Dissecting the pre-placodal transcriptome to reveal presumptive direct targets of Six1 and Eya1 in cranial placodes

Nick Riddiford[1,2], Gerhard Schlosser[1,2]*

[1]School of Natural Sciences, National University of Ireland, Galway, Ireland; [2]Regenerative Medicine Institute (REMEDI), National University of Ireland, Galway, Ireland

Abstract The pre-placodal ectoderm, marked by the expression of the transcription factor Six1 and its co-activator Eya1, develops into placodes and ultimately into many cranial sensory organs and ganglia. Using RNA-Seq in *Xenopus laevis* we screened for presumptive direct placodal target genes of Six1 and Eya1 by overexpressing hormone-inducible constructs of Six1 and Eya1 in pre-placodal explants, and blocking protein synthesis before hormone-inducing nuclear translocation of Six1 or Eya1. Comparing the transcriptome of explants with non-induced controls, we identified hundreds of novel Six1/Eya1 target genes with potentially important roles for placode development. Loss-of-function studies confirmed that target genes encoding known transcriptional regulators of progenitor fates (e.g. Sox2, Hes8) and neuronal/sensory differentiation (e.g. Ngn1, Atoh1, Pou4f1, Gfi1) require Six1 and Eya1 for their placodal expression. Our findings provide insights into the gene regulatory network regulating placodal neurogenesis downstream of Six1 and Eya1 suggesting new avenues of research into placode development and disease.

DOI: 10.7554/eLife.17666.001

*For correspondence: gerhard.schlosser@nuigalway.ie

Introduction

The cranial placodes give rise to many sense organs of the vertebrate head (including nose, ear and lateral line) and contribute to the anterior pituitary and sensory ganglia of the cranial nerves. Together with the neural crest, which also contributes to cranial ganglia as well as the head skeleton, they originated as an evolutionary novelty in stem vertebrates, on the adoption of a more active and exploratory life style (*Northcutt and Gans, 1983*; *Schlosser, 2015*). Defects in placode development underlie many congenital diseases of sensory organs and the endocrine system (*Petit et al., 2001*; *Davis et al., 2013*; *Xu, 2013*), however, despite this central importance of placodes in the evolution and development of the vertebrate head, they have been much less well studied than the neural crest, and little is known about the gene regulatory networks (GRNs) driving early placode development.

Fate mapping studies have shown that all cranial placodes develop from a common precursor region, the pre-placodal ectoderm (PPE) (*Streit, 2002*; *Bhattacharyya et al., 2004*; *Xu et al., 2008*; *Pieper et al., 2011*). In neural plate stage embryos, the PPE is located as a horseshoe-shaped domain around the anterior neural plate (and abutting the cranial neural crest laterally) which subsequently breaks up into individual placodes (*Schlosser, 2010*; *Grocott et al., 2012*; *Saint-Jeannet and Moody, 2014*). Molecularly, the PPE is characterised by the expression of *Six1* and *Eya1*, which also continues in most placodes derived from the PPE (*Schlosser and Ahrens, 2004*). Whereas *Six1* encodes a transcription factor, *Eya1* encodes a transcriptional co-activator that also has phosphatase activity (*Kumar, 2009*; *Tadjuidje and Hegde, 2013*), and Six1 and Eya1 have been shown to form a protein complex and synergistically activate transcription (*Ohto et al., 1999*;

eLife digest Animals that possess a backbone – also known as vertebrates – have several paired sense organs in their heads, such as the eyes and the olfactory system. These organs are thought to have arisen as vertebrates evolved from their filter-feeding ancestors and adopted an increasingly active and predatory lifestyle. The cranial placodes are tissues in the vertebrate embryo that give rise to many of these sense organs early in development. The sensory neurons that transmit information from the organs to the brain – including those that process hearing and smell – also develop from these tissues.

While much is known about how the sense organs work, relatively little is known about the early processes involved in their development. Two genes have been established as crucial for the formation and later development of the sense organs. These genes encode two proteins called Six1 and Eya1 that regulate other genes, although the identity of the genes that they target in placodes was not known.

Using computational and experimental approaches in the African clawed frog (*Xenopus laevis*), Riddiford and Schlosser identified hundreds of genes that are regulated by both Six1 and Eya1 in placodes. Many of these targeted genes regulate the activity of other genes and direct important cell decisions, such as whether a stem cell should develop into a neuron. After validating several of these targets in the laboratory, Riddiford and Schlosser proposed a network of gene regulation, activated by Six1 and Eya1, that drives sense organ development in vertebrates.

While Riddiford and Schlosser provide strong evidence that Six1 and Eya1 directly regulate many of the newly identified genes, further work is required to firmly establish this. Future studies could also explore how Six1 and Eya1 drive seemingly contradictory cellular decisions, encouraging some stem cells to develop into neurons and others to maintain a stem-like state.

DOI: 10.7554/eLife.17666.002

Li et al., 2013). However, both Six1 and Eya1 also interact with other protein interaction partners; Six1, for example, has been shown to act as a transcriptional repressor after binding to the co-repressor Groucho (*Brugmann et al., 2004*) whereas Eya1 is known to form protein complexes with other binding partners including the transcription factor Sox2 (*Ahmed et al., 2012a*; *Tadjuidje and Hegde, 2013*).

Loss of Six1 or Eya1 function in mouse, zebrafish, chick or *Xenopus* embryos leads to a similar spectrum of PPE and placodal defects, with altered expression of other PPE genes, decreased proliferation and increased apoptosis in many placodes, compromised morphogenetic movements (invagination or cell delamination) and a decreased production of sensory cells and neurons (*Xu et al., 1999*; *Laclef et al., 2003*; *Zheng et al., 2003*; *Brugmann et al., 2004*; *Zou et al., 2004*; *Kozlowski et al., 2005*; *Schlosser et al., 2008*; *Christophorou et al., 2009*; *Ahmed et al., 2012a, 2012b*). In human patients, mutations in both Six1 and Eya1 lead to branchio-oto-renal (BOR) and branchio-otic (BO) syndromes with congenital hearing loss (*Kochhar et al., 2007*). These findings suggest that these proteins are core regulators of placode development and promote multiple aspects of placode development synergistically, although Eya1-independent roles of Six1 have also been reported (*Brugmann et al., 2004*; *Bricaud and Collazo, 2011*). Specifically, Six1 and Eya1 have been shown to play central roles, during multiple steps, in the development of sensory cells (e.g. hair cells in the inner ear) as well as sensory neurons, and promote both the proliferation of sensory/neuronal progenitors as well as sensory and neuronal differentiation in a dosage dependent fashion (*Zou et al., 2004*; *Schlosser et al., 2008*; *Zou et al., 2008*; *Ahmed et al., 2012b, 2012a*). Recently Atoh1, an essential determination gene for hair cell development, has been shown to be directly transcriptionally activated by Six1/Eya1 binding to its enhancer (*Ahmed et al., 2012a*). Moreover, the neuronal progenitor genes *Sox2* and *Sox3* have been shown to be up-regulated by Six1 and Eya1 in the absence of protein synthesis, suggesting that they are also direct target genes (*Schlosser et al., 2008*). Several other direct target genes of Six1 have been identified (*Kumar, 2009*; *Xu, 2013*), but no specific screen for direct target genes of Six1 and Eya1 in the PPE and the developing placodes has yet been conducted.

Here, using RNA-Seq in *Xenopus laevis*, we present the first comprehensive screen for presumptive direct target genes of Six1 and Eya1 in the developing placodes in any vertebrate. Hormone-inducible constructs of Six1 and Eya1 (fused with the human glucocorticoid receptor [GR]) were overexpressed, either alone or in combination, in *Xenopus* embryos. We then explanted the PPE at neural fold stages and activated nuclear translocation of Six1 or Eya1 in these explants by the addition of dexamethasone (DEX) after blocking protein synthesis by cycloheximide (CHX). This approach has previously been shown to reliably activate direct targets of GR-fusion constructs only in the presence of DEX (*Kolm and Sive, 1995*; *Seo et al., 2007*). We then analysed the transcriptome of placodal explants by RNA-Seq and compared this to control explants which were not hormone induced, in order to specifically survey target genes directly activated or repressed by Six1 or Eya1 in the PPE and developing placodes. Using this method, we were able to identify a large number of novel target genes with potentially important roles for cranial placode development. We were further able to show in loss of function studies that several target genes encoding known regulators of progenitor fates (e.g. *Sox2*, *Hes8*) and neuronal/sensory differentiation (e.g. *Ngn1*, *Atoh1*, *Pou4f1.2*, *Gfi1a*) required both Six1 and Eya1 for their expression in the developing placodes. Our findings provide pioneering insights into the GRNs regulating placode development downstream of Six1 and Eya1, and suggest exciting new avenues of research for understanding placode development and disease.

Results

The pre-placodal transcriptome

RNA was extracted from explants cut from the PPE of un-injected embryos and characterised using RNA-Seq to provide a complete transcriptome of the PPE. After removing genes expressed at low levels (FPKM < 1) and annotation against a *Xenopus* mRNA database (see Materials and methods), we assembled a transcriptome comprising 15,794 transcripts, and the top 1000 expressed genes are shown in *Supplementary file 1*. Gene Set Enrichment Analysis (GSEA) on these genes revealed that RNA processing/splicing was very highly enriched in the PPE transcriptome (enrichment score [E]: 43), suggesting that RNA-binding proteins and mRNA splicing mechanisms may play an important role in placodal development as has also been reported for the neural crest (*Simões-Costa et al., 2014*). Translation elongation and ribosomal proteins were also highly enriched (E: 32), perhaps reflecting the high rate of protein turnover in the rapidly changing PPE (*McCabe et al., 2004*).

Identification of direct targets of Six1 and Eya1 in PPE

To identify presumptive direct targets of Six1 and Eya1, Six1-GR and Eya1-GR fusion proteins were overexpressed either alone or together in the PPE. In combination with a protein synthesis inhibitor (CHX), nuclear translocation of Six1 and Eya1 was induced by adding DEX for 2.5 hr, and gene expression was analysed using RNA-Seq (*Figure 1*). Presumptive direct targets of Six1 and Eya1 were determined by comparing Six1-GR-, Eya1-GR- or Six1-GR+Eya1-GR-injected embryos treated with CHX alone (as controls) against CHX+DEX-treated samples. Resultant data sets from such individual treatment groups (each with two biological replicates) are henceforth referred to as Six1$_i$, Eya1$_i$ and Six1+Eya1$_i$. In this paradigm, the expression of target genes for which either Six1 or Eya1 concentrations are limiting in the PPE should be affected in Six1$_i$ and Eya1$_i$ treatment groups, respectively (and potentially also in Six1+Eya1$_i$), while the expression of target genes limited by both Eya1 and Six1 concentrations in the PPE should be modulated only in the Six1+Eya1$_i$ treatment group.

Using this approach, we identified 365 genes up-regulated at least twofold that satisfied all criteria for differential expression (log$_2$ fold change [FC] $\geq$ 1; FPKM $\geq$ 1; FC < 0.5 in un-injected control) in Six1$_i$, 508 in Eya1$_i$ and 836 in Six1+Eya1$_i$ treatment groups, while 292 genes were down-regulated in Six1$_i$, 218 in Eya1$_i$ and 490 in Six1+Eya1$_i$ treatment groups (*Figure 2A and B*; *Supplementary file 2*). As an initial means of estimating data quality, we searched for targets of Six1 established in previous studies (*Atoh1* (*Ahmed et al., 2012a*); *Slc12a2* (*Ando et al., 2005*); *CyclinA1* (*Coletta et al., 2004*); *CyclinD1* (*Li et al., 2013*); *c-Myc* (*Li et al., 2003*); *Ezrin* (*Yu et al., 2006*); *Gdnf* (*Li et al., 2003*); *Sox3* (*Schlosser et al., 2008*); *Sox2* (*Schlosser et al., 2008*); *Sall1* (*Chai et al., 2006*); and *MyoD1* (*Liu et al., 2013*)) in the Six1$_i$ and Six1+Eya1$_i$ data sets. With the exception of *c-Myc,* all genes were present in the transcriptome, and most were found in either Six1$_i$ (*CyclinD1 [ccndx]* FC:
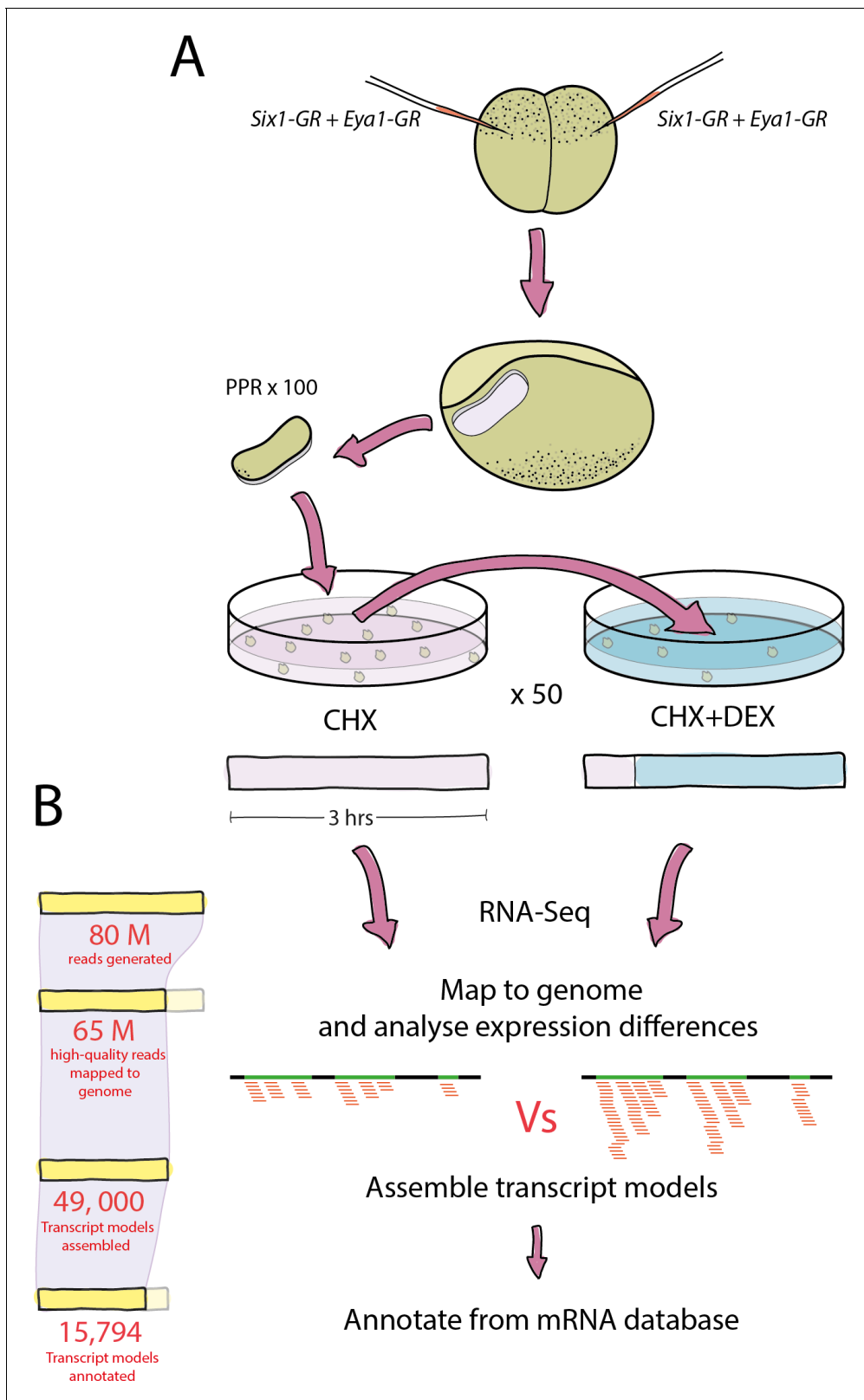
Figure 1. Experimental pipeline and overview of bioinformatic analysis. (**A**) Both blastomeres of two-cell stage embryos were injected with Six1-GR, Eya1-GR or Six1-GR+Eya1-GR and explants were cut from pre-placodal ectoderm. Explants were incubated in CHX for 30 min before being split into two groups; 50% were kept in CHX for 2.5 hr and 50% were transferred to CHX+DEX for 2.5 hr. RNA was extracted from both treatment groups and submitted to RNA-Sequencing. (**B**) On average, 80 million reads were generated in sequencing for each treatment group, and 65 million quality-
Figure 1 continued on next page

7.48; *Slc12a2*, FC: -2.75; *CyclinA1*, FC: -3.68; *Sox2*, FC: 1.2; *MyoD, FC: 3.4)* or Six1+Eya1$_i$ (*Sox3*, FC: 0.9; *Atoh1*, FC: 1.4; *Sall1*, FC: 0.99) data sets, confirming the utility of our approach in identifying direct targets. Moreover, *Atoh1, Sox2* and *MyoD1* were found both in our Six1+Eya1$_i$ and Eya1$_i$ datasets as expected based on the known coregulation of these Six1 target genes by Eya1 (*Ahmed et al., 2012a*; *Grifone et al., 2007*; *Schlosser et al., 2008*). We suggest that overexpression of Eya1 alone may upregulate such genes in those parts of the ectoderm where *Six1* is already expressed at high levels but *Eya1* at relatively low levels in vivo.

