changes in marker gene expression in the ectoderm after Hes8 gain and loss of function.

Injection	**Cont. MO**	***hes8* MO (1 ng)**[1]	***hes8* mRNA (67.5–250 pg)**
Phenotype	% (n)	% (n)	% (n)
hes8			
Reduced	15 (26)	28 (36)	nd
Increased/ectopic	0 (16)	64** (36)	nd
neurog1			
Reduced	13 (15)	36 (39)	70 (82)
Increased/ectopic	7 (15)	64** (39)	21 (82)
neurog2			
Reduced	5 (19)	25 (20)	100 (12)
Increased/ectopic	0 (19)	40* (20)	0 (12)
delta1			
Reduced	24 (17)	39 (23)	72 (54)
Increased/ectopic	0 (17)	30* (23)	0 (54)
pou4f1.2			
Reduced	24 (21)	48 (23)	88 (16)
Increased/ectopic	0 (21)	35* (23)	12 (16)
neurod1			
Reduced	5 (19)	63** (24)	67 (12)
Increased/ectopic	10 (19)	4* (24)	0 (12)
tubb2b			
Reduced	19 (26)	71** (28)	51 (86)
Increased/ectopic	0 (11)	4 (28)	24 (86)
sox3			
Reduced	0 (24)	47* (30)	40 (88)
Increased/ectopic	0 (24)	0 (30)	8 (88)
six1			
Reduced	10 (31)	38* (47)	100 (24)
Increased/ectopic	0 (31)	19* (47)	0 (24)
eya1			
Reduced	3 (29)	19* (52)	100 (10)
Increased/ectopic	0 (29)	23* (52)	0 (10)

n: number of embryos analyzed at neural plate (stage 14–16); some embryos could only be checked for reduced or for increased expression and numbers analyzed for reduced expression are, thus, not always identical to numbers analyzed for increased expression.

[1] Significant differences (Fisher's exact test; *: $p < 0.05$, **: $p < 0.001$) to control MO injections are indicated.

3.4. Six1 and Eya1 promote hes8 in a dose-dependent fashion

We have previously shown that *hes8* is expressed in all neurogenic placodes and requires Six1 and Eya1 for placodal expression (Table 1; Riddiford and Schlosser, 2016). To elucidate its function downstream of Six1 and Eya1, we first checked whether *hes8* shows a dose-dependent response to Six1 and Eya1 like other Six1/Eya1 target genes. Previous studies found the effects of Six1 and Eya1 on placodal neurogenesis to be strongly dose-dependent, with high doses (1000–2000 pg) promoting expression of progenitor markers (*sox3*, *p27^{Xic1}*) and lower doses (250–500 pg) leading to ectopic expression of neuronal determination and differentiation genes (*neurog2*, *neurod1*) at early tailbud stages (Schlosser et al., 2008).

To test whether Six1 and Eya1 also affect *hes8* expression in a dose-dependent way, we co-injected different levels of mRNAs encoding hormone-inducible forms of GR-Eya1 and GR-Six1 (Kolm and Sive, 1995; Seo et al., 2007) into one of two blastomeres and induced nuclear translocation of the proteins at the end of gastrulation by dexamethasone (DEX) treatment. Whereas the normal expression domains of *hes8* in the developing placodes at neural plate stages were frequently reduced at all doses injected, Six1/Eya1 promoted increased expression of *hes8* in the non-neural ectoderm (expanded placodal or ectopic expression) with increasing frequency and intensity at higher doses (Fig. 4A). This is also observed in the presence of CHX suggesting that *hes8* may be directly transcriptionally regulated by Six1/Eya1 in a dose-dependent manner (Fig. S3). To verify that *Hes8* is upregulated cell-autonomously, we injected plasmids encoding myc-tagged forms of Six1 or Eya1 resulting in a mosaic overexpression of these proteins. Ectopic *hes8* expression was observed exclusively in a subset of the ectodermal cells immunopositive for myc (or for coinjected lacZ) indicating that *hes8* is upregulated cell-autonomously in cells that overexpress Six1 (not shown) or Eya1 (Fig. 5A).

3.5. Six1 and Eya1 promote neurog1 but arrest neuronal differentiation in a dose-dependent fashion

Confirming our previous results at tailbud stages (Schlosser et al., 2008), increasing doses of Six1/Eya1 also result in increasing ectopic expression of another progenitor gene, *sox3*, in the preplacodal region and adjacent ectoderm at neural plate stages (Fig. 4D), while placodal expression of the proneural gene *neurog1* (Fig. 4B) and of the neuronal differentiation genes *neurod1* and *tubb2b* is increasingly reduced (Fig. 4E,F) possibly as a consequence of increased *hes8* expression. Low doses of Six1/Eya1, conversely, promote increased placodal expression of *neurod1* but not of *tubb2b* (Fig. 4, E,F). While placodal *neurog1* expression was repressed in some embryos, it was also upregulated and/or broadened in others. Surprisingly the prevalence and intensity of *neurog1* upregulation increased with higher doses of Six1 and Eya1, similar to *hes8* and different from *neurod1* (Fig. 4, A,B,E). As described for *hes8* above, this dose-dependent upregulation of *neurog1* expression is also observed in the presence of CHX suggesting that *neurog1* may be directly transcriptionally regulated by Six1/Eya1 in a dose-dependent manner (Fig. S3).

To verify that *neurog1* is upregulated cell-autonomously, we injected plasmids encoding myc-tagged forms of Six1 or Eya1. Ectopic *neurog1* (but not *tubb2b*) expression was observed exclusively in a subset of the ectodermal cells immunopositive for myc mostly near