Six1 and Eya1 co-regulate many but not all PPE target genes

Comparison between our different treatment groups allows us to distinguish genes likely co-regulated by Six1 and Eya1 from those that are not and, thus, may be regulated by Six1 or Eya1 alone or in conjunction with other protein-binding partners. Since ectodermal expression of *Six1* and *Eya1* is widely overlapping in vivo but not completely congruent, genes co-regulated by Six1 and Eya1 may be differentially expressed not only after coinjection of Six1 and Eya1 (Six1+Eya1$_i$ treatment group) but also after injection of Six1 or Eya1 alone (Six1$_i$ and Eya1$_i$ treatment groups, respectively) because elevation of Six1 or Eya1 levels will produce higher levels of the coregulatory complex in those parts of the ectoderm where the respective protein is expressed at much lower levels than its binding partner. Hence, a subset of target genes with high response thresholds to the Six1-Eya1 coregulatory complex (e.g. due to low affinity binding sites) will respond to overexpression of Six1 or Eya1 alone with differential expression in these parts of the ectoderm while another subset of genes with low response thresholds (e.g. due to high affinity binding sites) will not. The latter subset will, thus, only be differentially expressed after overexpression of both Six1 and Eya1, creating expanded areas of Six1 and Eya1 coexpression in the ectoderm. Notably, the false discovery rate is expected to be lower for the former subset, which is supported by three independent treatment groups (Six1$_i$, Eya1$_i$ and Six1+Eya1$_i$), than in the latter subset, supported only by one (Six1+Eya1$_i$).

About half of all genes differentially expressed in the PPE in our various treatment groups show evidence of co-regulation by Six1 and Eya1. This includes 690 (633+57) up-regulated and 444 (440+4) down-regulated genes (*Figure 2A and B*). Indeed, the top 10% of transcripts (ranked by FC; post DEX-filtering) up-regulated in Six1$_i$, Eya1$_i$ or Six+Eya1$_i$ treatment groups were each highly enriched for the top 10% of transcripts up-regulated in any of the other experimental treatment groups (p<0.0001; Fisher's exact test). More genes co-regulated by Six1 and Eya1 were up-regulated than were down-regulated (690/1134 = 60.8% for all co-regulated genes, 57/61 = 93.4% for co-regulated genes identified in each treatment group; *Figure 2A and B*), corroborating previous findings that Six1 and Eya1 typically act synergistically to activate transcription (*Ahmed et al., 2012b*, *2012a*; *Brugmann et al., 2004*; *Christophorou et al., 2009*; *Li et al., 2003*; *Ruf et al., 2004*). However, our identification of a subset of genes synergistically down-regulated by Six1 and Eya1 suggests that Eya1 may not always act as a co-activator of Six1.

In contrast, there is no support for co-regulation for genes that are differentially expressed only in Six1$_i$ but not Eya1$_i$ treatment groups (and vice versa) even for those genes that are also differentially expressed after Six1+Eya1$_i$ treatment. We identified 283 (190+93) genes up-regulated and 270 (233+37) genes down-regulated by Six1 but not Eya1, indicating that these are regulated by Six1 in an Eya1 independent way but possibly dependent on other co-factors. Conversely, we identified 426 (373+53) genes up-regulated and 196 (187+9) genes down-regulated by Eya1 but not Six1 (*Figure 2A,B*) suggesting that these are regulated by Eya1 in conjunction with transcription factors other than Six1.

To add statistical power to our analysis, we next merged treatment groups and determined significantly differentially expressed genes (q<0.05) in these merged groups. We first created a data set Six1+Eya1$_m$ in which all replicates that involved overexpression of either Six1 or Eya1 were
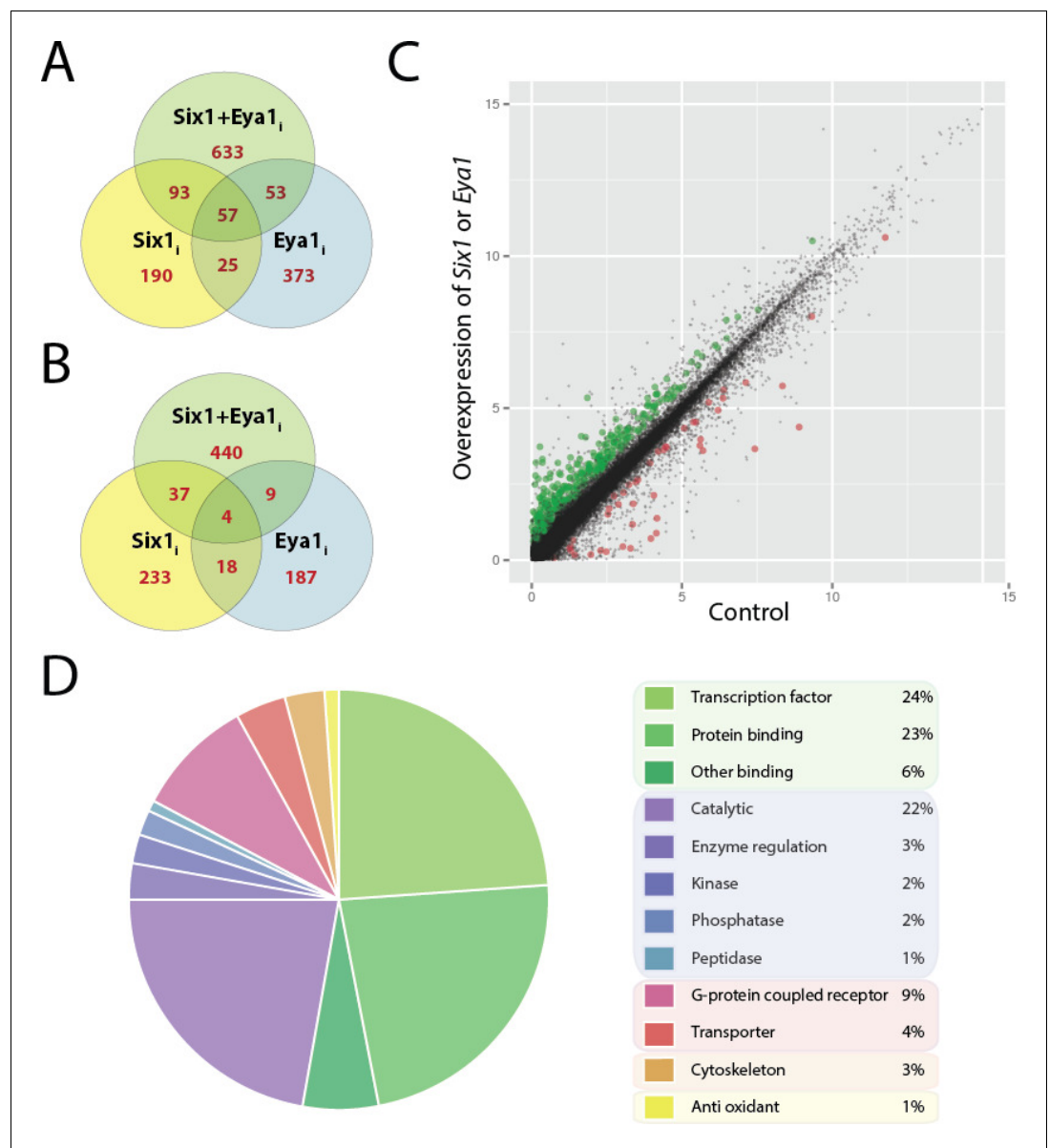
Figure 2. Differentially expressed genes after overexpression of *Six1* and *Eya1* in the PPE. Plots **A** and **B** show number of genes differentially regulated after overexpression of *Six1* alone (Six1$_i$; yellow), *Eya1* alone (Eya1$_i$; blue) or *Six1* and *Eya1* combined (Six1+Eya1$_i$; green). Each Venn diagram shows the number of genes (red) unique for each treatment group or shared between them. (**A**) Number of genes up-regulated and (**B**) down-regulated after injection with *Six1, Eya1* or *Six1+Eya1*. (**C**) The merged analysis resulted in hundreds of significantly differentially expressed genes in the PPE data set. Plot shows log$_2$ transformed (FPKM+1) values after overexpression of *Six1 or Eya1* (combination of all treatment groups; Six1+Eya1$_m$). Green points represent significantly (q<0.05) up-regulated genes and red points show significantly down-regulated genes. Plot **D** shows the enrichment of molecular function terms after overexpression of *Six1 or Eya1* based on significantly differentially expressed genes from the merged data set (Six1+Eya1$_m$; ***Supplementary file 3,*** Table 5). The area of the pie represents the total number of functional terms contained in the analysis, with each slice representing the percentage of genes against this total. Molecular functions shown can be broadly divided into five categories: Green slices are related to binding functions (53%); purple/blue represents enzyme activity (30%); pink/red shows transmembrane proteins (13%); orange cytoskeleton (3%) and yellow anti-oxidant (1%).

DOI: 10.7554/eLife.17666.004

considered as equivalent (injection of Six1-GR, Eya1-GR or Six1-GR+Eya1-GR; 6 replicates in total). This allowed us to identify genes that are significantly differentially expressed across all treatment groups. We also created a data set Six1$_m$, in which all replicates that involved Six1 overexpression were considered as equivalent (injection of Six1-GR or Six1-GR+Eya1-GR; 4 replicates). This allowed us to identify genes with significant differential expression after Six1 upregulation. Similarly, we created data set Eya1$_m$ based on all replicates that involved Eya1 overexpression (injection of Eya1-GR or Six1-GR+Eya1-GR; 4 replicates) allowing us to identify genes differentially expressed after Eya1 upregulation. We found 181 significantly (q<0.05) up-regulated genes in the Six1+Eya1$_m$ group, 149 in Six1$_m$ and 112 in Eya1$_m$ (*Supplementary file 3*, Tables 1–3). Substantially fewer genes were negatively regulated in these merged groups, with only 14 significantly down-regulated genes found in Six1+Eya1$_m$, 11 in Six1$_m$ and 13 in Eya1$_m$ (*Supplementary file 3*, Tables 4–6), re-enforcing the notion that together, Six1 and Eya1 act primarily as transcriptional activators (*Figure 2C*).

Target genes of Six1 and Eya1 are implicated in sensory neurogenesis

Presumptive direct targets that were significantly up-regulated in our merged data set (Six1+Eya1$_m$) were analysed using Panther (*Mi et al., 2013*) to examine the representation of genes grouped by molecular function (*Figure 2D*). Transcription factors and protein binding together accounted for the largest fraction of up-regulated genes (53% in total), followed by enzymes (30%) and transporter molecules (13%), suggesting a developmental function of many of the genes up-regulated by either Six1 or Eya1. GSEA was then conducted using DAVID (*Huang et al., 2009*) on the sets of significantly up- or down-regulated genes in our merged data sets, as well as in various combinations of subsets of differentially expressed genes from our individual treatment groups (*Figure 3* and *Figure 3—figure supplement 1*). This analysis showed that genes directly up-regulated by Six1, Eya1 or Six1+Eya1 were highly enriched for terms associated with sense organ development, inner-ear development, mechanoreceptor differentiation, eye morphogenesis, neurogenesis and axon guidance consistent with their synergistic role in sensory development (*Grocott et al., 2012*; *Schlosser, 2010*) and neurogenesis (*Maier et al., 2014*; *Schlosser and Northcutt, 2000*). Apart from genes encoding transcription factors involved in sensory development (see below), genes encoding cell cycle regulators (*CyclinD, RGCC*), cell surface receptors and adhesion molecules (e.g. *CXCR7, EDAR, Protocadherin11, Claudin3, Fzd1, Fzd4*,), secreted proteins (e.g. *FGF3, FGF19, Dkk1, Neurotrophin3*) and cytoskeletal regulators (e.g. *RhoV, Espin*) with known or potential roles in placode development were also up-regulated.

GSEA analysis of discrete subsets of genes exclusively regulated by Six1 or Eya1 suggested that they also regulate some categories of genes independently of one another. A particularly interesting finding was the extreme enrichment of *Hox* genes (specifically of the Antennapedia-type) in the Eya1-specific subset of up-regulated genes (*Figure 3F*), suggesting that Eya1 may play a previously un-identified role in regulating Hox gene expression independently of Six1.

Characterisation of transcriptional regulators activated by Six1 or Eya1

To verify our RNA-Seq data, we selected a number of target genes for further characterisation and, in order to gain insight into the GRN downstream of Six1 and Eya1, we restricted candidates to transcription factors or co-factors up-regulated by Six1 or Eya1. First, we generated a list of well-supported target genes containing all genes with at least a two-fold up-regulation in at least two of our three treatment groups (*Table 1*). From the 228 genes in this list we selected all 30 transcription factors or co-factors for further analysis. However, we were unable to amplify two genes from this list (*Egr3, Fbxo41*) from cDNA and therefore omitted these genes from further characterisation. We additionally included *Sox3* and *Ngn1* - which were found to be slightly below our threshold of two-fold up-regulation in at least two treatment groups - because previous studies have implicated these genes in the regulation of placodal neurogenesis downstream of Six1 and Eya1 (*Ma et al., 1996*, *1998*; *Schlosser et al., 2008*; *Ahmed et al., 2012b*) (*Table 2*).

The expression of genes previously undescribed in *Xenopus* (*Crem, FosB, Hes8, Isl2, Znf214*) was fully characterised in neural fold, and early and late tail bud stages, along with those for which expression has been described for relatively few stages (*Atoh1, Emx1.2, Gfi1a, Hes2, Hes9, Lhx5, Mab21l2b, Pou3f2b, Pou4f1.2, Ripply3, Sim1, Sox21, Tbx6, Tlx1*) (summarised in *Figure 4A–T*; *Figure 4—figure supplements 1–5*). Genes with extensively characterised expression patterns

A

Legend: Six1 (yellow), Eya1 (blue), Six1+Eya1 (green)

Functional cluster [1]	Enrichment Ind.[2]	Merg.[3]
Homeobox	7.5	3.0
DNA-binding	4	2.0
Transducer	3.3	-
Positive regulation of transcription	3	2.0
Rhodopsin-like receptor	2.9	-
Inner-ear development	2.8	2.5
bHLH motif	2.8	-
Female sex differentiation	2.5	1.6
Mechanoreceptor differentiation	1.8	3.2
Axon guidance	1.8	1.1
Regulation of neurogenesis	1.7	2.9
Eye morphogenesis	1.6	-

B

Functional cluster [1]	Enrichment Ind.[2]	Merg.[3]
Inner-ear development	2.7	1.8
Transducer	2.7	-
Homeobox	2.5	1.5
Rhodopsin-like receptor	2.3	-
Eye morphogenesis	2	-
HMG	1.9	1.7
bHLH motif	1.9	-
Transmembrane	1.8	-
Regulation of neurogenesis	1.8	1.9
Positive regulation of transcription	1.7	1.1
Frizzled	1.6	-

C

Functional cluster [1]	Enrichment Ind.[2]	Merg.[3]
Antennapedia-type homeobox	6.2	-
Homeobox	6.1	3.7
Embryonic organ development	3.5	1.8
DNA-binding	3.1	2.4
Positive regulation of transcription	2.8	-

D

Functional cluster [1]	Enrichment [4]
Extracellular matrix	2.4
HMG	2.4
Rhodopsin-like receptor	1.6
Positive regulation of transcription	1.5

E

Functional cluster [1]	Enrichment [4]
Homeobox	3.1
Sensory perception	2
Regulation of transcription	2
Inner-ear development	1.9

F

Functional cluster [1]	Enrichment [4]
Antennapedia-type homeobox	7.1
Homeobox	6.8
Regulation of transcription	4
Embryonic organ development	3.7
Positive regulation of transcription	2.3

[1] Functional cluster of terms that show enrichment
[2] Enrichment for individual treatment groups; $Six1_i$, $Eya1_i$ and $Six1+Eya1_i$
[3] Enrichment for merged treatment groups; $Six1_m$, $Eya1_m$ and $Six1+Eya1_m$
[4] Enrichment for individual treatment groups only
* Genes in these subsets were excluded from GSEA analyses of individual treatment groups
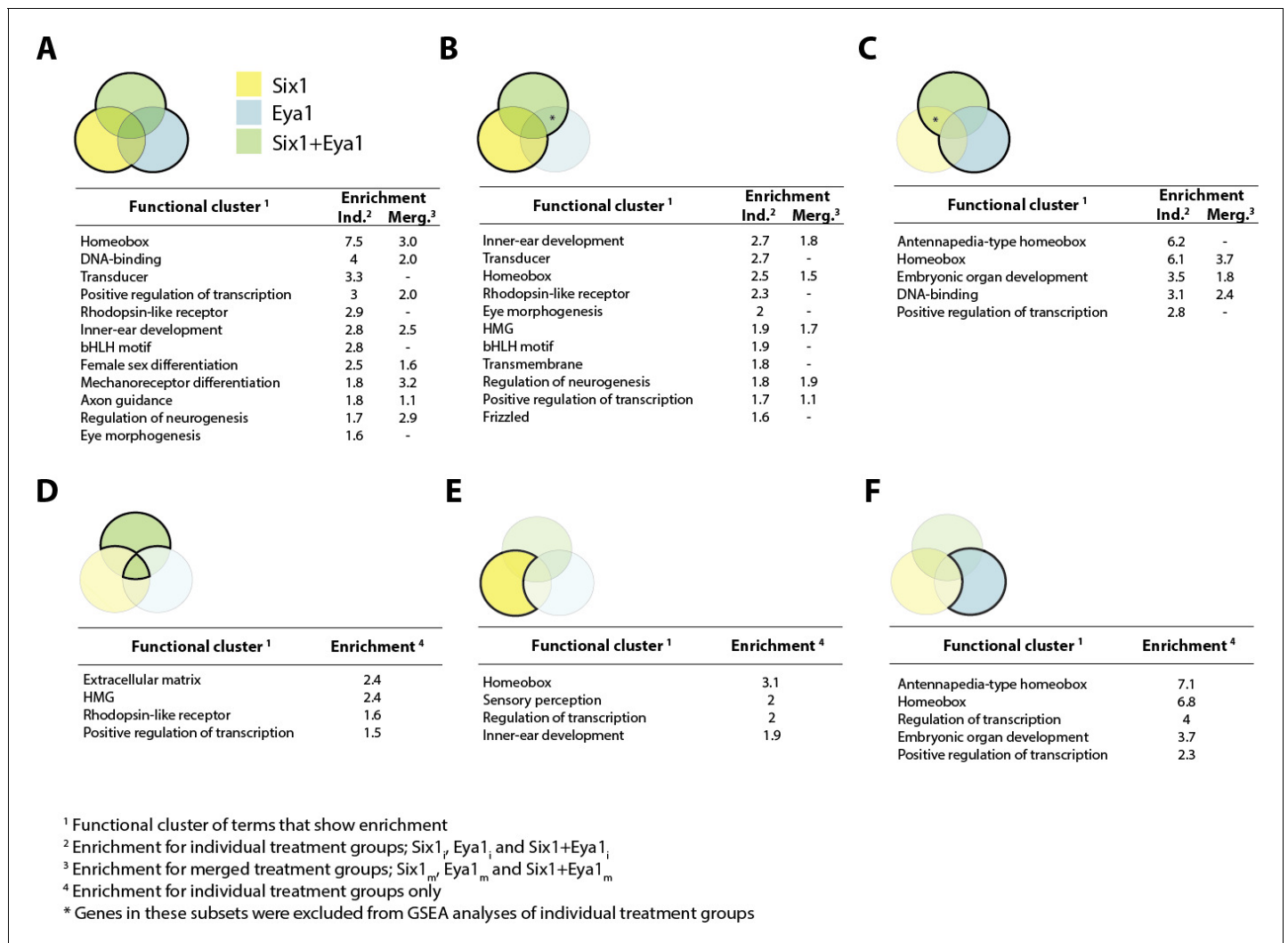
Figure 3. Gene set enrichment analysis (GSEA) showing enriched clusters of functional terms for up-regulated genes in different treatment groups. In each case, treatment groups considered are highlighted and outlined in bold in the accompanying Venn diagram. Yellow colouring indicates Six1 treatment; blue shows Eya1 and green Six1+Eya1. Enrichment scores ≥1.5 are reported for individual treatment groups (Ind.) and, where available, ≥0.5 for merged treatment groups (Merg.). (A) Up-regulated genes from all treatment groups included in analysis; (B) *Six1* overexpression only; (C) *Eya1* overexpression only. (D) Genes differentially expressed after overexpression of both *Six1* and *Eya1*; (E) exclusively after *Six1* overexpression; (F) exclusively after *Eya1* overexpression.
DOI: 10.7554/eLife.17666.005
The following figure supplement is available for figure 3:

Figure supplement 1. Gene set enrichment analysis (GSEA) showing enriched clusters of functional terms for down-regulated genes in different treatment groups.
DOI: 10.7554/eLife.17666.006

(*Ngn1* (**Nieber et al., 2009**); *Six1* (**Pandur and Moody, 2000**); *Six2* (**Ghanbari et al., 2001**); *Sox2* (**Mizuseki et al., 1998**); *Sox3* (**Penzel et al., 1997**); *Sox17* (**Hudson et al., 1997**), *MyoD1* (**Hopwood et al., 1989**), *Sox1* (**Nitta et al., 2006**), *Ets2a* (**Salanga et al., 2010**), *Mafa* (**Coolen et al., 2005**)) are not shown here.

We found 19/30 (63.3%) of these transcriptional regulators to be expressed in PPE or placodal derivatives, while 11/30 (*Sox17, MyoD1, Sox1, Ets2a, Mafa, Emx1.2, Lhx5, Pou3f2b, Tbx6, Tbx15, Sim1*) were not expressed in either the PPE or any placodal derivatives. However, many of the genes in the latter group were expressed in the adjacent neural folds or other tissues. Thus, it is possible that such genes may be direct targets of Six1 or Eya1 in domains surrounding the PPE, likely to have

Table 1. Genes with at least two-fold up-regulation in at least two out of three individual treatment groups (Six1$_i$; Eya1$_i$; Six1+Eya1$_i$).

	Annotation*	Accession	Six1 FC[†]	Eya1 FC[‡]	Six1+Eya1 FC[§]
	Chromosome unknown open reading frame	XM_002938866.2	6.2	-	7.9
	cDNA clone IMAGE:7022272	BC094950.1	5.6	5.1	7.5
	X. laevis cyclin Dx (ccndx)	NP_001087887.1	7.5	-	5.2
	Calcitonin gene-related peptide-like	XM_002941675.2	7	-	3
	X. laevis tripartite motif containing 63, E3 ubiquitin protein ligase (trim63)	NM_001093214.1	5.3	3.6	6.3
	ATP-sensitive inward rectifier potassium channel 11-like	XM_004916278.1	5.1	-	6.1
	Leucine rich repeat containing 52 (lrrc52)	XM_002933773.2	6.1	-	2.8
#	SIX homeobox 2 (six2)	NM_001100275.1	5	3.5	5.9
	Potassium voltage-gated channel shaker-related subfamily member 2 (kcna2)	XM_004910736.1	5.1	-	4.9
	Butyrophilin subfamily 2 member A1 (btn2a1)	NM_001094508.1	-	1.2	4.9
	Glutathione peroxidase 2 (gpx2)	NM_001256315.1	-	2.5	4.8
#	*X. laevis* for Xsox17-alpha protein	AJ001730.1	3.6	2.6	4.8
	X. laevis ectodysplasin A receptor (edar)	NM_001087047.1	2.8	2.5	4.7
	Uncharacterized (LOC101734405)	XM_004918247.1	4.4	0.8	3.5
	Glutathione peroxidase 2 (gpx2)	NM_001256315.1	3.4	2.1	4.4
	X. laevis cytochrome P450, family 2, subfamily D, polypeptide 6 (cyp2d6)	NM_001093574.1	1.1	-	4.4
	Calcium/calmodulin-dependent protein kinase kinase 2beta (camkk2)	XM_002937701.2	4.4	2.6	3.5
	Cytochrome P450 family 26 subfamily B polypeptide 1 (cyp26b1)	NM_001079187.2	3.3	4	4.3
	Troponin I type 1 (skeletal, slow)	BC061268	1.8	-	4.3
	72 kDa inositol polyphosphate 5-phosphatase-like (LOC101734556)	XM_004916572.1	-	4.2	1.3
	Chemokine (C-X-C motif) receptor 7 (cxcr7)	NM_001030434.1	3	2.8	4.1
#	*X. laevis* xSox17 alpha 2	AB052691.1	1.7	1.4	4
	Espin (espn) transcript variant X3	XM_004916193.1	-	2.1	4
	B-cell CLL/lymphoma 11B (zinc finger protein) (bcl11b)	XM_004917116.1	-	1.9	4
	C-X-C motif chemokine 10-like	XM_002940578.2	1.9	4	3.5
	X. laevis hedgehog-interacting protein	BC046952.1	-	2.7	4
	X-linked inhibitor of apoptosis (xiap)	NM_001030412.1	4	3.1	2.3
	X. laevis uncharacterized (LOC496300)	NM_001095458.1	1.4	3.9	1.1
	X. laevis RDC1 like protein	BC098974.1	3.6	2.1	3.9
	X. laevis for frizzled 4 protein (fz4 gene)	AJ251750.1	1.3	0.6	3.8
	Espin (espn) transcript variant X1	XM_002933856.2	3.1	2.7	3.7
	Paired box 1 (pax1) transcript variant X1	JQ929179.1	-	3	3.7
	Potassium voltage-gated channel subfamily F member 1 (kcnf1)	NM_001102926.1	3.6	-	2.1
	Echinoderm microtubule-associated protein-like 1-like	XM_004917169.1	-	3.6	2.8
	Leucine rich adaptor protein 1-like (lurap1l)	XM_002940127.2	3.6	1.4	2.4
	Sine oculis binding protein homolog (Drosophila)	BC154687.1	2.7	1.6	3.4
	RNA-directed DNA polymerase homolog	XM_004916122.1	3.4	-	2.1
	Kinesin family member 3C (kif3c) transcript variant X1	XM_004914940.1	1.4	0.8	3.4
	Anoctamin 2 (ano2)	XM_002932297.2	2.2	1.3	3.4
	X. laevis natriuretic peptide C (nppc)	NM_001112924.1	2.1	-	3.3
	Uncharacterized (LOC101734952)	XM_004916172.1	2.5	-	3.3
	Poly (ADP-ribose) polymerase 14-like (LOC101731378)	XM_004920062.1	3.1	-	3.3
	Protocadherin-11 X-linked-like (LOC100493938)	XM_004916890.1	-	3.2	1.4
	Uncharacterized (LOC101733225)	XM_004919937.1	2.5	3.2	1.6