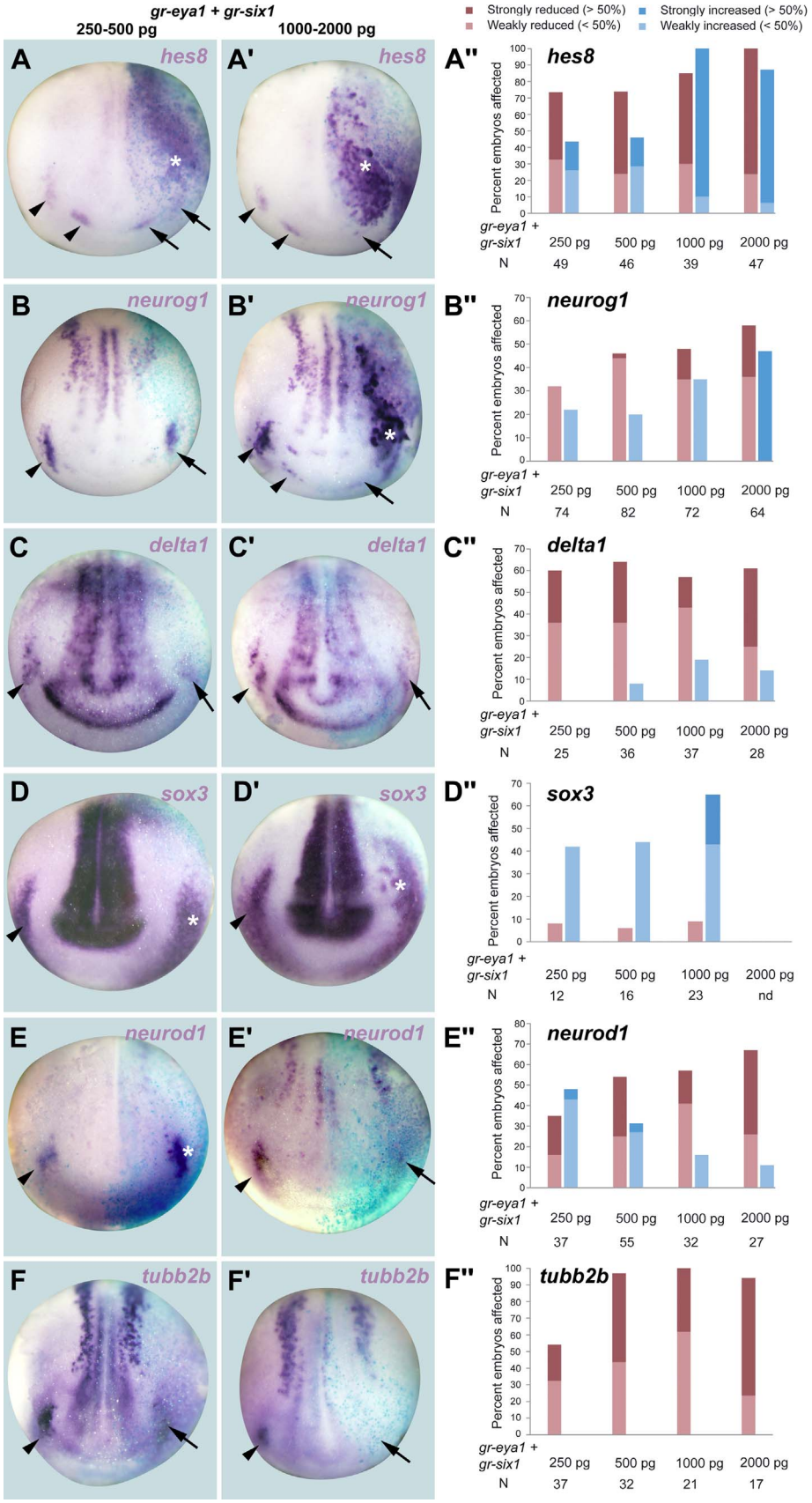

Fig. 4. Dose-dependent activation or repression of neurogenesis genes by Six1 and Eya1 at neural plate stages. A-F, A′-F′: Expression of various neurogenesis genes in neural plate stage *Xenopus* embryos after injection of low (first column, **A-F**) or high doses (second column, **A′-F′**) of *gr-six1* and *gr-eya1* mRNA followed by DEX activation at end of gastrulation. Control side is shown on the left and injected side on the right (as indicated by blue LcZ staining). Arrowheads indicate expression domains in developing placodes. Arrows indicate reductions in placodal expression while asterisks indicate increased expression in placodes or adjacent non-neural ectoderm. Note that embryos may have reductions in some placodes but increased expression elsewhere (**A′,B′**). **A′′-F′′**: Frequency of embryos with reductions of neurogenesis genes in PPR/placodes (red) or increases of neurogenesis genes in PPR/placodes or adjacent non-neural ectoderm (blue) after injection of different doses of *gr-six1* and *gr-eya1* mRNA. N: number of embryos analyzed.

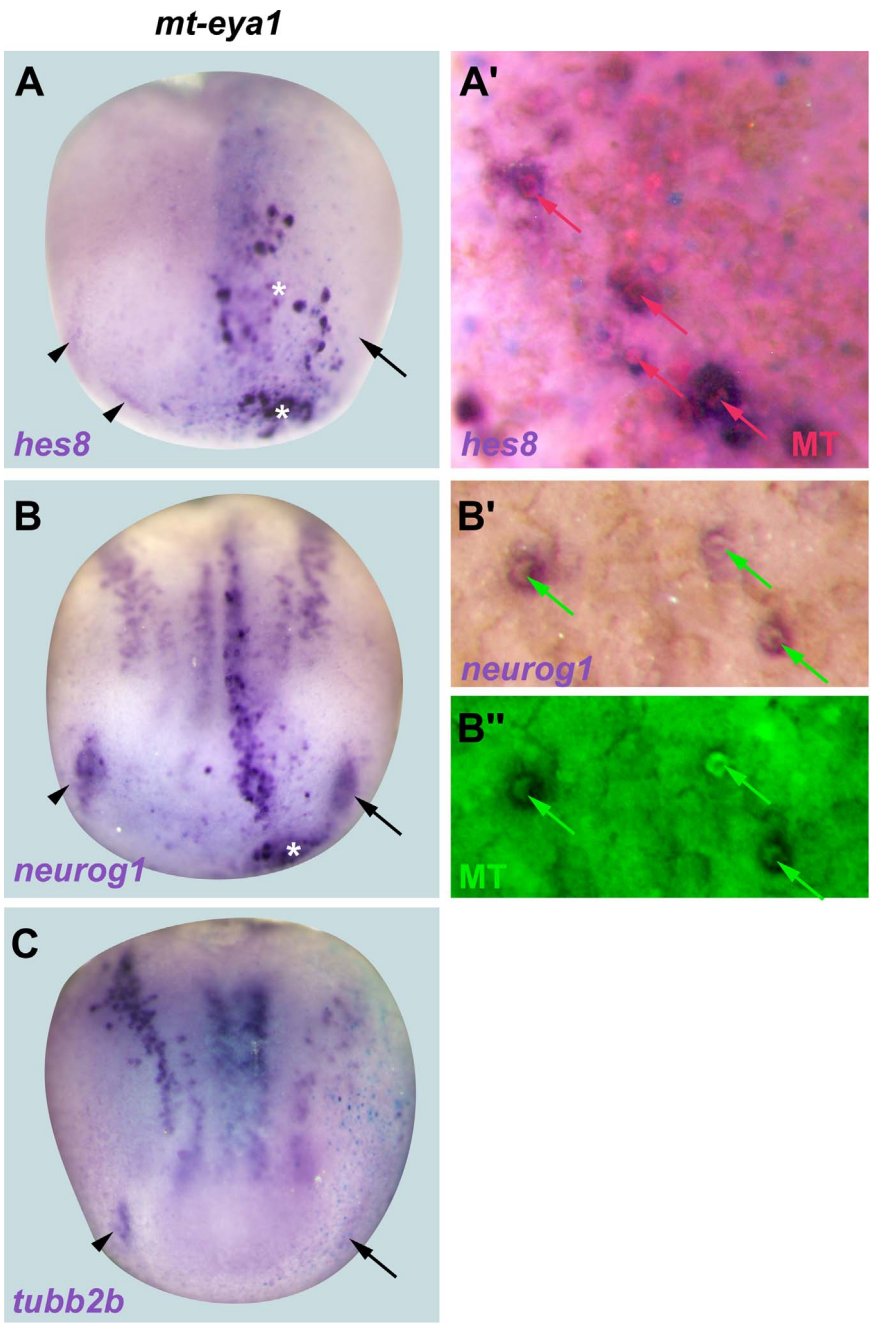

Fig. 5. Cell autonomous effects of Six1 and Eya1. A-C: Expression of *hes8, neurog1* and *tubb2b* in neural plate stage *Xenopus* embryos after injection of a myc-tagged (mt) *eya1* plasmid. Control side is shown on the left and injected side on the right (as indicated by blue LacZ staining). Arrowheads indicate expression domains in developing placodes. Arrows indicate reductions in placodal expression while asterisks indicate increased expression in placodes or adjacent non-neural ectoderm. Both *hes8* and *neurog1* but not *tubb2b* show ectopic expression. **A′, B′, B′′**: Ectopic expression of *hes8* and *neurog1* in non-neural ectoderm of embryos injected with *mt-eya1* at higher magnification together with immunostaining for myc (MT). Colored arrows indicate *hes8* and *neurog1* expression in MT immunopositive cells.

the normal *neurog1* expression domains (Fig. 5B). High levels of Six1 and Eya1 thus appear to cell-autonomously upregulate both *neurog1* and its repressor *hes8* but are not sufficient to drive ectopic *tubb2b* expression (Fig. 5C). Importantly, *neurog1* and *hes8* expression was only observed in a subset of cells overexpressing *six1* (not shown) or *eya1* (Fig. 5A,B). This raises the possibility that *neurog1* and *hes8* may be upregulated in mutually exclusive subsets of Six1/Eya1 expressing cells via lateral inhibition. The salt and pepper like expression of both genes after overexpression of high doses of Six1/Eya1 (Fig. 4A,B) supports this hypothesis, but further studies are needed to show this conclusively. Since *delta1* - a known target gene of *neurog1* and mediator of lateral inhibition - showed little evidence for dose-dependent regulation by Six1/Eya1 (Fig. 4C), other Notch ligands are likely to be involved.

3.6. Six1 and Eya1 synergize with Sox3 to activate neurog1 expression

Although we previously identified *neurog1* as a putative direct target of Six1 and Eya1 (Riddiford and Schlosser, 2016), embryos injected with *six1* or *eya1* plasmids displayed relatively mild increases of *neurog1* expression, or expansions into the vicinity of the normal placodal expression domains, suggesting that Six1 and Eya1 depend on additional factors for *neurog1* activation. *SoxB1* genes have recently

Table 3
Changes in marker gene expression in the placodal and non-neural ectoderm after injection of various constructs.

Injection	*eya1* plasmid	*six1* plasmid	*eya1+six1* plasmid +gr-*sox3* cont.	*eya1+six1* plasmid +gr-*sox3* DEX11	*gr-sox3* DEX11
Phenotype	% (n)	% (n)	% (n)	% (n)	% (n)
hes8					
Reduced	67 (15)	82 (28)	54 (13)	25 (8)	33 (12)
Increased/ ectopic	84 (25)	75 (28)	89 (19)	87 (15)	0 (12)
neurog1					
Reduced	49 (41)	54 (48)	44 (18)	56 (16)	47 (19)
Increased/ ectopic	60[a] (42)	48[a] (48)	44[a] (18)	75[b] (20)	5[a] (19)
tubb2b					
Reduced	95 (38)	75 (28)	76 (17)	85 (13)	67 (15)
Increased/ ectopic	3 (38)	0 (28)	59 (17)	53 (15)	0 (15)

n: number of embryos analyzed at neural plate (stage 14–16) stage; some embryos could only be checked for reduced or for increased expression and numbers analyzed for reduced expression are, thus, not always identical to numbers analyzed for increased expression.

[a] Weak increases/few ectopically expressing cells.

[b] Strong increases/many ectopically expressing cells.

been shown to synergize with Six1/Eya1 to promote neuronal differentiation (Ahmed et al., 2012b). While *sox3* is itself upregulated after Six1 und Eya1 overexpression, the observed increase in *sox3* expression is relatively modest and often confined to the vicinity of the PPR. To test whether Sox3 co-operates with Six1 and Eya1 to promote *neurog1* expression we therefore coexpressed these transcription factors more broadly by co-injecting *gr-sox3* mRNA with *six1* and *eya1* plasmids and activating nuclear translocation of Sox3 by DEX treatment at the end of gastrulation. Control embryos, which were *six1/eya1/gr-sox3* injected but did not receive DEX treatment, showed an increase in scattered ectopic *tubb2b* expression but no changes in *neurog1* or *hes8* expression compared to embryos that were injected with *six1* or *eya1* alone, suggesting that Six1 and Eya1 synergize in promoting neuronal differentiation downstream of or in parallel to *neurog1* (Fig. S4D-F, Table 3). However, embryos that were *six1/eya1/gr-sox3* injected and DEX treated, showed more frequent and much stronger ectopic *neurog1* expression but no changes in *tubb2b* or *hes8* expression compared to control embryos, indicating that Sox3 indeed synergizes with Six1 and Eya1 in *neurog1* activation but not in *hes8* activation or in neuronal differentiation pathways downstream of or parallel to *neurog1* (Fig. 5 S4; Table 3).

3.7. Activation of neurog1 by Six1 and Eya1 is transient

Our observation that increasing doses of Six1 and Eya1 promoted activation of *neurog1* as well as *hes8* and *sox3* but inhibited neuronal differentiation raised the possibility that neuronal differentiation genes would be initially repressed (e.g. by high Hes8 and Sox3 levels) but upregulated by ectopic Neurog1 at later stages. To clarify whether this is the case, we analyzed embryos co-injected with *gr-six1* and *gr-eya1* and DEX-induced at the end of gastrulation at tailbud stages. Neuronal differentiation genes (*neurod1* and *tubb2b*) were still increasingly repressed with higher doses of Six1/Eya1, while ectopic expression of both genes was more frequent and stronger at lower doses similar to neural plate stages (confirming and extending our previous findings: (Schlosser et al., 2008)) (Fig. 6C-D). Similarly, increased or ectopic expression of *hes8* was more frequent at higher doses of Six1 and Eya1 (Fig. 6A) similar to neural plate stages. However, *neurog1* now was no longer increasingly upregulated with increasing doses of Six1 and Eya1 as it was at neural plate stages, but instead showed the opposite trend resembling *neurod1* and *tubb2b* (Fig. 6B). This suggests that the increasing upregulation of *neurog1* by higher doses of Six1 and Eya1 at neural plate stages is transient and this transient activation of *neurog1* is not sufficient to activate neuronal differentiation genes. At subsequent stages this dose-dependency is reversed (decreasing activation of *neurog1* with increasing concentration of Six1/Eya1) mirroring the dose-dependency of *neurod1* and *tubb2b*.

3.8. Six1 and Eya1 activate hes8 and arrest neuronal differentiation in a Notch-independent manner

We have shown that *hes8* can be activated either by Six1 or Eya1 or by Notch signaling (even in the absence of Six1 and Eya1). To test whether Six1 and Eya1 are able to activate *hes8* in a Notch-independent fashion, we injected either Six1 or Eya1 into one of two blastomeres and subsequently treated embryos with the Notch inhibitor DAPT from the end of gastrulation. Around 30% of the embryos showed increased or ectopic expression of *hes8* on the injected side after either Six1 or Eya1 injection in DAPT treated embryos, not significantly different from DMSO treated control embryos (Fig. 7, Fig. S4, Table 1). Conversely, placodal *neurog1* expression and neuronal differentiation (as indicated by *tubb2b* expression) was reduced in the majority of DAPT treated embryos, again not significantly different from DMSO treated controls (Fig. 7, Fig. S4, Table 1). This suggests that Six1 and Eya1 can activate *hes8* and arrest neuronal differentiation even in the absence of Notch signaling.

To corroborate this, we injected mRNA for *su(h)-DBM,* a dominant negative inhibitor of Notch signaling (Wettstein et al., 1997) either alone or in conjunction with *six1* or *eya1* mRNA into one out of two blastomeres and subsequently analyzed the embryos at neural plate stages. Whereas *su(h)-DBM* alone leads to reduction of *hes8* and expansion of *neurog1* and *tubb2b* expression in both placodes and neural plate in most embryos, the opposite phenotypes (increased *hes8* and reduced *neurog1* and *tubb2b*) are almost never observed. After co-injection of *six1* or *eya1*, however, *hes8* is increased or ectopically expressed in the majority of embryos, and *neurog1* and *tubb2b* are frequently reduced, in particular after co-injection of *six1* (Fig. 7, Fig. S4, Table 1).

Taken together this clearly indicates, that Six1 and Eya1 can activate *hes8* in the absence of Notch signaling and reduce *neurog1* expression and neuronal differentiation as reflected in *tubb2b* expression. Six1 and Eya1 may arrest or delay neuronal differentiation indirectly via *hes8* activation but this remains to be confirmed in further studies.

To test whether *hes8* is essential to mediate the arrest of neuronal differentiation downstream of Six1 and Eya1, we co-injected *hes8* MO (1 ng) with *gr-six1* and *gr-eya1* (500 pg, DEX at stage 11). This did not prevent the reduction of placodal *neurog1* (60%, N = 30 with *hes8* MO; 46%, N = 82 without Hes8 MO) or *tubb2b* expression (91%, N = 23 with *hes8* MO; 97%, N = 32 without *hes8* MO) at neural plate stages suggesting that other repressors of neuronal differentiation activated by Six1 and Eya1 (e.g. other *Hes* genes) may be able to substitute for *hes8* loss of function.

4. Discussion

4.1. Six1 and Eya1 promote progenitor states by exploiting the Notch pathway

Previous functional studies implicated Six1 and Eya1 in promoting both proliferative placodal progenitors and sensory/neuronal differentiation (Ahmed et al., 2012b; Chen et al., 2009; Schlosser et al., 2008; Zou et al., 2004) and our recent screen has identified multiple candidate target genes involved in these processes (Riddiford and