Table 1 continued on next page

Table 1 continued

	Annotation*	Accession	Six1 FC†	Eya1 FC‡	Six1+Eya1 FC§
	Calcium channel voltage-dependent beta 4 subunit (cacnb4)	NM_001142151.1	3.1	-	1.9
	F-box protein 32 (fbxo32) transcript variant X1	XM_002941397.2	1.8	-	3.1
	cDNA clone TEgg026p17	CR761997.2	3	2.6	-
	X. laevis transforming growth factor beta-induced (tgfbi)	NM_001095238.1	1.3	-	3
	Mucin-2-like (LOC100494747)	XM_002936043.2	3	2	1.7
	X. laevis uncharacterized protein (MGC68450)	NM_001089841.1	2.2	-	2.8
	X. laevis neuregulin alpha-1	AF076618.1	1.4	0.8	2.7
	Potassium voltage-gated channel Isk-related (kcne1)	XM_004912135.1	2.2	1.5	2.7
	Olfactory receptor 5G3-like (LOC100492086)	XM_002942220.1	1.9	-	2.7
	Alpha-kinase 2 (alpk2)	XM_004910401.1	1.1	2.2	2.7
	X. laevis arginyl amino peptidase (amino peptidase B) b (rnpep-b)	NM_001092079.1	-	2.7	1.7
#	*X. laevis* SRY-box containing protein (Sox1)	EF672727.1	-	2.6	2.1
	Copine II (cpne2) transcript variant X1	XM_004913481.1	1	1.2	2.6
	X. laevis hemoglobin, gamma A (hbg1)	NM_001096347	1.2	-	2.6
	KIAA0895 protein (kiaa0895)	NM_001114073.1	1.6	2.6	-
#	*X. laevis* empty spiracles homeobox 1gene 2 (emx1.2)	NM_001093430.1	2.6	1.9	1.1
	Homeobox B8 (hoxb8) transcript variant X1	XM_002938021.2	1.1	2.5	-
	Monocyte to macrophage differentiation-associated (mmd)	XM_004918560.1	-	1.2	2.5
	X. laevis uncharacterized (LOC100036933)	NM_001097704.1	1.5	1.5	2.5
	Finished cDNA clone TNeu143f19	CR760056.2	2.2	2.5	-
	Chromosome unknown open reading frame C2orf80	XM_002937119.2	1.4	2.1	2.4
#	Single-minded homolog 1 (sim1) transcript variant X2	XM_004914545.1	-	1.4	2.4
	Transmembrane protein 2-like (LOC100491930)	XM_002932255.2	2.4	1.9	1.3
	PX domain containing 1 (pxdc1)	NM_001130262.1	1.4	-	2.4
	Aldehyde dehydrogenase 1 family member L2 (aldh1l2)	XM_002938070.2	0.9	1.3	2.3
	Uncharacterized (LOC100490228)	XM_002942932.2	1.8	-	2.3
	Beta-1 3-galactosyltransferase 2-like (LOC101732799)	XM_004918863.1	1.6	2.3	-
	Alpha-2 3-sialyltransferase ST3Gal V (st3gal5)	FN550108.1	1.8	-	2.3
	X. laevis uncharacterized protein (MGC64538)	NM_001086337.1	-	1.6	2.3
	Transmembrane channel-like protein 7-like (LOC100493700)	XM_002932222.2	1.4	0.9	2.3
	Kinase insert domain receptor (a type III receptor tyrosine kinase) (kdr)	XM_002934669.2	1.9	0.9	2.3
	Serine/threonine kinase 32A (stk32a)	XM_002936707.2	1.3	2.2	-
	Pancreatic lipase-related protein 2 (pnliprp2)	NM_001089647.1	2.1	0.7	2.2
	X. laevis nephrin (NPHS1)	AY902238.1	-	2.2	1.1
	Poly (ADP-ribose) polymerase 14-like (LOC100485144)	XM_002943546.2	2	2.2	1.2
	Frizzled family receptor 4 (fzd4)	XM_002936543.2	1.4	0.7	2.1
	Neuropeptide Y receptor Y2 (npy2r)	XM_004911153.1	2.1	-	1.6
	Deoxyribonuclease gamma-like (LOC100497175)	XM_002938386.2	1.8	2.1	2
	X. laevis dehydrogenase/reductase (SDR family) member 11 (dhrs11)	NM_001094963.1	-	1.5	2.1
	X. laevis gamma-glutamyl hydrolase (ggh)	NM_001092691.1	2.1	1.3	2
	Opsin-3-like	XM_002932623.2	1	1.2	2
	X. laevis transmembrane protein 56 (tmem56-b)	NM_001086447.1	-	1.1	2
	X. laevis pyruvate dehydrogenase phosphatase catalytic subunit 1 (pdp1)	NM_001094221.1	1.5	2	1
	ArfGAP with SH3 domain ankyrin repeat and PH domain 3 (asap3)	XM_002939360.2	1.7	-	1.9

Table 1 continued on next page

Table 1 continued

	Annotation*	Accession	Six1 FC†	Eya1 FC‡	Six1+Eya1 FC§
#	Early growth response 3 (egr3)	XM_002932703.2	1.6	0.8	1.9
#	POU class 4 homeobox 1 (pou4f1.2)	NM_001097307.1	1.3	1	1.9
	Activin beta B subunit	S61773.1	-	1.7	1.8
	Monocyte to macrophage differentiation-associated (mmd)	XM_002937811.2	1.7	1.1	1.8
	X. laevis ribosomal protein S2e	BC130122.1	-	1.8	1.7
	X. laevis ras homolog family member V (rhov)	NM_001128659.1	1.2	0.8	1.6
	X. laevis adenomatosis polyposis coli down-regulated 1 (apcdd1)	NM_001094109.1	1.2	1	1.6
#	X. laevis zinc finger protein 214 (znf214)	NM_001097042.1	1.2	0.8	1.5
	X. laevis cdc25Ba	AB363840.1	1.2	-	1.5
	X. laevis apelin (apln-a)	NM_001097924.1	0.9	1.3	1.5
	Suppressor of cytokine signaling 2 (socs2)	NM_001095760.1	-	1.1	1.5
#	cAMP responsive element modulator (crem)	XM_002935162.2	-	1.4	1.5
	X. laevis clone IMAGE:4684003	BC042305.1	1.4	-	1.2
#	X. laevis ets-2a proto-oncogene	BC133183.1	1.3	1	1.4
	X. laevis similar to envoplakin	BC045116.1	1.4	1.4	-
	Ras homolog family member V (rhov)	NM_001095566.1	1.4	1	1.2
	Samd9l protein (samd9l)	XM_002943522.2	-	1.2	1.3
	Flocculation protein FLO11-like (LOC100490389)	XM_002942555.2	1.2	-	1.3
	c-Jun-amino-terminal kinase-interacting protein 4-like (LOC100493724)	XM_002939963.2	1.1	-	1.2
	X. laevis Dickkopf-1 (Xdkk-1)	AF030434.1	1	1.2	1.1
	X. laevis ectoderm neural cortex related-3 (Encr-3)	AY216793.1	1.1	0.8	1.2
	Uncharacterized (LOC101730819)	XM_004915204.1	0.9	1.1	1.2
#	X. laevis LIM class homeodomain protein	BC084744.1	1.1	0.7	1.1
	Ceramide kinase-like (cerkl)	XM_002932015.2	1.4	1.3	2
	Mannosyl (alpha-1 3-)-glycoprotein beta-1 4-N-acetylglucosaminyltransferase (mgat4b)	NM_001091975.1	2	-	1.8
	Fibroblast growth factor 19 (fgf19)	NM_001142825.1	-	2	1.5
#	F-box protein 41 (fbxo41)	NM_001079043.1	1.3	0.6	2
	Avidin-like (LOC100487365)	XM_002939983.2	2	1.6	-
	Four and a half LIM domains 2 (fhl2)	NM_001126761.1	-	1.1	1.9
	Metalloprotease TIKI1-like (LOC100491951)	XM_002936336.2	1.1	1.4	1.9
	X. laevis Kazal-type serine peptidase inhibitor domain 1 (kazald1)	NM_001092073.1	1.6	1.1	1.9
	Uncharacterized (LOC101734664)	XM_004910525.1	1.2	0.6	1.9
	X. laevis similar to calsequestrin 2 (cardiac muscle)	BC097545.1	1.8	1.5	1.9
	X. laevis COMM domain containing 3 (commd3)	NM_001095386.1	1.1	1.9	0.6
	X. laevis alcohol dehydrogenase iron containing1 (adhfe1)	NM_001127802.1	-	1.9	1.2
	X. laevis ectonucleoside triphosphate diphosphohydrolase 1 (entpd1)	NM_001092268.1	1.8	0.6	1.3
#	Protein fosB-like transcript variant X2	XM_004916957.1	-	1.7	1.4
	Tocopherol (alpha) transfer protein (ttpa)	NM_001008184.1	-	1.7	1.6
	X. laevis tetratricopeptide repeat domain 39B (ttc39b)	NM_001094701.1	1.1	-	1.7
#	X. laevis Tbx6 (Tbx6)	DQ355794.1	1.4	1.7	1
	X. laevis uncharacterized (LOC100036989)	NM_001097746.1	-	1.3	1.7
	X. laevis cDNA clone IMAGE:6947552	BC093552.1	1.3	1.7	-
	B-cell CLL/lymphoma 10 (bcl10)	NM_001015777.2	1.7	-	1.2
	Uncharacterized (LOC100494710)	XM_002939048.2	1.4	1.6	-

Table 1 continued on next page

Table 1 continued

	Annotation*	Accession	Six1 FC[†]	Eya1 FC[‡]	Six1+Eya1 FC[§]
	X. laevis keratin 17 (krt17)	NM_001094941.1	-	1.2	1.6
	Membrane metallo-endopeptidase-like 1 (mmel1)	NM_001127095.1	0.9	1.1	1.6
	Putative methyltransferase KIAA1456 homolog	XM_002934674.2	1.1	-	1.6
	Phospholipase Cdelta 3 (plcd3)	XM_002935518.2	1.1	1.5	1.6
	IdnK gluconokinase homolog (E. coli) (idnk)	NM_001126592.1	1.4	0.9	1.5
	Uncharacterized (LOC100486093) transcript variant X2	XM_002939117.2	1.5	-	1.5
	X. laevis similar to calsequestrin 2 (cardiac muscle)	BC041283.1	1.1	-	1.5
	Piwi-like RNA-mediated gene silencing 2 (piwil2)	NM_001112999.1	1.1	-	1.5
	Zinc finger and BTB domain containing 20 (zbtb20)	XM_002939649.2	1.4	-	1.1
#	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (mafa)	NM_001032304.1	1.4	0.9	1.1
	X. laevis uncharacterized protein (MGC81120)	NM_001091225.1	1.4	0.9	1.3
#	Single-minded homolog 1 (*Drosophila*) (sim1) transcript variant X3	XM_004914546.1	1.1	1.3	1.2
	Xenopus laevis alpha-2-macroglobulin-like 1 (a2ml1)	NM_001135077.1	1.1	-	1.1
	X. laevis chromogranin A (parathyroid secretory protein 1) (chga)	NM_001094724.1	1.6	1.4	2.2
	X. laevis lipaseendothelial (lipg)	NM_001090061.1	1.2	1.3	0.6
	G protein-coupled receptor 56 (gpr56)	XM_002931653.2	1.7	-	1.6
	X. laevis family with sequence similarity 101member B (fam101b)	NM_001093870.1	1.5	0.8	1.5
	X. laevis CD81 protein (cd81-a)	NM_001086613.1	0.7	1.1	1.9
	X. laevis calbindin D28k	BC170542.1	2.2	-	3.1
	X. laevis ATPaseNa+/K+ transportingbeta 1 polypeptide (atp1b1)	NM_001086759.1	1.2	1	1.7
	X. laevis 7-transmembrane receptor frizzled-1	AF231711.1	1.4	1	2
	X. laevis prostaglandin reductase 2 (ptgr2)	NM_001079334.1	1.4	1.5	-
	X. laevis TGF-beta2 for transforming growth factor-beta2	X51817.1	1.3	-	1.1
#	SRY (sex determining region Y)-box 2 (sox2)	NM_213704.3	1.1	1.3	1.9
#	*X. laevis* for enhancer of split related 9 (esr9 gene)	AJ009282.1	1.7	1.6	-
	X. laevis mal T-cell differentiation protein (mal)	NM_001086577.1	-	1.2	1.4
	Transmembrane proteaseserine 13 (tmprss13)	XM_002932904.2	1.5	1.1	1.9
	X. laevis Ras-related associated with diabetes (rrad)	NM_001092750.1	8	4.6	4.2
	Integrin beta 4 (itgb4) transcript variant X1	XM_002939974.2	1.4	-	2.2
	Xenopus (Silurana) tropicalis FERM domain containing 4A (frmd4a)	XM_002935243.2	1.1	0.6	1.3
	X. laevis complement factor I (cfi-a)	NM_001085952.1	1.4	1.2	1.6
#	*X. laevis* SIX homeobox 1 (six1)	NP_001082027.1	1.4	1.2	2.3
	FH2 domain-containing protein 1-like (LOC100496216)	XM_002934907.2	1.9	0.9	1.9
#	*X. laevis* mab-21-like 2 (mab21l2-b)	NM_001096770.1	-	2.8	2.9
	X. laevis regulator of cell cycle (rgcc)	NM_001093976.1	1.3	1.1	1.7
	X. laevis Cep63	FJ464988.1	-	1.4	2.3
	X. laevis CD81 antigen (target of anti proliferative antibody 1)	BC041217.1	1.7	1.1	2
	Transmembrane serine protease 9	BC087611.1	1.1	1.1	1.2
#	*X. laevis* POU class 3 homeobox 2 (pou3f2-b)	NM_001096751.1	3	2.3	2.9
	G protein-coupled receptor 153 (gpr153)	NM_001128052.1	2.5	1.1	1.5
#	*X. laevis* Myoblast determination protein 1 homolog A	BC041190.1	3.5	2.7	4.7
#	T-cell leukemia homeobox 1 (tlx1) transcript variant 1	XM_002936768.2	2.6	2.3	2.6
	X. laevis neurotrophin 3 (ntf3)	NM_001092740.1	1.4	1.5	1.9
	X. laevis p21 GTPase-associated kinase 1 (PAK1)	AF000239.1	1.2	-	2.1

Table 1 continued on next page

Table 1 continued

	Annotation*	Accession	Six1 FC[†]	Eya1 FC[‡]	Six1+Eya1 FC[§]
#	*X. laevis* hairy and enhancer of split 9, gene 1 (hes9.1-b)	NP_001089097.1	1.8	1.5	1.6
	X. laevis tetraspanin 1 (tspan1)	NM_001095473.1	1.2	0.7	1.3
	X. laevis uncharacterized protein (MGC83079)	NM_001091250.1	2	1.5	-
	X. laevis cDNA clone IMAGE:5085355	BC073731.1	1.3	-	1.4
	Family with sequence similarity 198member A (fam198a)	XM_002937853.2	1.7	0.7	1.3
	Progestin and adipoQ receptor family member IX (paqr9)	XM_004914351.1	1.7	-	1.2
#	Hairy and enhancer of split 8 (hes8)	XM_002933849.2	2.8	1.7	3.6
	X. laevis p21 GTPase-associated kinase 1	BC081113.1	1.3	0.8	1.7
	Finished cDNA clone TNeu008g03	CR761907.2	1.2	1.1	0.7
	WD repeat domain 27 (wdr27)	XM_002931515.2	1.2	2.2	1.1
#	Growth factor independent 1 transcription repressor (gfi1)	XM_002933803.2	1.8	1.8	3.2
	Protein phosphatase 2 regulatory subunit B'beta (ppp2r5b)	NM_001100279.1	2.4	1.4	4.2
	Ornithine decarboxylase antizyme 2 (oaz2), transcript variant 2	NP_001106583.2	1.8	-	1.5
	X. laevis fast troponin T (TNNT3)	AY114144.1	-	1.1	1.5
#	*X. laevis* xRipply3 for xRipply3 protein	AB455086.1	0.9	1.1	2
	RAS-like family 11member B (rasl11b)	NM_001015774.1	-	1.2	1.4
	X. laevis for thimet oligopeptidase	BC070748.1	3.8	-	2
	X. laevis fibroblast growth factor 3 (fgf3)	NM_001008153.1	2	1.2	2
	X. laevis cDNA clone IMAGE:8332229	BC155363.1	1.5	0.9	1.4
	Proline rich 15 (prr15)	XM_002933381.2	1.6	-	1.3
	Integrin beta 6 (itgb6)	NM_001097306.1	2.3	0.6	2.8
#	*Xenopus laevis* empty spiracles homeobox 1, gene 2 (emx1.2)	NM_001093430.1	2.6	1.4	2.1
	X. laevis p21-activated kinase (PAK1)	AF169794.1	1.4	1.8	2.6
#	ISL LIM homeobox 2 (isl2)	NM_001166041.1	1.5	-	1.7
#	Atonal homolog 1 (Drosophila) (atoh1)	XM_004911085.1	0.9	1.1	1.5
	Ectodysplasin A receptor (edar)	NM_001087047.1	4.3	-	3.3
	X. laevis degr03	DQ096846.1	2.1	2.2	2
	Calcyphosine (caps)	NM_001097320.1	-	1.4	3.7
	X. laevis kiaa0930	NM_001086221.1	1.5	1	1.6
	Putative N-acetyltransferase 16-like (LOC100490742)	XM_002943189.1	2.1	1	1.7
#	T-box 15 (tbx15)	XM_002940981.2	2	1	1.8
#	SRY (sex determining region Y)-box 1 (sox1)	NM_001080996.1	0.6	1.5	1.2
	Cytochrome P450 family 2 subfamily C polypeptide 18 (cyp2c18)	NM_001091776.1	2.1	1.4	1.6
	X. laevis calcitonin receptor-like (calcrl)	NM_001086737.1	1.1	0.8	1.6
	X. laevis claudin 3 (cldn3)	NM_001005709.1	2.1	1.3	1.5
	Atlastin GTPase 1 (atl1)	NM_001078754.1	1.8	2	1.7
	Rho GTPase activating protein 9 (arhgap9), transcript variant X2	XM_012957829	1.8	1.2	3.4
#	*X. laevis* Hes2	BC084134.1	1.7	0.9	1.3
	X. laevis U3 snRNA	X07318.1	1	2.8	1.1
	Uncharacterized (LOC101732195)	XM_004912378.1	2	-	1.5
	Tumor necrosis factor receptor superfamilymember 21 (tnfrsf21)	NM_001079136.1	1.1	0.8	1.2
	X. laevis arginase 3	U08408.1	-	1.3	1.8
	ChaC cation transport regulator homolog 1 (chac1)	XM_002939546.2	1.2	1.3	1.5
	X. laevis DIRAS familyGTP-binding RAS-like 3 (diras3)	NM_001095243.1	0.8	1.7	1.4
	X. laevis DnaJ (Hsp40) homolog subfamily C member 27 (dnajc27-b)	NM_001095422.1	1.1	0.8	1.1