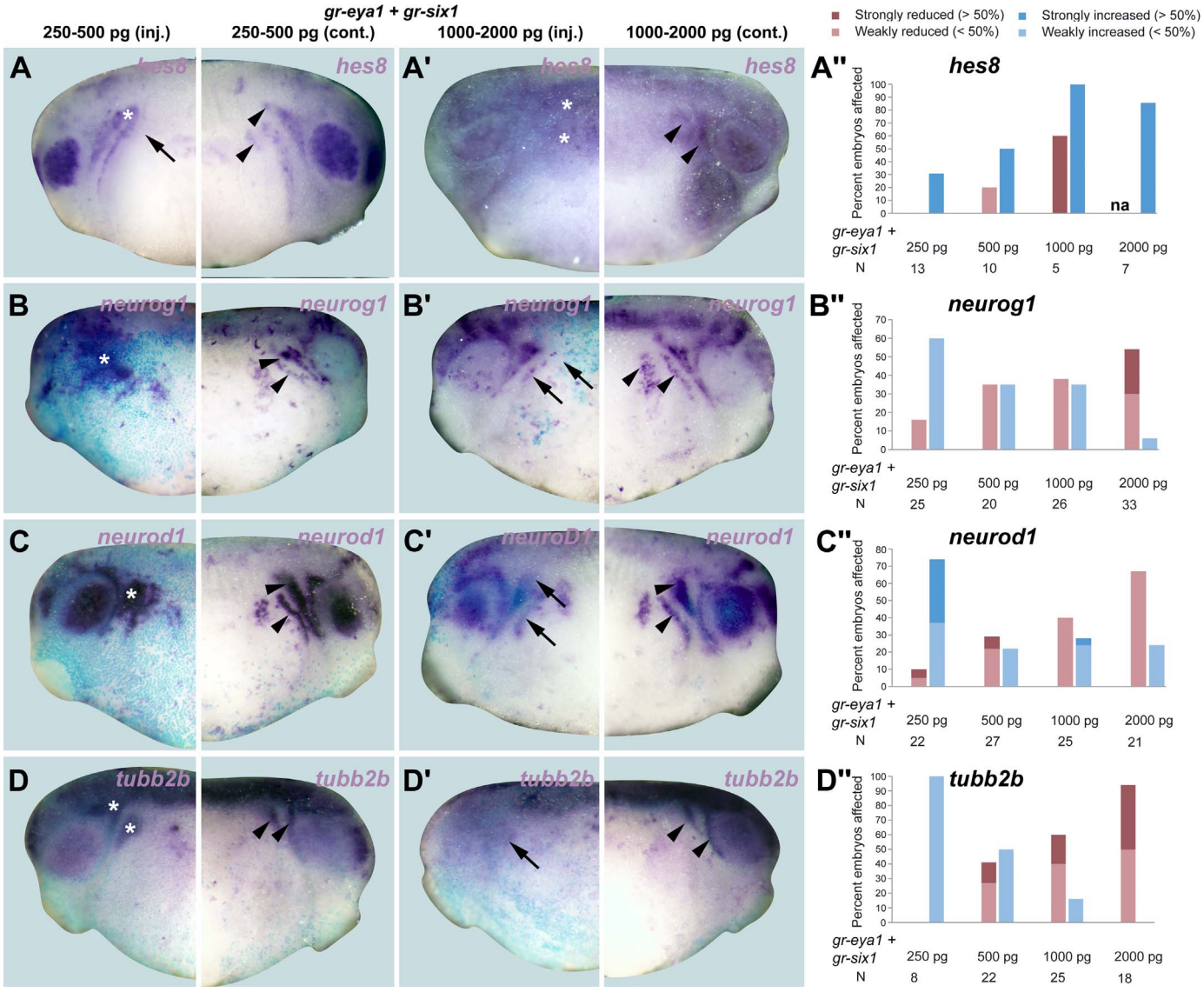

Fig. 6. Dose-dependent activation or repression of neurogenesis genes by Six1 and Eya1 at tailbud stages. A-D, A′-D′: Expression of various neurogenesis genes in tailbud stage *Xenopus* embryos after injection of low (**A-D**) or high doses (**A′-D′**) of *gr-six1* and *gr-eya1* mRNA followed by DEX activation at end of gastrulation. Control side is shown in the right and injected side in the left panels (as indicated by blue LacZ staining). Arrowheads indicate expression domains in developing placodes. Arrows indicate reductions in placodal expression while asterisks indicate increased expression in placodes or adjacent non-neural ectoderm. **A′′-D′′**: Frequency of embryos with reductions of neurogenesis genes in PPR/placodes (red) or increases of neurogenesis genes in PPR/placodes or adjacent non-neural ectoderm (blue) after injection of different doses of *gr-six1* and *gr-eya1* mRNA. N: number of embryos analyzed.

Schlosser, 2016). Two of these candidates, *hes8* and *neurog1*, are related to *Hes* genes and proneural genes which play crucial roles in Notch-mediated lateral inhibition. Proneural genes (e.g. *neurog1* or *neurog2*) on the one hand promote cell cycle exit and upregulate neuronal differentiation genes (e.g. *neuroD1*), that activate batteries of genes driving various aspects of neuronal differentiation (e.g. *POU4* class genes, *islet1/2*) and ultimately neuron specific structural genes (incl. *tubb2b*) (Dykes et al., 2011; Guillemot, 2007; Ma et al., 1996). On the other hand, they promote transcription of ligands of the Notch pathway like Delta1 (Ma et al., 1996). These ligands bind to Notch receptors on neighboring cells triggering cleavage of the intracellular domain (ICD) of Notch. The latter translocates to the nucleus where it binds to transcription factor Suppressor of hairless (Su(H)) and activates transcription of genes encoding the transcriptional repressors Hes1 and Hes5 (Contreras-Cornejo et al., 2016; Pierfelice et al., 2011; Wettstein et al., 1997). These repress neuronal determination genes thereby blocking neuronal differentiation in these neighboring cells (Hatakeyama et al., 2004; Holmberg et al., 2008; Kobayashi and Kageyama, 2014; Ma et al., 1998; Ohtsuka et al., 1999). This "lateral inhibition" mechanism ensures the maintenance of progenitor cells in the vicinity of differentiating neurons.

In the present study we confirm that *hes8* - like *hes1* and *hes5* - is activated by Notch signaling. Furthermore, our gain of function (GOF) studies also show that *hes8* is able to transcriptionally repress a wide spectrum of genes promoting neuronal differentiation while our loss of function (LOF) studies reveal that *hes8* is required to limit expression of *neurog1*, *neurog2*, its target *delta1* and *pou4f1.2* during normal placode development. Taken together this suggests that *hes8* plays similar roles to *hes1* and *hes5* in Notch-mediated lateral inhibition. However, our findings also show that *hes8* can be activated by Six1 and Eya1 in the absence of Notch signaling. Six1 and Eya1 thus maintain progenitor states at least partly by exploiting the Notch pathway even without signals from adjacent cells.

Both our GOF and LOF studies also indicate that *hes8* auto-represses its own transcription. Similar auto-repression has previously been reported for *hes1* and *hes5* and has been shown to drive

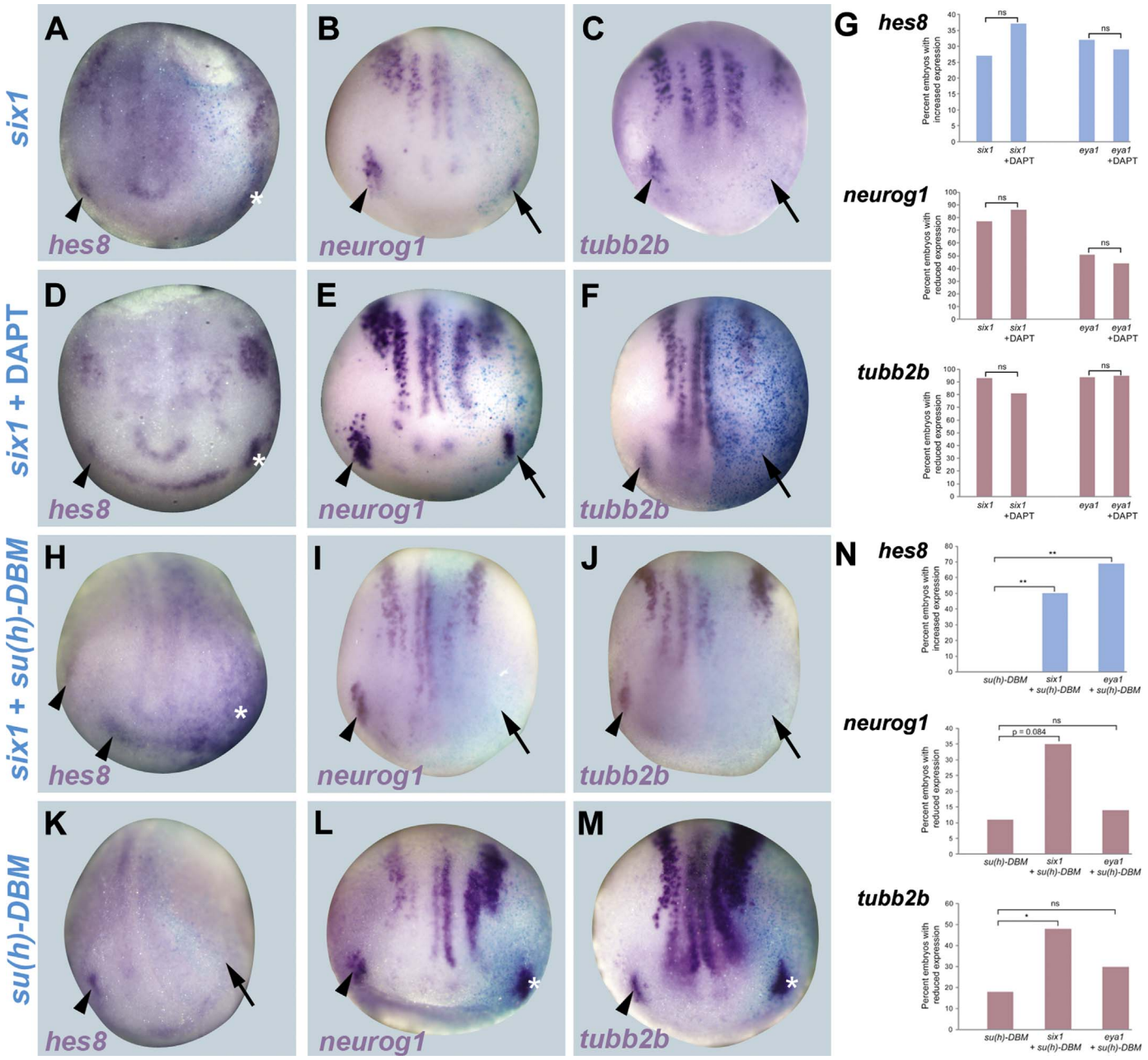

Fig. 7. Six1 activates *hes8* and represses neuronal differentiation independent of Notch. A-F, H-M: Expression of *hes8*, *neurog1* and *tubb2b* in neural plate stage *Xenopus* embryos after injection of *six1* mRNA (A-C), *six1* mRNA followed by DAPT treatment (D-F), *six1* plus *su(h)-DBM* mRNA (H-J) or *su(h)-DBM* mRNA (K-M) alone. Control side is shown on the left and injected side on the right (as indicated by blue lacZ staining). Arrowheads indicate expression domains in developing placodes. Arrows indicate reductions in placodal expression while asterisks indicate increased expression in placodes or adjacent non-neural ectoderm. Overexpression of Six1 upregulates *hes8* and represses *neurog1* and *tubb2b* even when Notch signaling is blocked by DAPT or *su(h)-DBM*. **G**: Frequency of increased *hes8* expression in PPR/placodes or adjacent non-neural ectoderm (blue) or reduced *neurog1* or *tubb2b* expression in PPR/placodes in embryos injected with *six1* and *eya1* is not significantly altered by DAPT treatment (for numbers see Table 1). **N**: Frequency of increased *hes8* expression in embryos injected with *su(h)-DBM* is significantly increased by coinjection of *six1* and *eya1* while frequency of reduced *neurog1* or *tubb2b* expression in embryos injected with *su(h)-DBM* is reduced after coinjection of *six1* but not *eya1* (for numbers see Table 1).

oscillations of Hes1 protein (Hirata et al., 2002; Takebayashi et al., 1994). These result in out of phase oscillations of proneural genes including *neurog2* and keep cells in a progenitor state (Imayoshi et al., 2013; Shimojo et al., 2008). Due to lateral inhibition, neighboring cells undergo similar, but phase-shifted oscillations (with high *neurogenin* peaks in one cell corresponding to high *hes* peaks in neighboring cells) (Shimojo et al., 2016, 2008). Only when oscillations stop and *neurog2* expression is sustained, does it promote cell cycle exit and activation of neuronal differentiation (Imayoshi et al., 2013; Kobayashi and Kageyama, 2014). Our findings indicate that the interaction between *hes8* and *neurog1* in cranial placodes may play a similar role and keep placodal progenitors in an undifferentiated but primed state, ready to undergo rapid differentiation when oscillations stop.

However, we also found that (different from *pou4f1.2*) the neuronal differentiation genes *neurod1* and *tubb2b* were decreased rather than increased in our Hes8 knockdown experiments suggesting that Hes8 is required for their activation during normal placode development. Since *Hes* genes act as transcriptional repressors this most likely will involve repression of a repressor. Due to the known ability of *Hes* genes to auto- and cross-repress each other (Hatakeyama et al., 2004; Hirata et al., 2002), other *Hes* genes that are co-expressed with *hes8* in placodes such as *hes5*, *hes1*, and *hes9* (Lamar and Kintner, 2005;

Riddiford and Schlosser, 2016; Vega-Lopez et al., 2015) are promising candidates. Our findings could be explained if, due to mutual cross-repression, different *Hes* genes oscillate in synchrony during normal placode development but have partly non-overlapping sets of target genes, e.g. with *pou4f1.2* being repressed predominantly by *hes8*, but *neurod1* and *tubb2b* by other *Hes* genes. At present this scenario remains speculative and further studies are needed to test it.

4.2. Six1 and Eya1 promote neuronal differentiation in a dose-dependent fashion

Previous studies have shown that Six1 and Eya1 activate proneural genes such as *atoh1* and *neurog1* which are subject to *Hes* repression (Ahmed et al., 2012a; Riddiford and Schlosser, 2016) and have supported a role for Six1 and Eya1 in promoting neuronal differentiation (Ahmed et al., 2012b; Schlosser et al., 2008; Zou et al., 2004). This raises the question how such a role of Six1 and Eya1 is balanced with their apparently conflicting role in promoting progenitor status via the activation of *hes8* or other *Hes* genes. Our previous study revealed that this balance is strongly dose-dependent with high doses of Six1 and Eya1 promoting proliferation and expression of progenitor genes *sox2* and *sox3* in tailbud stage embryos and low doses promoting expression of *neurog2*, *neurod1* and $p27^{Xic1}$, which initiate cell cycle exit and neuronal differentiation (Schlosser et al., 2008). Here we confirm this dose-dependence of *sox3* and *neurod1* for earlier, neural plate stage embryos and also show that high doses of Six1 and Eya1 promote both *hes8* and *neurog1* expression at that stage.

Before discussing this further, a potential caveat has to be considered. Since the dose-dependent effects in our study were observed after overexpression of various doses of Six1 or Eya1, it is possible that some of the effects observed may be due to abnormal protein-protein or protein-DNA interactions due to exceptionally high doses of Six1 or Eya1 which normally do not occur in the embryos. While our study doesn't allow to rule this out completely, several observations suggest that the responses of target genes to various injected doses of Six1 or Eya1 overall reflect their dose-dependent responses during normal placode development. First, dose-dependent decreases of *sox2*, *neurog1* and *neurod1* expression in the inner ear have also been found in Eya1 loss of function studies using allelic series of mouse mutants and both Eya1 and Six1 mutants are haploinsufficient (Friedman et al., 2005; Kochhar et al., 2007; Zou et al., 2008). Second, we observe ectopic neuronal differentiation predominantly after injection of low rather than high doses of Eya1 and Six1. And finally, uneven distribution of the injected *eya1* and *six1* mRNA often results in a spectrum of effects on target genes in single embryos which mimics the normal situation, for example with reduced/low expression of neuronal differentiation genes in areas of high Eya1 or Six1 levels but increased/high expression of neuronal differentiation genes in areas of low Eya1 or Six1 levels (Schlosser et al., 2008).

Taken together with our previous findings that Six1 and Eya1 are required for both maintenance of proliferating progenitors and neuronal differentiation in placodes, and that they directly activate both progenitor and differentiation genes (Schlosser et al., 2008; Riddiford and Schlosser, 2016), our current study suggests that Six1 and Eya1 may be able to activate progenitor genes such as *hes8* or *sox2* only at high doses, but neuronal differentiation genes already at a lower dose threshold. At the same time, *hes8* expression in placodal domains is often reduced after Six1 and Eya1 overexpression suggesting that these may also activate repressors of *hes8* (such as other *Hes* genes), but this remains to be investigated further.

In this model, in cells with high Six1 and Eya1 levels, neuronal differentiation is prevented by the repressive effects of *hes8* or other progenitor genes, while cell cycle exit and neuronal differentiation is promoted when Six1 and Eya1 levels have declined below the threshold required to support activation of *hes8*, *sox2* or other progenitor genes. Since co-injection of *hes8* MOs with Six1 and Eya1 alone did not prevent the repression of *neurog1* and *tubb2b* expression in placodes, *hes8* probably acts in conjunction with other progenitor genes to arrest neuronal differentiation downstream of Six1 and Eya1.

Surprisingly, we only observed ectopic expression of *neurog1* and *neurod1* but never of *tubb2b* after overexpression of low doses of Six1 and Eya1 mRNA at neural plate stages, while we did observe ectopic expression of both *neurog1* and *tubb2b* after Six1 and Eya1 plasmid injections. One possible explanation for this finding relates to the more mosaic distribution of Six1/Eya1 expressing cells after plasmid injection, which implies that a smaller fraction of neighboring cells may also overexpress Six1/Eya1 and thus may inhibit terminal neuronal differentiation in their neighbors by lateral inhibition.

The dose-dependent regulation of *neurog1* by Six1 and Eya1 is complex and combines features observed for progenitor genes (e.g. *hes8*) and neuronal differentiation genes (e.g. *neurod1*). At neural plate stages, high doses of Six1 and Eya1 increasingly repress native placodal expression of *neurog1* possibly due to increasing repression by progenitor genes activated by Six1/Eya1 such as *hes8*. At the same time, however, higher doses of Six1 and Eya1 lead to increasingly ectopic expression of *neurog1* despite the concomitant upregulation of *hes8*.