been included in our screen as a result of non-PPE contamination during dissection. Of the transcriptional regulators identified in our list of well-supported targets and expressed in the PPE or placodal derivatives 79% (15/19) were statistically supported in the analyses of merged datasets (*Table 2*). These included genes broadly expressed in cranial ectoderm including the PPE (*Crem*, *FosB*, *Znf214*, *Ripply3*), and genes expressed in the PPE or subdomains of the PPE and subsequently in some placodes (*Hes2*, *Hes8*, *Hes9*, *Mab21l2b*, *Six1*, *Six2*, *Sox2*, *Sox3*, *Sox21*, *Atoh1*, *Ngn1*, *Gfi1a*, *Isl2*, *Pou4f1.2*, *Tlx1*) (*Figure 4A–T*).

To begin to elucidate the GRN downstream of Six1 and Eya1 we chose ten transcription factors showing expression in posterior placodes (i.e. those derived from the posterior placodal area; the lateral line, otic and epibranchial placodes) for additional functional studies including genes implicated in the maintenance of neuronal progenitors (*Sox2*, *Sox3*, *Hes8* and *Hes9*) as well as genes implicated in the regulation of sensory or neuronal differentiation (*Atoh1*, *Gfi1a*, *Isl2*, *Ngn1*, *Pou4f1.2* and *Tlx1*). Selected genes were independently verified as being direct targets of either Six1 (*Isl2*) or of both Six1 and Eya1 (all other targets; *Sox3* not analysed) in the PPE by qPCR, and the results were broadly consistent with the RNA-Seq data (*Figure 4U and V*).

Six1 and Eya1 are required for expression of transcriptional regulators of neurogenesis in the PPE and placodes

To explore whether Six1 or Eya1 were required for the expression of selected target genes, the expression of each target was analysed by in-situ-hybridisation after MO-mediated knockdown of *Six1* or *Eya1*. The efficacy and specificity of both co-injected *Six1*-MOs (*Six1*-MO1 and *Six1*-MO2; *Brugmann et al., 2004*) and *Eya1*-MOs (*Eya1*-MO1 and *Eya1*-MO2; *Schlosser et al., 2008*) have been previously reported. Compared to injection with a control MO (*Eya1*-mmMO with 5 mismatches relative to Eya1-MO2), knockdown of either *Six1* or *Eya1* significantly reduced the expression of all direct target genes in PPE or placodes, demonstrating that both Six1 and Eya1 are required for their expression (*Figure 5* and *Figure 5—figure supplement 1*; *Table 3*). To control for off-target effects associated with MO use, target gene expression was also analysed after overexpression of a dominant-negative version of *Six1* (*Six1-EnR*; *Brugmann et al., 2004*). Expression patterns of all target genes were highly similar to those seen after MO-knockdown of either *Six1* or *Eya1*, suggesting that the observed reductions in expression were caused by *Six1* or *Eya1* knockdown as opposed to being an artefact of MO use (*Figure 5—figure supplement 2*). Taken together, these findings show that Six1 and Eya1 are essential direct upstream regulators of multiple genes encoding transcription factors that promote neuro- and sensorigenesis in the PPE and placodes.

Six1 and Eya1 affect expression of presumptive direct target genes in complex ways

To complement the loss-of-function studies, and to examine the spatial distribution of presumptive direct targets of Six1 and Eya1 in gain-of-function experiments, we injected Six1-GR and Eya1-GR individually and, to ensure that overexpression did not affect early embryogenesis, induced their nuclear translocation by adding DEX at neural fold stage (stages 16–18), after PPE commitment (*Ahrens and Schlosser, 2005*). Surprisingly, although injection of Six1-GR or Eya1-GR resulted in upregulation of direct targets in a minority of cases (*Table 3*; *Figures 6* and *7*), the dominant observed phenotype was down-regulation of target gene expression in the PPE or placodes (*Table 3*; *Figures 8* and *9*). Considering that here, unlike in the initial RNA-Seq screen and qPCR experiments, CHX was not used to block protein synthesis, these results indicate that Six1 and Eya1 additionally affect expression of many of their direct target genes in indirect and partly opposing ways.

Table 2. Transcription factors and co-factors selected for characterisation by in-situ-hybridisation ranked by FC value in individual treatment.

Annotation	Gene	Accession	Individual			Merged		
			Six1*	Eya1[†]	Six1 +Eya1[‡]	Six1[§]	Eya1[#]	Six1 +Eya1[¶]
SIX homeobox 2 (Six2)	Six2	NM_001100275.1	5	3.5	5.9	5.4**	5**	4.9**
X. laevis for Xsox17-alpha protein	Sox17	AJ001730.1	3.6	2.6	4.8	4.4**	3.3**	3.5**
X. laevis Myoblast determination protein 1 homolog A	MyoD1	BC041190.1	3.5	2.7	4.7	4.1**	4.2**	3.9**
Hairy and enhancer of split 8 (Hes8)	Hes8	XM_002933849.2	2.8	1.7	3.6	3.2**	3.2**	3.1**
Growth factor independent 1 transcription repressor (Gfi1)	Gfi1a	XM_002933803.2	1.8	1.8	3.2	2.4**	2.6**	4.1**
X. laevis POU class 3 homeobox 2 (Pou3f2-b)	Pou3f2b	NM_001096751.1	3	2.3	2.9	3**	2.6**	2.7**
X. laevis Mab-21-like 2 (Mab21l2-b)	Mab21l2b	NM_001096770.1	-	2.8	2.9	-	-	-
T-cell leukemia homeobox 1 (Tlx1) transcript variant 1	Tlx1	XM_002936768.2	2.6	2.3	2.6	2.4**	2.4**	2.4**
X. laevis empty spiracles homeobox 1 gene 2 (Emx1.2)	Emx1.2	NM_001093430.1	2.6	1.9	1.1	-	-	1.7**
X. laevis SRY-box containing protein (Sox1)	Sox1	EF672727.1	-	2.6	2.1	-	2**	-
Single-minded homolog 1 (Sim1) transcript variant X2	Sim1	XM_004914545.1	-	1.4	2.4	-	-	-
X. laevis SIX homeobox 1 (Six1)	Six1	AF279254.1	1.4	1.2	2.3	1.9**	1.6**	1.6**
F-box protein 41 (Fbxo41)	Fbxo41	NM_001079043.1	1.3	0.6	2	-	-	-
T-box 15 (Tbx15)	Tbx15	XM_002940981.2	2	1	1.8	2**	1.4**	1.7**
X. laevis xRipply3 for xRipply3 protein	Ripply3	AB455086.1	0.9	1.1	2	1.6**	1.4**	1.3**
Early growth response 3 (Egr3)	Egr3	XM_002932703.2	1.6	0.8	1.9	1.7**	1.3**	1.9**
SRY (sex determining region Y)-box 2 (Sox2)	Sox2	NM_213704.3	1.1	1.3	1.9	1.6**	1.6**	1.5**
POU class 4 homeobox 1 (Pou4f1.2)	Pou4f1.2	NM_001097307.1	1.3	1	1.9	1.6**	1.5**	1.5**
X. laevis for enhancer of split related 9 (Esr9 gene)	Hes9.1a	AJ009282.1	1.7	1.6	-	-	-	-
ISL LIM homeobox 2 (Isl2)	Isl2	NM_001166041.1	1.5	-	1.7	1.6**	1.1**	1.4**
X. laevis Tbx6 (Tbx6)	Tbx6	DQ355794.1	1.4	1.7	1	-	-	-
Protein FosB-like transcript variant X2	FosB	XM_004916957.1	-	1.7	1.4	-	1.4**	1.2**
X. laevis Hes2	Hes2	BC084134.1	1.7	0.9	1.3	-	-	-
cAMP responsive element modulator (Crem)	Crem	XM_002935162.2	-	1.4	1.5	-	1.4**	1.2**
X. laevis zinc finger protein 214 (Znf214)	Znf214	NM_001097042.1	1.2	0.8	1.5	1.2**	5.9**	5.8**
Xenopus laevis SRY (sex determining region Y)-box 21 (Sox21)	Sox21	NM_001172213.1	1.2	0.6	1.5	1.4**	1.2**	1.2**
Atonal homolog 1 (Drosophila) (Atoh1)	Atoh1	XM_004911085.1	0.9	1.1	1.5	1	1	1
X. laevis Ets-2a proto-oncogene	Ets2a	BC133183.1	1.3	1	1.4	1.3**	1.2**	1.2**
V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (Mafa)	Mafa	NM_001032304.1	1.4	0.9	1.1	1.9**	-	1.8**
X. laevis LIM class homeodomain protein	Lhx5	BC084744.1	1.1	-	1.1	-	-	-
Xenopus (Silurana) tropicalis neurogenin 1 (Neurog1)	Ngn1	NM_001123423.1	0.8	0.9	0.8	0.8	0.8	0.8**
Xenopus laevis SOX3 protein	Sox3	BC072222.1	0.5	-	0.9	0.7	0.7	0.6

*Log$_2$ fold change values after *Six1* overexpression (Six1$_i$).

[†] Log$_2$ fold change values after *Eya1* overexpression (Eya1$_i$).

[‡] Log$_2$ fold change values after *Six1+Eya1* overexpression (Six1+ Eya1$_i$).

[§] Log$_2$ fold change values after overexpression of *Six1* or *Six1+Eya1* (Six1$_m$).

[#] Log$_2$ fold change values after overexpression of *Eya1* or *Six1+Eya1* (Eya1$_m$).

[¶] Log$_2$ fold change values after overexpression of *Six1* or *Eya1* or *Six1+Eya1* (Six1+Eya1$_m$).

** Denotes statistically supported data ($q < 0.05$).

DOI: 10.7554/eLife.17666.008
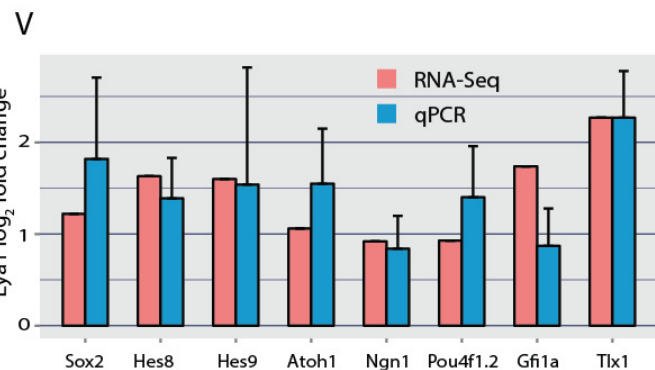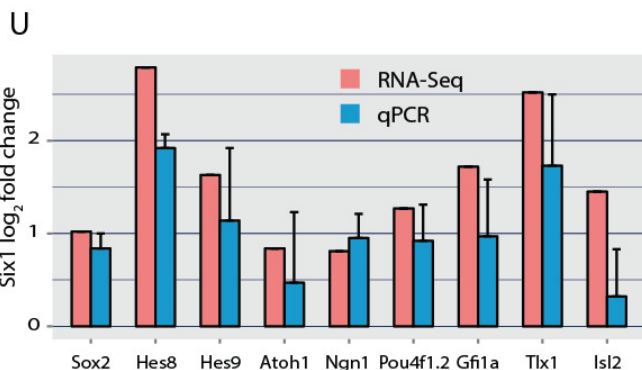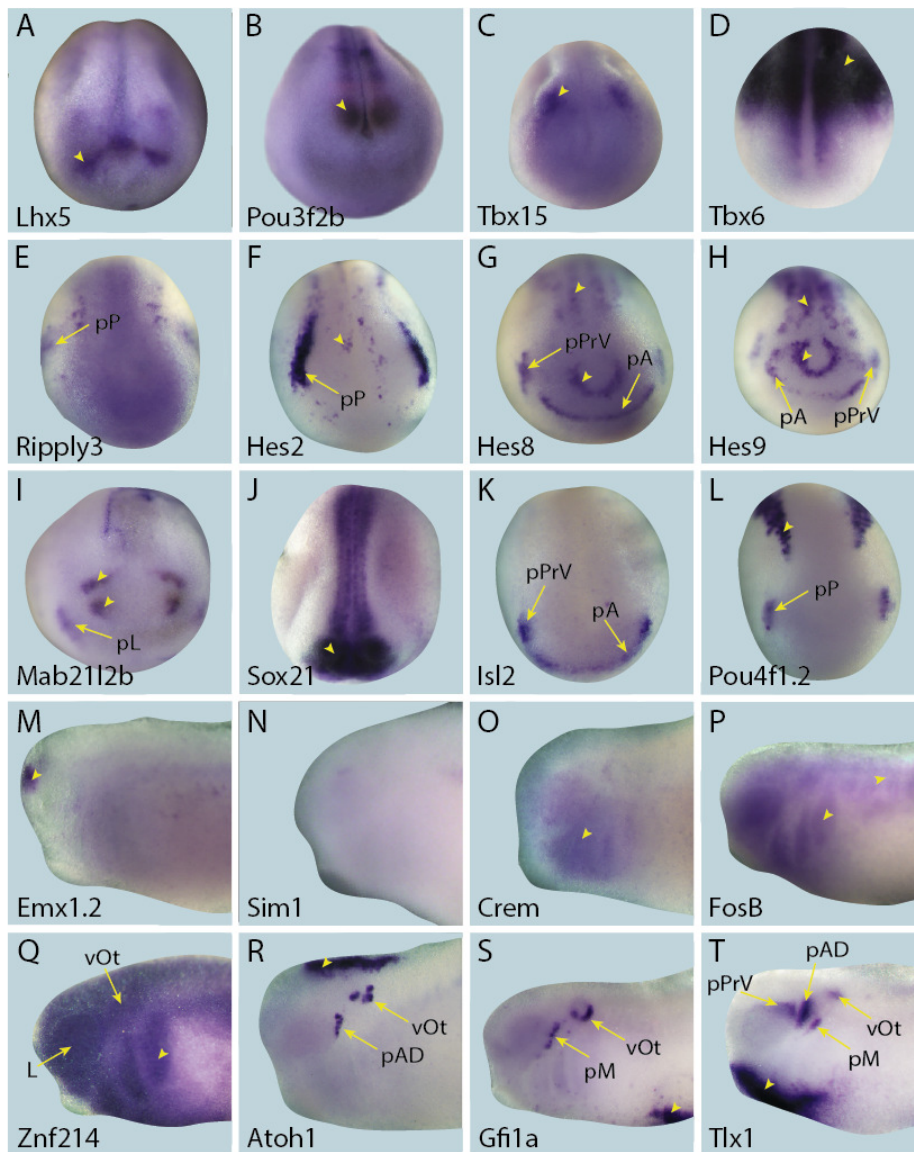
Figure 4. Expression of selected presumptive direct targets of Six1/Eya1 in whole-mount *Xenopus* embryos. Genes expressed at neural plate stages (stages 14–18) are shown in panels A–L, and those only expressed at later stages are shown at mid/late tail bud stage (stages 28–32) in panels M–T. Several of the genes surveyed (*Lhx5, Pou3f2b, Tbx15, Tbx6, Emx1.2* and *Sim1* (A–D, M and N), were not expressed in the PPE, nor any placodal derivatives in later stages. Instead, such genes were expressed in the adjacent neural folds (*Lhx5, Pou3f2b and Tbx15*), ectoderm (*Tbx6),* or in the