This seeming paradox can be resolved under the assumption that *neurog1* and *hes8* are expressed in complementary cells, which interact by lateral inhibition. Notch ligands other than Delta1 may be involved here, since we did not observe *delta1* to be upregulated in parallel to *neurog1*. Out of phase oscillations of Hes8 and Neurog1 in these cells would keep all of these cells in a non-differentiating progenitor state and prevent neuronal differentiation similar to what has been described for Hes1 and Neurog2 or Ascl1 (Imayoshi et al., 2013; Shimojo et al., 2008). Previous findings suggest that only cells that are able to escape these oscillatory cycles and maintain proneural gene expression will initiate neuronal differentiation (Kageyama et al., 2008; Shimojo et al., 2008). The underlying mechanisms are poorly understood, but our data suggest that low levels of Six1 and Eya1 could contribute to destabilising *hes8* levels allowing sustained *neurog1* expression, which is known to initiate cell cycle exit and neuronal differentiation (Farah et al., 2000; Ma et al., 1998, 1996). Conversely, high levels of Six1 and Eya1 promote multiple progenitor genes such as *sox2* in addition to *hes8*, which are known to block neuronal differentiation downstream of *neurog1* and *neurog2* (Bylund et al., 2003; Holmberg et al., 2008) but have also shown to repress expression of *neurog1* and *neurog2* in *Xenopus* ((Schlosser et al., 2008); present study). Further experiments are required to test the details of this scenario, but our observation that the dependence of *neurog1* expression on Six1/Eya1 dosage becomes reversed at tailbud stages with lower doses of Six1/Eya1 supporting increasingly ectopic expression of *neurog1* would support this hypothesis.

4.3. Six1 and Eya1 synergize with Sox3 to promote neuronal determination genes

We show here that Six1 and Eya1 synergize with Sox3 in the regulation of *neurog1* but not of *hes8* or *tubb2b* expression. Moreover, increasing levels of Six1 and Eya1, increasingly promote both ectopic *sox3* and transient ectopic *neurog1* expression. Together with previous studies demonstrating that Six1 and Eya1 physically interact with Sox2 and cooperate with it in the activation of *atoh1* in hair cell precursors as well as in activating neuronal differentiation (Ahmed et al., 2012a, 2012b) this indicates an important role of SoxB1 transcription factors (Sox2, Sox3) in activating neuronal determination genes in conjunction with Six1 and Eya1. However, previous studies also indicate that SoxB1 factors ultimately have to be downregulated to permit neuronal differentiation (Bylund et al., 2003; Graham et al., 2003; Rogers et al., 2009). A previous study suggested that SoxB1 proteins act as pioneer factors, which keep enhancers of neuronal determination genes poised for activation by maintaining bivalent chromatin marks but that need to be replaced by other transcription factors (e.g. Sox11) to allow transcriptional activation to proceed (Bergsland et al., 2011). Similarly, a

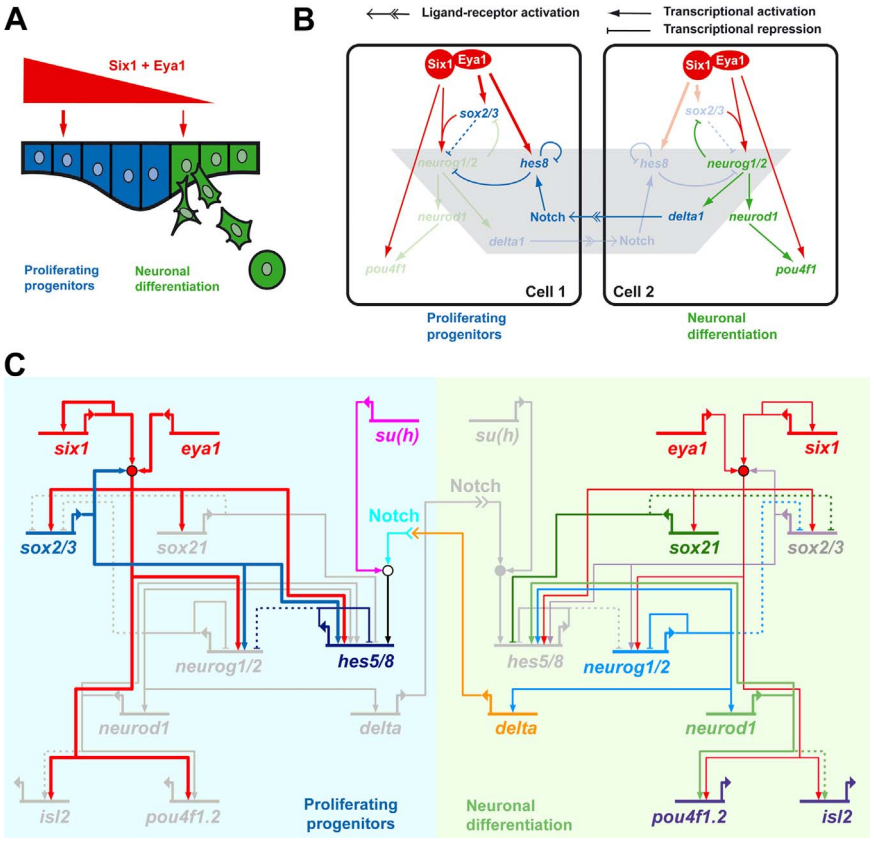

Fig. 8. Model for Six1 and Eya1 functions in placodal neurogenesis. A: While high doses of Six1 and Eya1 promote expansion of proliferating progenitors, declining levels of Six1 and Eya1 when cells migrate away from placodes promote neuronal differentiation. **B**: Interaction of Six1 and Eya1 with genes and proteins involved in the Notch mediated lateral inhibition pathway (*hes8*, *neurog1*/2, *delta1*, Notch; grey area). Thick red arrows indicate that target genes are activated only by high levels of Six1 and Eya1, while thin red arrows indicate that target genes can be activated by low levels of Six1 and Eya1. In progenitors, high levels of Six1 and Eya1 transcriptionally activate *sox2/3* and *hes8* - which may be activated even in the absence of Notch signaling - thereby repressing the transcription of *neurog1/2* and neuronal differentiation. In differentiating neurons, low levels of Six1 and Eya1, which are not sufficient to activate *sox2/3* and *hes8*, transcriptionally activate *neurog1/2* and *pou4f1.2*, thereby promoting neuronal differentiation. While Sox2/3 alone inhibits neuronal differentiation upstream (hatched line) and downstream (not shown) of *neurog1/2* it poises *neurog1/2* for activation in conjunction with Six1 and Eya1. In addition to the interactions depicted, Sox21 has been reported to either repress (Sandberg et al., 2005) or promote (Whittington et al., 2015) neuronal differentiation genes; due to these conflicting reports, we do not show these interactions here. **C**: Detailed gene regulatory network designed using BioTapestry (Longabaugh et al., 2005, 2009) for regulation of neurogenesis downstream of Six1 and Eya1 based on this and previous studies. References supporting the interactions shown are given in Table S2. Arrows indicate direct (solid line) and indirect (dotted line) activation; barred lines show direct (solid line) and indirect (dotted line) repression. Thin lines indicate a low level of expression, whereas thick lines show high levels of expression. Greyed out genes represent those inactive in current cell (e.g. neuronal differentiation genes in progenitors). Low level activation of Sox2/3 in differentiating neurons is shown as faded violet. See text for details.

complex of SoxB1, Six1 and Eya1 may poise *neurog1* and other neuronal determination genes for activation in placodal progenitor cells, allowing *neurog1* to be transcribed and neuronal differentiation to proceed when SoxB1 levels subside. One important open question left by this model is whether (and if so, how) oscillatory *neurog1* expression can be maintained in progenitors in the presence of SoxB1 factors.

Interestingly, the synergism of SoxB1 and Six1/Eya1 allows us to account for the placodal expression domains of *neurog1*, *neurog2* as well as some of their targets (*delta1*, *islet2*, *neurod1*), which are confined to the intersection of Sox3 and Six1/Eya1 expression domains in neural plate stage embryos (Nieber et al., 2009; Schlosser and Ahrens, 2004; Schlosser and Northcutt, 2000). While the prospective olfactory placode is found at the intersection of *sox3* expression in the anterior neural ridge (just anterior to the neural plate) and the *six1/eya1* expressing PPE, the prospective profundal/trigeminal placodes and posterior placodal area (giving rise to otic, lateral line and epibranchial placodes) are located at the intersection of the lateral domains of *sox3* expression with the PPE (Fig. S6).