Figure 4 continued on next page

Figure 4 continued

forebrain at later stages (*Emx1.2*). Several other genes were expressed broadly across the cranial ectoderm, at least partially overlapping with the PPE at neural plate stages (*Ripply3, Crem, FosB* and *Znf214*; **E**,**O**–**Q**), some of which are also maintained in placodal derivatives such as *Znf214* in the otic vesicle (**Q**). The remaining genes (**F**–**L**, **R**–**T**) are expressed in parts of the PPE and maintained in some placodes (*Hes2, Hes8, Hes9, Mab21l2b, Sox21, Isl2, Pou4f1.2,* and *Tlx1*) or are expressed in a subset of placodes only (*Atoh1, Gfi1a*) (see *Figure 4—figure supplements 1–4* for additional stages). Yellow arrows mark placodal expression. Arrowheads mark non-placodal expression. Abbreviations: pA: anterior placodal region; pAD: anterior lateral line placode; pE: epibranchial placode; pL: lens placode; L: lens; pM: middle lateral line placode; pO: olfactory placode; pP: posterior placodal region; pPrV: profundal/trigeminal placodes; vOt: otic vesicle. Plots **U** and **V** show qPCR after *Six1* or *Eya1* overexpression. Log$_2$ fold change values were calculated from qPCR data obtained after overexpression of Six1-GR (**U**) or Eya1-GR (**V**) in placodal explants and are shown next to corresponding fold change values obtained from the RNA-Seq data. In all cases shown, qPCR values broadly corroborate those from the RNA-Seq data - showing up-regulation of target genes after either *Six1* or *Eya1* overexpression. Vertical error bars show the standard deviation of the mean of biological triplicates.
DOI: 10.7554/eLife.17666.009

The following figure supplements are available for figure 4:

Figure supplement 1. Expression of targets not expressed in placodes in whole-mount *Xenopus* embryos.
DOI: 10.7554/eLife.17666.010

Figure supplement 2. Expression of targets that broadly overlap with PPE in whole-mount *Xenopus* embryos.
DOI: 10.7554/eLife.17666.011

Figure supplement 3. Expression of targets with dynamic/transient expression pattern in placodes in whole-mount *Xenopus* embryos.
DOI: 10.7554/eLife.17666.012

Figure supplement 4. Expression of targets with persistent expression in placodes in whole-mount *Xenopus* embryos.
DOI: 10.7554/eLife.17666.013

Figure supplement 5. Expression of selected targets in pre-placodal ectoderm (PPE) in sections through neural plate stage *Xenopus* embryos.
DOI: 10.7554/eLife.17666.014

Discussion

Overexpression of GR-fusion constructs followed by DEX-induced nuclear translocation in the presence of protein synthesis inhibitors has been previously used successfully to screen for direct target genes of transcription factors or cofactors in *Xenopus* (*Kolm and Sive, 1995*; *Taverner et al., 2005*; *Seo et al., 2007*). Here, we combine this approach with high-throughput sequencing of tissue-specific RNA to identify several hundred novel presumptive direct target genes of Six1 and Eya1 in the PPE. We show that this strategy indeed recovers the majority of direct Six1 target genes known from previous studies, indicating its reliability. Our in situ and qPCR analyses of target genes predicted from the RNA-Seq screen also provided independent verification of selected target genes suggesting a low false discovery rate. Moreover, the expression of all genes selected for detailed analysis proved to be dependent on Six1 and Eya1 in the PPE, indicating that many genes our screen predicted as Six1/Eya1 targets are also functionally dependent on these upstream regulators in the PPE. A comparison of our data set with recently identified direct target genes of *sine oculis*, the *Six1* orthologue in the developing eye of *Drosophila* (*Jusiak et al., 2014*; *Jin et al., 2016*) also reveals that homologues to six out of the 12 *sine oculis* target genes identified with high confidence in *Jin et al. (2016)* are differentially expressed in our Six$_i$ or Six1+Eya1$_i$ (and often also in Eya1$_i$) treatment groups, viz. *Six1* and *Six2; Eya4; Shh*; various matrix metalloproteases (e.g. *MMP9*); *Ets2*; and *Frizzled1* and *Frizzled4*. This suggests that a relatively high proportion of Six1 target genes may be evolutionarily conserved.

Our finding that many of the presumptive direct target genes of Six1 or Eya1 are not up-regulated in the absence of CHX indicates that without blocking protein synthesis it is not possible to reliably identify direct target genes, presumably due to the existence of indirect interactions with such targets. We believe that this is one of the reasons why our findings differ substantially from the study of *Yan et al. (2015)*, which analysed differentially expressed genes in *Xenopus* animal cap explants after overexpression of Six1 without first blocking protein synthesis. None of the transcription factors in our prioritised list was identified in the study by *Yan et al. (2015)*; and we found none of the transcription factors differentially expressed in their study. A second likely reason for the discrepancy between the results presented here and in *Yan et al. (2015)* is that, while we specifically analysed PPE tissue (presumably containing tissue-specific cofactors required for the activation or repression of Six1 and Eya1 target genes specific for the developing placodes), they analysed target genes in animal cap tissue, known to be composed of pluripotent cells.
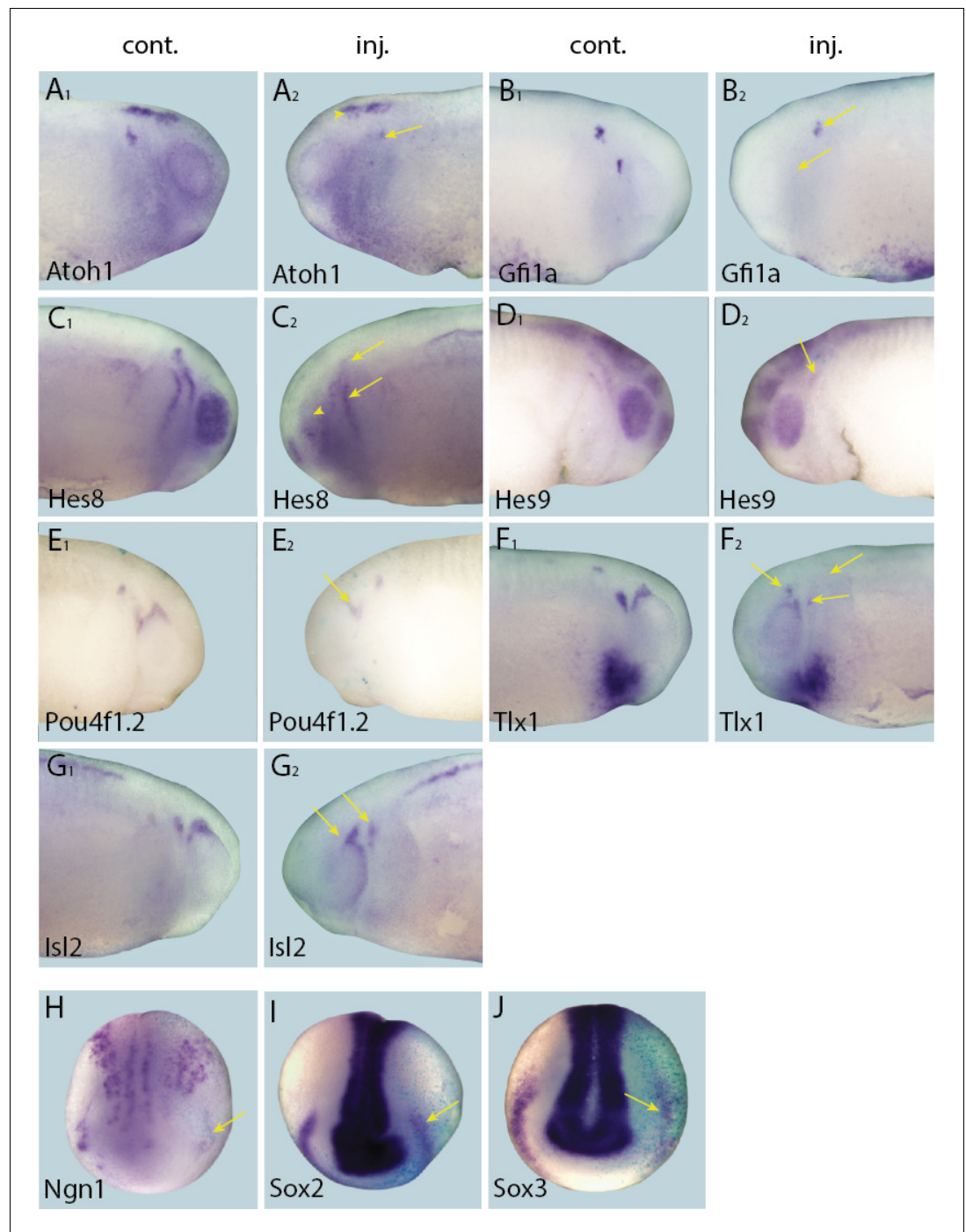
Figure 5. Effects of *Eya1* knockdown on target genes. Tail bud (**A–G**) and neural plate (**H–I**) stage embryos after unilateral injection of *Eya1*-MO1+2. In each case, *lacZ* was co-injected as a lineage tracer and panels **A₁–G₁** show the control (un-injected) side and **A₂–G₂** show the injected side (*lacZ* staining out of frame in most specimens). The injected side is positioned to the right in **H–J**, as marked by blue *lacZ* staining. Arrows and arrowheads mark reductions in marker gene expression in placodal and non-placodal derivatives, respectively.
DOI: 10.7554/eLife.17666.015

The following figure supplements are available for figure 5:

Figure supplement 1. Effects of Six1 knockdown on target genes.
DOI: 10.7554/eLife.17666.016

Figure supplement 2. Repression of Six1 target genes by *Six1-EnR* injection.
DOI: 10.7554/eLife.17666.017

Table 3. Changes in marker gene expression in the placodes after injection of various constructs.

	Injection	Six1-MO*	Eya1-MO*	Six1-EnR	Eya1-mmMO	Six1-GR§	Eya1-GR§
		%	%	%	%	%	%
	Phenotype	(n)	(n)	(n)	(n)	(n)	(n)
Atoh1	Reduced	77**	90‡	94	10	26	42
		(26)	(20)	(18)	(21)	(19)	(12)
	Increased	0	0	0	0	35	42
		(26)	(20)	(18)	(21)	(17)	(12)
Gfi1a	Reduced	82†	67†	69	31	57	36
		(27)	(17)	(16)	(26)	(14)	(14)
	Increased	0	0	0	0	7	43
		(27)	(17)	(16)	(26)	(14)	(14)
Hes8	Reduced	74‡	83‡	70	17	60	57
		(35)	(35)	(46)	(24)	(40)	(56)
	Increased	0	0	24	0	15	29
		(35)	(35)	(46)	(24)	(40)	(56)
Hes9	Reduced	73‡	76‡	84	11	75	29
		(45)	(33)	(38)	(27)	(12)	(29)
	Increased	0	0	8	0	0	0
		(45)	(33)	(38)	(27)	(12)	(29)
Isl2	Reduced	66†	100‡	nd	27	50	24
		(38)	(17)	nd	(22)	(18)	(17)
	Increased	6	0	nd	0	31	41
		(38)	(17)	nd	(22)	(16)	(17)
Ngn1	Reduced	65‡	49†	84	17	17	36
		(51)	(43)	(31)	(24)	(30)	(59)
	Increased	0	16	6	4	23	41
		(51)	(43)	(31)	(24)	(30)	(59)
Pou4f1.2	Reduced	67‡	63†	71	16	47	81
		(48)	(30)	(35)	(19)	(15)	(37)
	Increased	0	0	0	0	13	0
		(48)	(30)	(35)	(19)	(15)	(37)
Sox2	Reduced	74‡	78‡	87	6	90	48
		(19)	(18)	(30)	(16)	(21)	(33)
	Increased	0	0	23#	0	0	12
		(19)	(18)	(30)	(16)	(21)	(33)
Sox3	Reduced	68‡	54†	39	9	49	40
		(25)	(26)	(31)	(22)	(25)	(23)
	Increased	0	0	71#	0	16	17
		(25)	(26)	(31)	(22)	(25)	(23)
Tlx1	Reduced	84†	91‡	100	33	40	7
		(31)	(32)	(13)	(15)	(10)	(15)
	Increased	6	0	0	0	40	73
		(31)	(32)	(13)	(15)	(10)	(15)

* Significant differences (Fisher's exact test);

† $p<0.05$,

‡ $p<0.001$) to *Eya1*-mmMO injections are indicated.