4.4. A new model for the role of Six1 and Eya1 in placodal neurogenesis

Based on our findings we propose a new model of how Six1 and Eya1 regulate neurogenesis during development of placodes from the PPE (Fig. 8.) In the PPE, high levels of Six1 and Eya1 promote a neuronal progenitor state by directly activating *SoxB1* genes and *Hes* genes such as *hes8*. SoxB1 factors in conjunction with Six1/Eya1 prime cells for a neuronal fate by poising *neurog1* (and possibly *neurog2* as well) for activation but keeping initiation of neuronal differentiation repressed. *Hes8* represses both *neurog1/neurog2* and itself, thereby maintaining cells in a progenitor state defined by out of phase Hes8 and Neurog1 oscillations. Adjacent cells are locked into a similar, but out of phase, oscillatory cycle due to lateral inhibition by Notch ligands activated downstream of *neurog1/neurog2*.

As the individual placodes segregate from the PPE, Six1 and Eya1 levels decline by an as-yet-unknown mechanism. These declining levels fail to support expression of progenitor genes such as *SoxB1* genes and *hes8* but continue to promote expression of *neurog1/neurog2* and neuronal differentiation genes. The resulting decline of SoxB1 and Hes8 levels lifts transcriptional repression of *neurog1/neurog2* and terminates Hes8 cycling allowing sustained expression of Neurog1 leading to cell cycle exit and repression of *SoxB1* genes (Bylund et al., 2003; Evsen et al., 2013) and neuronal differentiation. While most of the interactions proposed in this model are supported by our present study, several important details, such as the predicted Hes8 and Neurog1 oscillations require further experimental validation. When

taken together with the gene regulatory network we previously proposed, (Riddiford and Schlosser, 2016) these interactions allow us to posit a comprehensive, predictive, model of gene regulation in placode neurogenesis (Fig. 8C).

Acknowledgments

Horst Grunz, Chris Kintner, Mike Klymkowsky, Sally Moody, Tomas Pieler, Monica Vetter and Doris Wedlich kindly provided plasmids. The c-myc antibody (9E10) developed by J.M. Bishop was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Dept. Biol. Sci., Iowa City, IA 52242. This work was supported by Science Foundation Ireland grant 11/RFP/EOB/3168 to Gerhard Schlosser.

Competing interests

The authors declare no competing interests.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2017.09.027.