Previous studies have shown that Six1 and Eya1 are essential for both the establishment of the PPE (*Brugmann et al., 2004*; *Christophorou et al., 2009*), as well as for the subsequent development of placode-derived sense organs (*Xu et al., 1999*; *Laclef et al., 2003*; *Zheng et al., 2003*; *Brugmann et al., 2004*; *Zou et al., 2004*; *Kozlowski et al., 2005*; *Schlosser et al., 2008*; *Ahmed et al., 2012b*, *2012a*) but the mechanisms through which they act are poorly understood. The continued expression of both genes in almost all placodes developing from the PPE (*Schlosser and Ahrens, 2004*), combined with the observed deficiencies in derivatives from most placodes after loss-of-function of either Six1 or Eya1, indicates that they play a role in generic aspects of placode development shared by all placodes. Indeed, our data show that genes revealed as presumptive direct targets of Six1 and Eya1 were highly enriched for GO terms associated with neurogenesis and placode development. Our screen also confirms previous studies suggesting that Six1 and Eya1 synergistically regulate many genes in the PPE, and that the Six1-Eya1 protein complex predominantly acts by activating transcription (*Li et al., 2003*; *Brugmann et al., 2004*). However, we also find support for independent action of Six1 and Eya1 in the PPE, possibly in
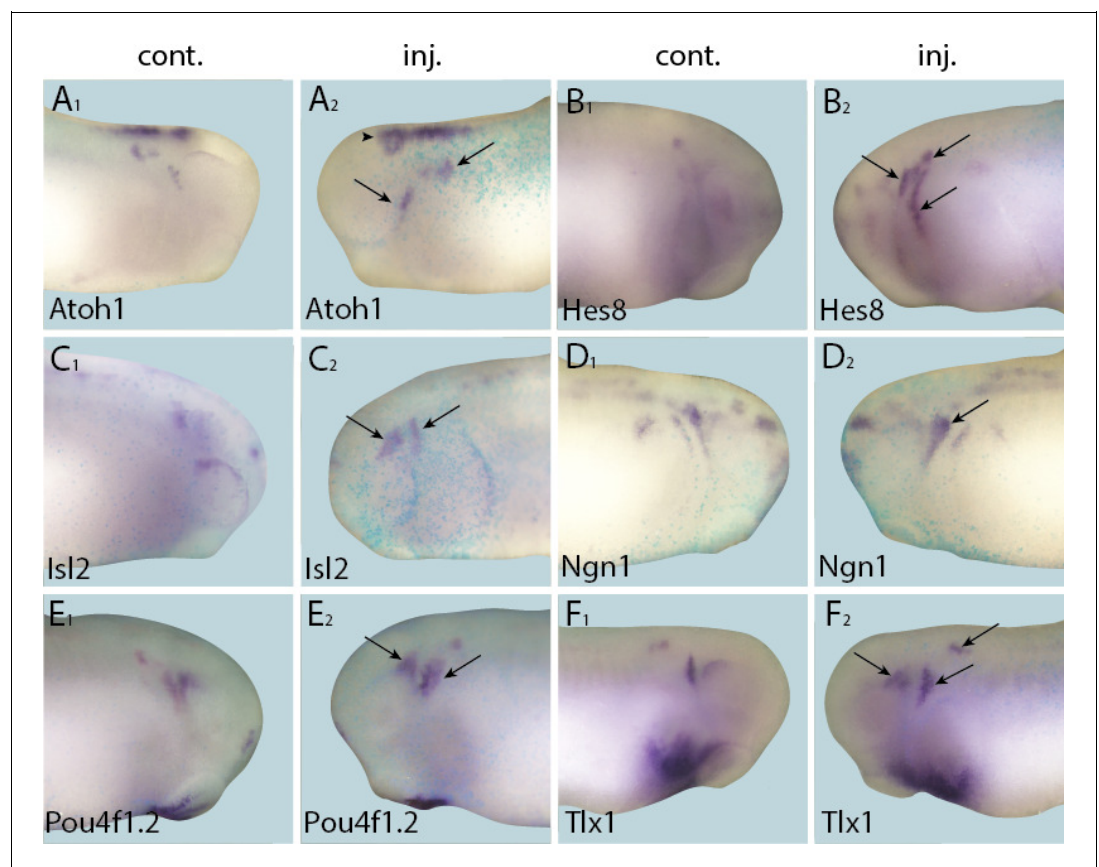
Figure 6. Up-regulation of target gene expression domains after overexpression of Six1. Tail bud stage embryos (**A–F**) after unilateral injection of *Six1*-GR and DEX induction at neural plate stage (16–18). In each case, *lacZ* was co-injected as a lineage tracer and panels A_1–F_1 show the control (un-injected) side and A_2–F_2 show the injected side. Arrows and arrowheads mark expansions in marker gene expression in placodal and non-placodal derivatives, respectively.
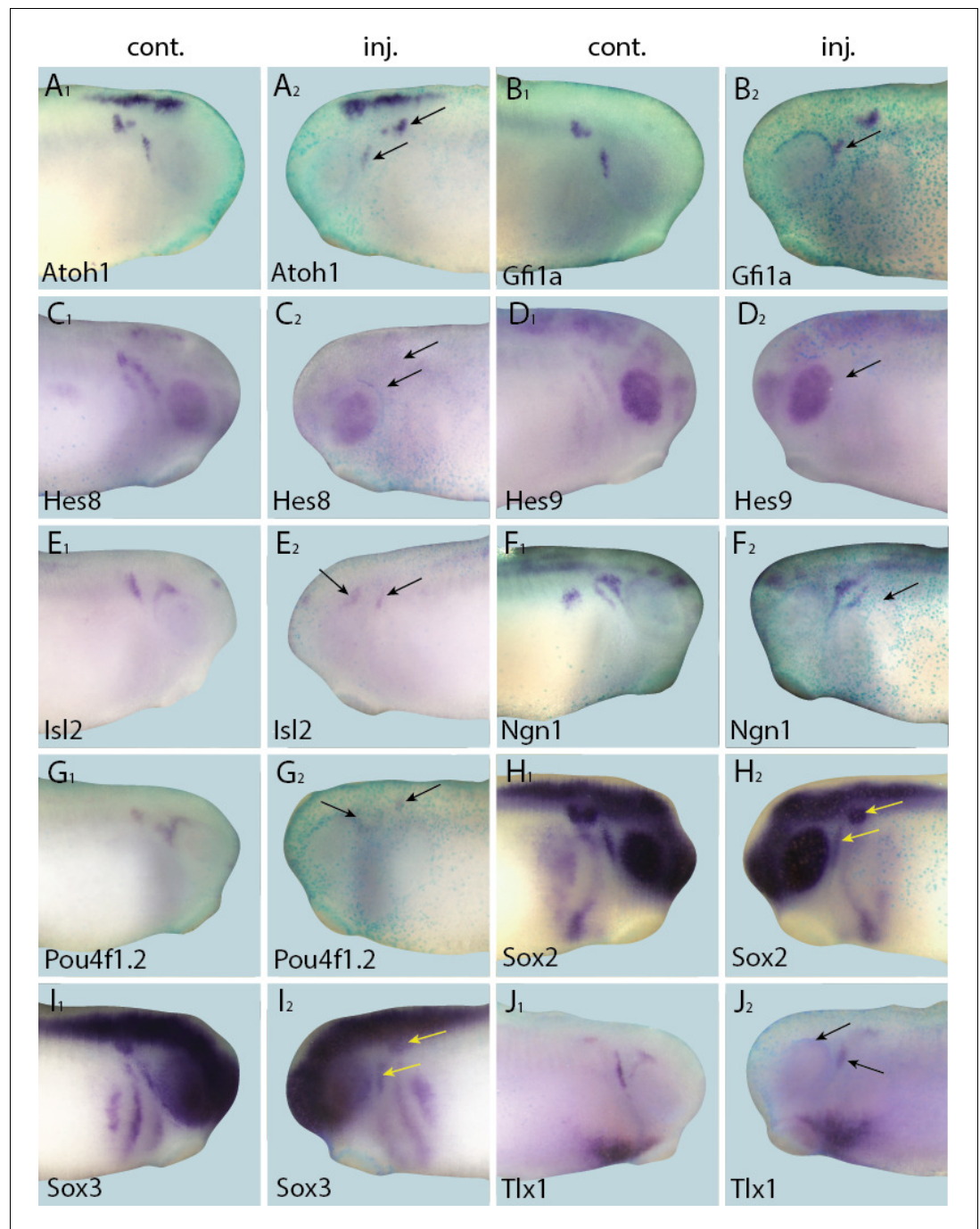DOI: 10.7554/eLife.17666.019
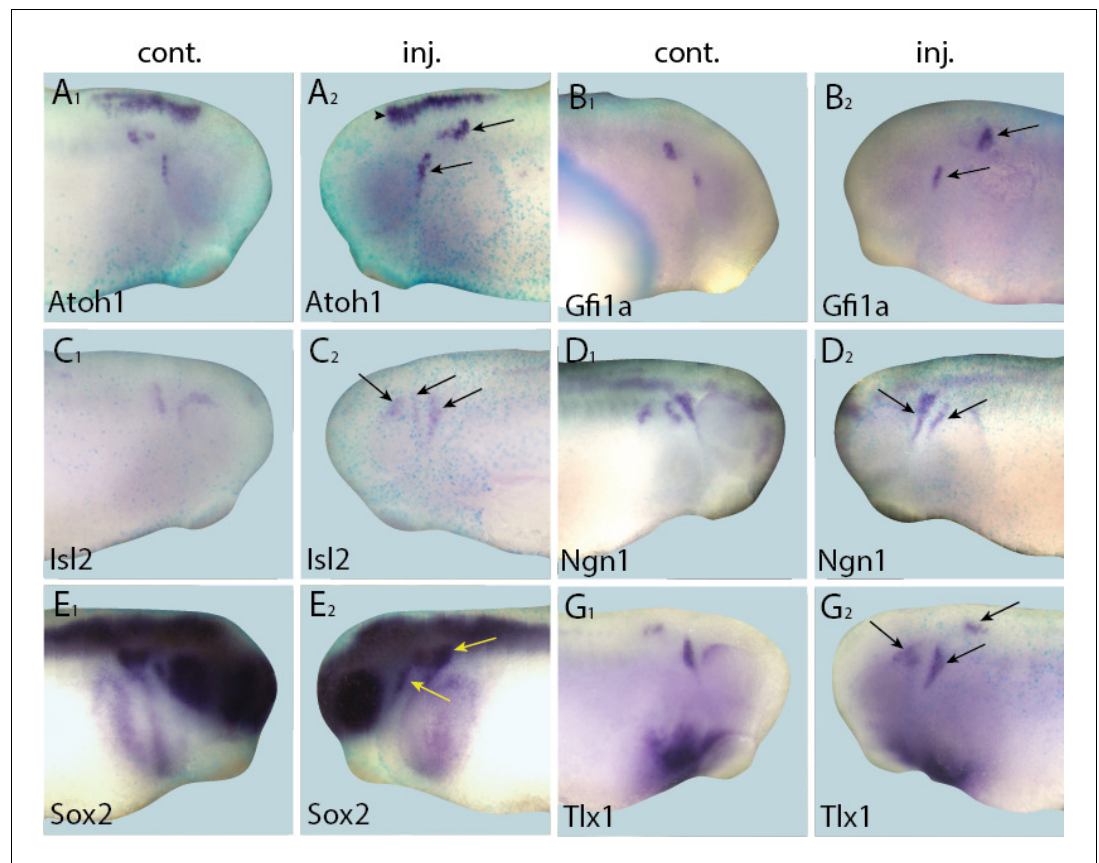
Figure 7. Up-regulation of target gene expression domains after overexpression of Eya1. Tail bud stage embryos (A–G) after unilateral injection of *Eya1*-GR and DEX induction at neural plate stage (16–18). In each case, *lacZ* was co-injected as a lineage tracer and panels A_1–G_1 show the control (un-injected) side and A_2–G_2 show the injected side. Arrows and arrowheads mark expansions in marker gene expression in placodal and non-placodal derivatives, respectively.

conjunction with other interacting partners (*Brugmann et al., 2004*; *Ahmed et al., 2012a*). Surprisingly, we found *Hox* genes to be strongly enriched in the list of target genes activated by Eya1 only. This deserves further study since Eya1 has not previously been recognised as an upstream regulator of *Hox* genes.

It has previously been suggested (*Schlosser, 2006*) that a generic role of Six1 and Eya1 for all placodes could be implemented in two possible ways: (1) By the direct contribution to the activation of genes regulating developmental processes shared between different placodes such as proliferation, morphogenetic movements and neuronal or sensory cytodifferentiation; or (2) by direct contribution to the activation of genes defining the identity of different individual placodes within the PPE. Our data strongly suggest that Six1 and Eya1 act predominantly in the first rather than in the second mode. A large number of transcription factor encoding genes, including several *Pax*, *Pitx*, *ANF* and *FoxI* genes, have been implicated in conferring identity to individual placodes, or groups of placodes, within the PPE (reviewed in *Schlosser, 2006*, *2010*; *Grocott et al., 2012*; *Saint-Jeannet and Moody, 2014*) however only a few of these genes were recovered as targets of Six1 or Eya1, e.g. *Gbx2* (FC 1.7 in Six1+Eya1$_i$) and *FoxI4* (FC 1.09 in Six1$_i$). In contrast, we found a large number of genes encoding transcription factors with roles in neuronal/sensory cytodifferentiation but also other proteins with likely roles for the maintenance of proliferating progenitors (e.g. *Cyclin D, RGCC*), the regulation of cell adhesion and morphogenetic movements (e.g. *EDAR, CXCR7, Protocadherin11, RhoV, Claudin3*) and cytodifferentiation (e.g. *Espin, Neurotrophin3*). This suggests that, similar to *Hox* or *Pax* genes, *Six1* and *Eya1* act as both master genes and micro-managers
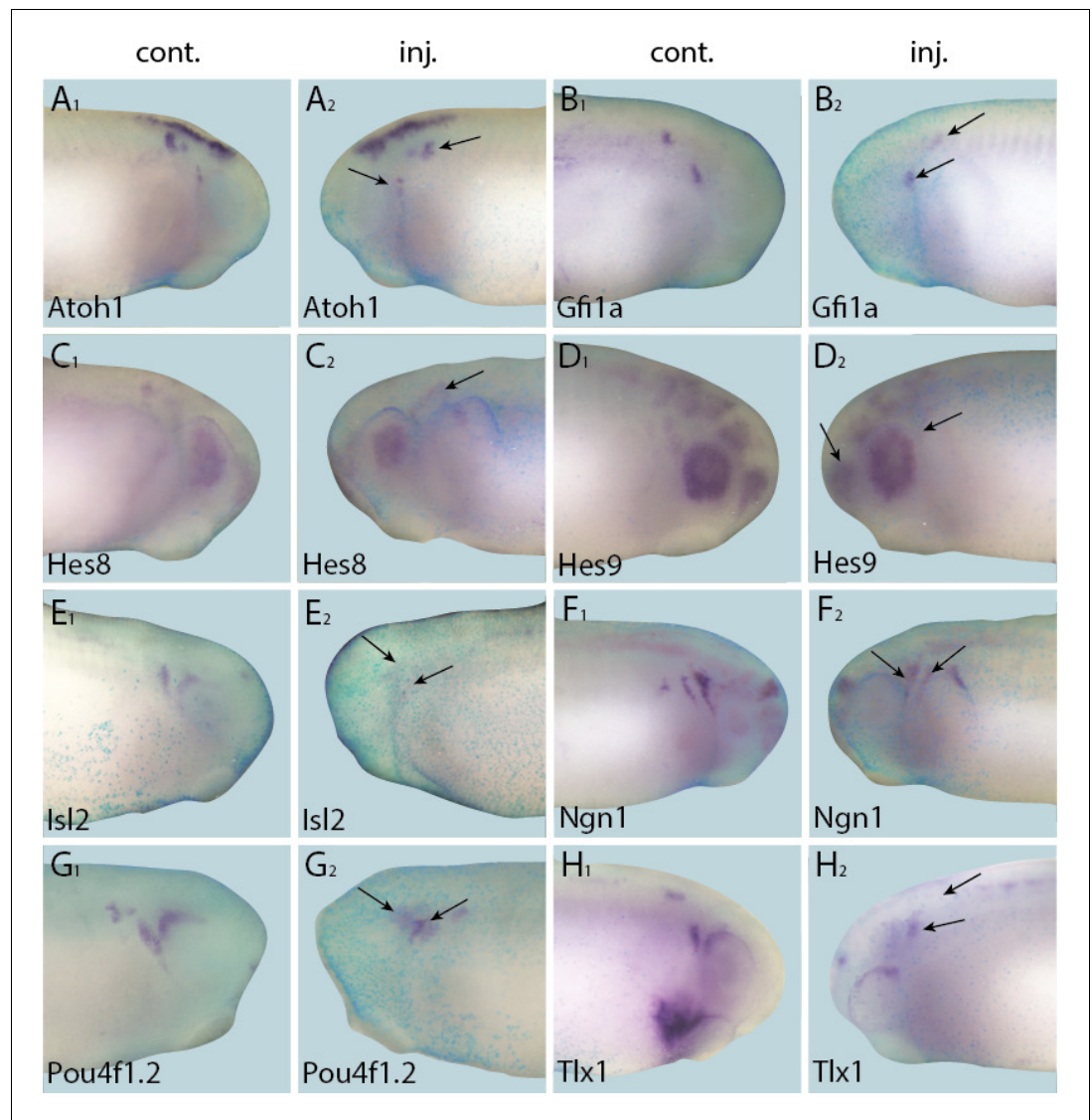
Figure 8. Down-regulation of target gene expression domains after overexpression of Six1. Tail bud stage embryos (A–H) after unilateral injection of *Six1*-GR and DEX induction at neural plate stage (16–18). In each case, *lacZ* was co-injected as a lineage tracer and panels A_1–H_1 show the control (un-injected) side and A_2–H_2 show the injected side. Arrows mark reductions in marker gene expression in placodal derivatives.
DOI: 10.7554/eLife.17666.021

(*Akam, 1998*; *Thompson and Ziman, 2011*; *Rezsohazy et al., 2015*), acting upstream of a GRN co-ordinating cell differentiation in the PPE as well as directly activating terminal differentiation gene batteries.