References

Ahmed, M., Wong, E.Y., Sun, J., Xu, J., Wang, F., Xu, P.X., 2012a. Eya1-six1 interaction is sufficient to induce hair cell fate in the cochlea by activating atoh1 expression in cooperation with sox2. Dev. Cell 22, 377–390.
Ahmed, M., Xu, J., Xu, P.X., 2012b. EYA1 and SIX1 drive the neuronal developmental program in cooperation with the SWI/SNF chromatin-remodeling complex and SOX2 in the mammalian inner ear. Development 139, 1965–1977.
Ahrens, K., Schlosser, G., 2005. Tissues and signals involved in the induction of placodal Six1 expression in Xenopus laevis. Dev. Biol. 288, 40–59.
Baker, C.V.H., Bronner-Fraser, M., 2001. Vertebrate cranial placodes. I. Embryonic induction. Dev. Biol. 232, 1–61.
Bergsland, M., Ramskold, D., Zaouter, C., Klum, S., Sandberg, R., Muhr, J., 2011. Sequentially acting Sox transcription factors in neural lineage development. Genes Dev. 25, 2453–2464.
Bricaud, O., Collazo, A., 2006. The transcription factor six1 inhibits neuronal and promotes hair cell fate in the developing zebrafish (Danio rerio) inner ear. J. Neurosci. 26, 10438–10451.
Brugmann, S.A., Pandur, P.D., Kenyon, K.L., Pignoni, F., Moody, S.A., 2004. Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a transcriptional activator and repressor. Development 131, 5871–5881.
Bylund, M., Andersson, E., Novitch, B.G., Muhr, J., 2003. Vertebrate neurogenesis is counteracted by Sox1-3 activity. Nat. Neurosci. 6, 1162–1168.
Chen, B., Kim, E.H., Xu, P.X., 2009. Initiation of olfactory placode development and neurogenesis is blocked in mice lacking both Six1 and Six4. Dev. Biol. 326, 75–85.
Chitnis, A.B., 1995. The role of notch in lateral inhibition and cell fate specification. Mol. Cell Neurosci. 6, 311–321.
Contreras-Cornejo, H., Saucedo-Correa, G., Oviedo-Boyso, J., Valdez-Alarcon, J.J., Baizabal-Aguirre, V.M., Cajero-Juarez, M., Bravo-Patino, A., 2016. The CSL proteins, versatile transcription factors and context dependent corepressors of the notch signaling pathway. Cell Div. 11, 12.
Dykes, I.M., Tempest, L., Lee, S.I., Turner, E.E., 2011. Brn3a and Islet1 act epistatically to regulate the gene expression program of sensory differentiation. J. Neurosci. 31, 9789–9799.
Evsen, L., Sugahara, S., Uchikawa, M., Kondoh, H., Wu, D.K., 2013. Progression of neurogenesis in the inner ear requires inhibition of Sox2 transcription by neurogenin1 and neurod1. J. Neurosci. 33, 3879–3890.
Farah, M.H., Olson, J.M., Sucic, H.B., Hume, R.I., Tapscott, S.J., Turner, D.L., 2000. Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. Development 127, 693–702.
Friedman, R.A., Makmura, L., Biesiada, E., Wang, X., Keithley, E.M., 2005. Eya1 acts upstream of Tbx1, Neurogenin 1, NeuroD and the neurotrophins BDNF and NT-3 during inner ear development. Mech. Dev. 122, 625–634.
Graham, V., Khudyakov, J., Ellis, P., Pevny, L., 2003. SOX2 functions to maintain neural progenitor identity. Neuron 39, 749–765.
Grocott, T., Tambalo, M., Streit, A., 2012. The peripheral sensory nervous system in the vertebrate head: a gene regulatory perspective. Dev. Biol. 370, 3–23.
Guillemot, F., 2007. Spatial and temporal specification of neural fates by transcription factor codes. Development 134, 3771–3780.
Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F., Kageyama, R., 2004. Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development 131, 5539–5550.
Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K., Kageyama, R., 2002. Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. Science 298, 840–843.
Holmberg, J., Hansson, E., Malewicz, M., Sandberg, M., Perlmann, T., Lendahl, U., Muhr, J., 2008. SoxB1 transcription factors and Notch signaling use distinct mechanisms to regulate proneural gene function and neural progenitor differentiation. Development 135, 1843–1851.
Hutcheson, D.A., Vetter, M.L., 2001. The bHLH factors Xath5 and XNeuroD can upregulate the expression of XBrn3d, a POU-homeodomain transcription factor. Dev. Biol. 232, 327–338.
Ikeda, K., Ookawara, S., Sato, S., Ando, Z., Kageyama, R., Kawakami, K., 2007. Six1 is essential for early neurogenesis in the development of olfactory epithelium. Dev. Biol. 311, 53–68.
Imayoshi, I., Isomura, A., Harima, Y., Kawaguchi, K., Kori, H., Miyachi, H., Fujiwara, T., Ishidate, F., Kageyama, R., 2013. Oscillatory control of factors determining multipotency and fate in mouse neural progenitors. Science 342, 1203–1208.
Kageyama, R., Ohtsuka, T., Kobayashi, T., 2008. Roles of Hes genes in neural development. Dev. Growth Differ. 50 (Suppl 1), S97–S103.
Kobayashi, T., Kageyama, R., 2014. Expression dynamics and functions of Hes factors in development and diseases. Curr. Top. Dev. Biol. 110, 263–283.
Kochhar, A., Fischer, S.M., Kimberling, W.J., Smith, R.J., 2007. Branchio-oto-renal syndrome. Am. J. Med. Genet. A 143, 1671–1678.
Kolm, P.J., Sive, H.L., 1995. Regulation of the Xenopus labial homeodomain genes, HoxA1 and HoxD1: activation by retinoids and peptide growth factors. Dev. Biol. 167, 34–49.
Kumar, J.P., 2009a. The molecular circuitry governing retinal determination. Biochim. Biophys. Acta 1789, 306–314.
Kumar, J.P., 2009b. The sine oculis homeobox (SIX) family of transcription factors as regulators of development and disease. Cell Mol. Life Sci. 66, 565–583.
Lamar, E., Kintner, C., 2005. The Notch targets Esr1 and Esr10 are differentially regulated in Xenopus neural precursors. Development 132, 3619–3630.
Lee, J.E., Hollenberg, S.M., Snider, L., Turner, D.L., Lipnick, N., Weintraub, H., 1995. Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science 268, 836–844.
Li, X., Oghi, K.A., Zhang, J., Krones, A., Bush, K.T., Glass, C.K., Nigam, S.K., Aggarwal, A.K., Maas, R., Rose, D.W., Rosenfeld, M.G., 2003a. Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. Nature 426, 247–254.
Li, Y., Fenger, U., Niehrs, C., Pollet, N., 2003b. Cyclic expression of esr9 gene in Xenopus presomitic mesoderm. Differentiation 71, 83–89.
Longabaugh, W.J., Davidson, E.H., Bolouri, H., 2005. Computational representation of developmental genetic regulatory networks. Dev. Biol. 283, 1–16.
Longabaugh, W.J., Davidson, E.H., Bolouri, H., 2009. Visualization, documentation, analysis, and communication of large-scale gene regulatory networks. Biochim. Biophys. Acta 1789, 363–374.
Ma, Q.F., Chen, Z.F., Barrantes, I.D., de la Pompa, J.L., Anderson, D.J., 1998. Neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron 20, 469–482.
Ma, Q.F., Kintner, C., Anderson, D.J., 1996. Identification of neurogenin, a vertebrate neuronal determination gene. Cell 87, 43–52.
Moody, S.A., LaMantia, A.S., 2015. Transcriptional regulation of cranial sensory placode development. Curr. Top. Dev. Biol. 111, 301–350.
Nieber, F., Pieler, T., Henningfeld, K.A., 2009. Comparative expression analysis of the neurogenins in Xenopus tropicalis and Xenopus laevis. Dev. Dyn. 238, 451–458.
Nieuwkoop, P.D., Faber, J., 1967. Normal Table of Xenopus laevis (Daudin). North-Holland, Amsterdam.
Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., Kageyama, R., 1999. Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J. 18, 2196–2207.
Oschwald, R., Richter, K., Grunz, H., 1991. Localization of a nervous system-specific class II beta-tubulin gene in Xenopus laevis embryos by whole-mount in situ hybridization. Int. J. Dev. Biol. 35, 399–405.
Ozaki, H., Nakamura, K., Funahashi, J., Ikeda, K., Yamada, G., Tokano, H., Okamura, H.O., Kitamura, K., Muto, S., Kotaki, H., Sudo, K., Horai, R., Iwakura, Y., Kawakami, K., 2004. Six1 controls patterning of the mouse otic vesicle. Development 131, 551–562.
Pandur, P.D., Moody, S.A., 2000. Xenopus Six1 gene is expressed in neurogenic cranial placodes and maintained in differentiating lateral lines. Mech. Dev. 96, 253–257.
Penzel, R., Oschwald, R., Chen, Y., Tacke, L., Grunz, H., 1997. Characterization and early embryonic expression of a neural specific transcription factor xSOX3 in Xenopus laevis. Int. J. Dev. Biol. 41, 667–677.
Pieper, M., Eagleson, G.W., Wosniok, W., Schlosser, G., 2011. Origin and segregation of cranial placodes in Xenopus laevis. Dev. Biol. 360, 257–275.
Pierfelice, T., Alberi, L., Gaiano, N., 2011. Notch in the vertebrate nervous system: an old dog with new tricks. Neuron 69, 840–855.
Rebay, I., 2015. Multiple functions of the Eya phosphotyrosine phosphatase. Mol. Cell Biol. 36, 668–677.
Riddiford, N., Schlosser, G., 2016. Dissecting the pre-placodal transcriptome to reveal presumptive direct targets of Six1 and Eya1 in cranial placodes. eLife 5.
Rogers, C.D., Harafuji, N., Cunningham, D.D., Archer, T., Casey, E.S., 2009. Xenopus Sox3 activates sox2 and geminin and indirectly represses Xvent2 expression to induce neural progenitor formation at the expense of non-neural ectodermal derivatives. Mech. Dev. 126, 42–55.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Saint-Jeannet, J.P., Moody, S.A., 2014. Establishing the pre-placodal region and breaking it into placodes with distinct identities. Dev. Biol. 389, 13–27.
Sandberg, M., Kallstrom, M., Muhr, J., 2005. Sox21 promotes the progression of vertebrate neurogenesis. Nat. Neurosci. 8, 995–1001.
Schlosser, G., 2010. Making senses: development of vertebrate cranial placodes. Int. Rev. Cell Mol. Biol. 283C, 129–234.
Schlosser, G., Ahrens, K., 2004. Molecular anatomy of placode development in Xenopus laevis. Dev. Biol. 271, 439–466.
Schlosser, G., Awtry, T., Brugmann, S.A., Jensen, E.D., Neilson, K., Ruan, G., Stammler, A., Voelker, D., Yan, B., Zhang, C., Klymkowsky, M.W., Moody, S.A., 2008. Eya1 and Six1 promote neurogenesis in the cranial placodes in a SoxB1-dependent fashion. Dev. Biol. 320, 199–214.
Schlosser, G., Northcutt, R.G., 2000. Development of neurogenic placodes in Xenopus laevis. J. Comp. Neurol. 418, 121–146.
Seo, S., Lim, J.W., Yellajoshyula, D., Chang, L.W., Kroll, K.L., 2007. Neurogenin and NeuroD direct transcriptional targets and their regulatory enhancers. EMBO J. 26, 5093–5108.
Shimojo, H., Isomura, A., Ohtsuka, T., Kori, H., Miyachi, H., Kageyama, R., 2016. Oscillatory control of Delta-like1 in cell interactions regulates dynamic gene expression and tissue morphogenesis. Genes Dev. 30, 102–116.
Shimojo, H., Ohtsuka, T., Kageyama, R., 2008. Oscillations in notch signaling regulate maintenance of neural progenitors. Neuron 58, 52–64.
Silver, S.J., Rebay, I., 2005. Signaling circuitries in development: insights from the retinal determination gene network. Development 132, 3–13.
Sive, H.L., Grainger, R.M., Harland, R.M., 2000. Early Development of Xenopus laevis Cold. Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Stamatakis, A., Ludwig, T., Meier, H., 2005. RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics 21, 456–463.
Tadjuidje, E., Hegde, R.S., 2013. The Eyes Absent proteins in development and disease. Cell Mol. Life Sci. 70, 1897–1913.
Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S., Kageyama, R., 1994. Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements. J. Biol. Chem. 269, 5150–5156.
Vega-Lopez, G.A., Bonano, M., Tribulo, C., Fernandez, J.P., Aguero, T.H., Aybar, M.J., 2015. Functional analysis of Hairy genes in Xenopus neural crest initial specification and cell migration. Dev. Dyn. 244, 988–1013.
Wettstein, D.A., Turner, D.L., Kintner, C., 1997. The Xenopus homolog of Drosophila Suppressor of hairless mediates Notch signaling during primary neurogenesis. Development 124, 693–702.
Whelan, S., Goldman, N., 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol. Biol. Evol. 18, 691–699.
Whittington, N., Cunningham, D., Le, T.K., De Maria, D., Silva, E.M., 2015. Sox21 regulates the progression of neuronal differentiation in a dose-dependent manner. Dev. Biol. 397, 237–247.
Wong, E.Y., Ahmed, M., Xu, P.X., 2012. EYA1-SIX1 complex in neurosensory cell fate induction in the mammalian inner ear. Hear. Res..
Zhang, C., Klymkowsky, M.W., 2007. The Sox axis, Nodal signaling, and germ layer specification. Differentiation 75, 536–545.
Zheng, W., Huang, L., Wei, Z.B., Silvius, D., Tang, B., Xu, P.X., 2003. The role of Six1 in mammalian auditory system development. Development 130, 3989–4000.
Zou, D., Erickson, C., Kim, E.H., Jin, D., Fritzsch, B., Xu, P.X., 2008. Eya1 gene dosage critically affects the development of sensory epithelia in the mammalian inner ear. Hum. Mol. Genet. 17, 3340–3356.
Zou, D., Silvius, D., Fritzsch, B., Xu, P.X., 2004. Eya1 and Six1 are essential for early steps of sensory neurogenesis in mammalian cranial placodes. Development 131, 5561–5572.
Zou, D., Silvius, D., Rodrigo-Blomqvist, S., Enerback, S., Xu, P.X., 2006. Eya1 regulates the growth of otic epithelium and interacts with Pax2 during the development of all sensory areas in the inner ear. Dev. Biol. 298, 430–441.