Considering that Six1 and Eya1 have previously been shown to promote a proliferative progenitor state at high doses but neuronal and sensory differentiation at lower doses (*Schlosser et al., 2008*), it is particularly interesting that we identified presumptive direct target genes encoding transcription factors previously implicated in progenitor maintenance (Sox2, Sox3, Hes8, Hes9) and differentiation (Ngn1, Atoh1, POU4f1, Gfi1a, Isl2, Tlx1). Both Hes (Hes8, Hes9) and SoxB1 (Sox2, Sox3) proteins are known to keep progenitor cells in an undifferentiated state, and must be down-regulated for neuronal differentiation to proceed. While Sox2 and Sox3 play multiple roles including activity as pioneer factors, which open up chromatin for transcription (*Bylund et al., 2003*; *Graham et al., 2003*; *Pevny and Placzek, 2005*; *Bergsland et al., 2011*), Hes proteins generally repress neuronal/sensory determination genes such as *Ngn1* or *Atoh1* as effectors of Notch signalling (*Kobayashi and*

Figure 9. Down-regulation of target gene expression domains after overexpression of Eya1. Tail bud stage embryos (**A–J**) after unilateral injection of *Eya1*-GR and DEX induction at neural plate stage (16–18). In each case, *lacZ* was co-injected as a lineage tracer and panels A_1–J_1 show the control (un-injected) side and A_2–J_2 show the injected side. Arrows mark reductions in marker gene expression in placodal derivatives.
DOI: 10.7554/eLife.17666.022

Kageyama, 2014; *Su et al., 2015*; *Abdolazimi et al., 2016*). Conversely, Ngn1 and Atoh1 are known to act as proneural factors that initiate differentiation of sensory neurons and hair cells, respectively (*Ma et al., 1996*, *1998*; *Bermingham et al., 1999*; *Millimaki et al., 2007*), whereas POU4f1 (previously known as Brn3a), Gfi1a, Isl2 and Tlx1 act further downstream in differentiation of sensory neurons (*Patterson and Krieg, 1999*; *Wallis et al., 2003*; *Cheng et al., 2004*; *Eng et al.,*

2004; *Lanier et al., 2009*; *Dykes et al., 2011*), and Gfi1a and the related POU domain factor POU4f3 (or Brn3c) are required for hair cell maintenance and survival (*Xiang et al., 1998*; *Wallis et al., 2003*). Our findings strongly indicate that Six1 and Eya1 directly promote multiple steps during sensory and neuronal development, and act to drive both progenitor maintenance and neuronal differentiation programmes in placodes (summarised in *Figure 10*), although further functional studies are needed to clarify the mechanism allowing Six1 and Eya1 to maintain the balance
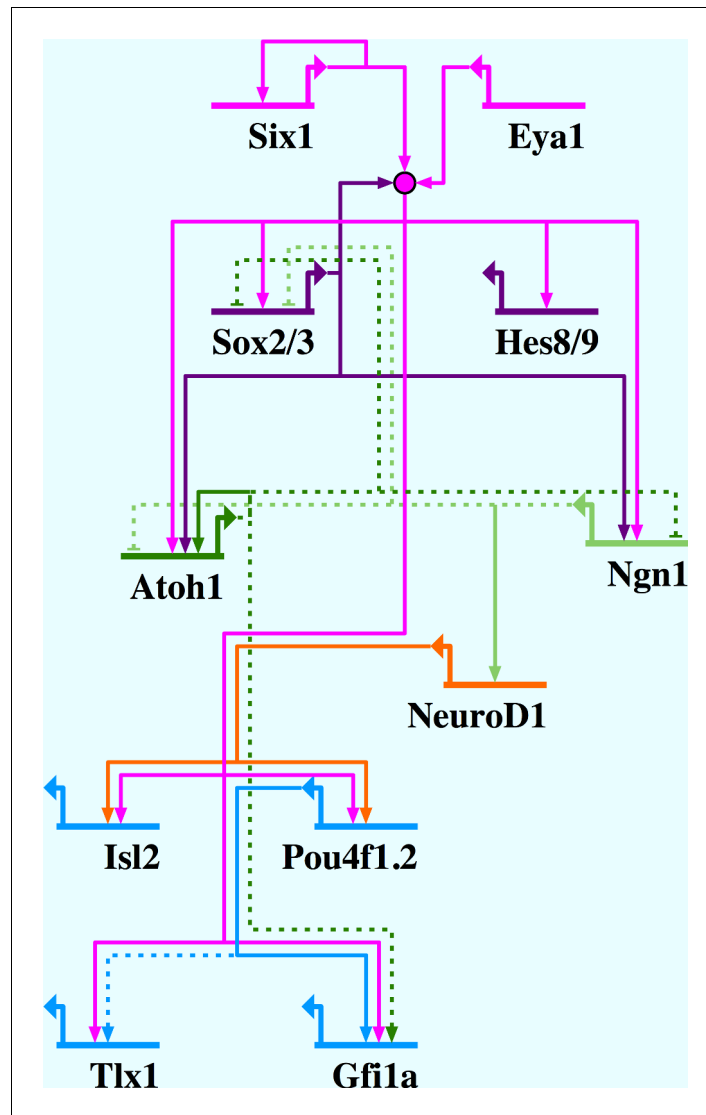
Figure 10. Network summary for Six1/Eya1-activated gene regulation in the PPE. Six1/Eya1 act to promote neuronal differentiation, by activation of pro-neural genes (*Ngn1, Atoh1*), as well as progenitor state maintenance, by activation of genes such as *SoxB1* and *Hes* genes. Arrows indicate direct (solid line) and indirect (dotted line) activation; barred lines show direct (solid line) and indirect (dotted line) repression. Evidence for interactions: Six1 positively autoregulates (*Sato et al., 2012*); Six1/Eya1 directly activate Sox2, Sox3, Hes8, Hes9, Ngn1, Atoh1, Isl2, Pou4f1.2, Tlx1 and Gfi1a (this study); Sox2 synergises with Six1/Eya1 (*Ahmed et al., 2012b*, *2012a*); Sox2 directly activates Atoh1 (*Ahmed et al., 2012a*) and Ngn1 (*Cimadamore et al., 2011*); Atoh1 and Ngn1 indirectly repress each other (*Gowan et al., 2001*); Ngn1 indirectly represses Sox2 (*Evsen et al., 2013*); Ngn1 directly activates NeuroD1 (*Seo et al., 2007*); Atoh1 positively autoregulates (*Helms et al., 2000*); Atoh1 indirectly represses Sox2 (*Neves et al., 2012*) and activates Gfi1 (*Wallis et al., 2003*); NeuroD1 directly activates Pou4f1.2 (*Hutcheson and Vetter, 2001*) and Isl1 (*Lee et al., 1995*); Pou4f1.2 directly activates Gfi1 (*Hertzano et al., 2004*) and indirectly activates Tlx1 (*Hutcheson and Vetter, 2001*).
DOI: 10.7554/eLife.17666.023

between activation of progenitor and differentiation genes. Additionally, direct binding of Six1 to regulatory regions of targets identified in this study should be confirmed by methods such as ChIP-Seq.

The analysis of Six1 and Eya1 presumptive direct target genes presented here establishes a GRN regulating the development of cranial vertebrate sensory organs and neurons from the PPE (*Figure 10*), and identifies a large number of novel putative direct target genes encoding a diverse array of proteins. Among these are many promising candidates potentially involved in mediating the effects of Six1 or Eya1 on proliferation, morphogenesis and cytodifferentiation in developing placodes. This makes our data an invaluable repository of information for designing further functional studies on early sensory development in vertebrates. Finally, while our study focussed on the role of Six1 and Eya1 during sensory development, cell proliferation, morphogenesis and cytodifferentiation are also known to be affected in human patients in which Six1 and Eya1 are dysregulated, leading to sensory deficits after *Six1* or *Eya1* loss of function mutations (*Kochhar et al., 2007*) or enhanced tumour progression after *Six1* or *Eya1* up-regulation (*Blevins et al., 2015*; *Liu et al., 2016*). This suggests that many target genes identified in our study may also be misregulated in these diseases, potentially opening up exciting new avenues for therapeutic intervention.

Materials and methods

Expression constructs and morpholinos

Capped RNAs of *Xenopus Six1-GR, Eya1-GR* and *Six1-EnR* were made by in vitro transcription using the mMessage mMachine SP6 kit (Ambion, Austin, Texas) from the following templates: pCS2$^+$-GR-myc-Six1, pCS2$^+$-GR-myc-Eya1α (*Schlosser et al., 2008*) and pCS2-EnR-Six1 (*Brugmann et al., 2004*).

Translation blocking morpholinos (MO) for Six1 (*Six1*-MO1: 5'-GGAAGGCAGCATAGACATGGC TCAG-3'; *Six1*-MO2: 5'-CGCACACGCAAACACATACACGGG-3') and Eya1 (*Eya1*-MO1: 5'-TACTA TGTGGACTGGTTAGATCCTG-3'; *Eya1*-MO2: 5'-ATATTTGTTCTGTCAGTGGCAAGTC-3') were previously described (*Brugmann et al., 2004*; *Schlosser et al., 2008*). An *Eya1*-MO carrying 5 mismatches was used as a control (*Eya1*-mmMO; mismatches shown in lower case: 5'-ATtTTaGTTCTGaCAGTGGgAAcTC-3').

Microinjection

Six1-GR (500 pg), *Eya1-GR,* (500 pg), *Six1-EnR* (100 pg) mRNAs and *Six1*-MO1+2 (2 ng), *Eya1*-MO1 +2 (2 ng), and *Eya1*-mismatch-MO (2 ng) were freshly prepared before each injection. *lacZ* (250 pg) or *myc-GFP* (125 pg) mRNAs were co-injected to mark the injected side. Embryos of *Xenopus laevis* were obtained by in vitro fertilisation, staged according to (*Nieuwkoop and Faber, 1967*) and injected unilaterally into two-cell blastomeres according to standard procedures (*Sive et al., 2000*). *Six1-EnR* was injected at the four-cell stage into single blastomeres that give rise to the dorsal ectoderm as previously described (*Brugmann et al., 2004*).

Conditional overexpression of GR-fusion constructs and isolation of placodal RNA

To obtain RNA for RNA-Seq or qPCR, both blastomeres of two-cell stage embryos were injected with either 1) *Six1-GR* (500 pg) + *myc-GFP* (125 pg), 2) *Eya1-GR* (500 pg) + *myc-GFP* (125 pg), or 3) *Six1-GR* (500 pg) + *Eya1-GR* (500 pg) + *myc-GFP* (125 pg). Each of these treatment groups was allowed to develop to early neural plate stage before being sorted under a fluorescent microscope. The lateral part of the preplacodal region (LPR of *Ahrens and Schlosser, 2005*) was explanted from GFP positive embryos (~100 per biological replicate) in 1 × MBSH (*Sive et al., 2000*) supplemented with 2 mM CaCl$_2$, 25 mg/l gentamycine (Sigma, St Louis, Missouri), 400 mg/l penicillin (Sigma), and 400 mg/l streptomycin sulphate (Sigma). Explants were pre-treated with 0.1 × modified Barth's solution (MBS; *Sive et al., 2000*) with cycloheximide (CHX; final concentration 10 µg/ml) for 30 min at 25°C. After pre-treatment, 50% of the explants were transferred to 0.1 × MBS with CHX + dexamethasone (DEX; final concentration 10 µM) and incubated for 2 hr 30 at 25°C (*Figure 1*) when control embryos had reached stage 20. Explants were immediately homogenised in Trizol

(Invitrogen, Carlsbad, California) and total RNAs extracted. Isolated RNA was quality assayed in an Agilent 2100 Bioanalyzer and all samples used for sequencing had an RIN >7.0.

RNA-sequencing, mapping and annotation

Libraries were prepared from 1 mg total RNAs and subjected to deep sequencing with Illumina Hi-Seq1000 at the EMBL Genecore facility. Paired-end (100 bp) sequence reads were quality-filtered using Trimmomatic (*Bolger et al., 2014*), and mapped to the *Xenopus laevis* genome (XL7.0) with Bowtie2 (version 2.2.5; *Langmead and Salzberg, 2012*) and Tophat2; (version 2.0.13; *Kim et al., 2013*). An average of 65 million reads (~80% of quality filtered reads) were mapped with 90% of reads properly paired in sequencing across treatment groups. Transcript models were assembled using Cufflinks2 (version 2.1.1; *Trapnell et al., 2012*), and differential expression was determined using Cuffdiff2 (version 2.1.1; *Trapnell et al., 2012*). Gene models were annotated against a combined *Xenopus* mRNA database (*X. laevis*: ftp://ftp.xenbase.org/pub/Genomics/Sequences/xlaevisMRNA.fasta; *X. tropicalis*: ftp://ftp.xenbase.org/pub/Genomics/Sequences/xtropMRNA.fasta) using blastn with an e-value cut-off of 1E-5. Using this approach we were able to annotate an average of 80% of mapped reads.

Differential expression analysis for individual treatment groups

Initially, two samples of CHX- and CHX+DEX-treated explants were independently collected, sequenced and mapped for each treatment group (injection of Six1-GR, Eya1-GR or Six1-GR+Eya1-GR), and were specified as two biological replicates in Cuffdiff. To preclude the inclusion of genes affected by DEX treatment alone, we also analysed explants taken from un-injected embryos and treated as above (CHX vs. CHX+DEX). Two biological replicates of this control treatment group were included in sequencing. Presumptive direct targets of Six1, Eya1 or Six1+Eya1 were determined by comparing Six1-GR, Eya1-GR or Six1-GR+Eya1-GR-injected embryos treated with CHX (as controls) against CHX+DEX-treated samples. Genes were considered to be differentially expressed if (1) the FPKM (Fragments Per Kilobase of exon per Million fragments mapped) for that gene was >1 in the CHX+DEX treatment group, (2) the gene was at least two-fold up-/down-regulated after CHX+DEX treatment compared to CHX treatment, (3) there was at least a two-fold difference between the control (un-injected) and experimental (injected with either Six1-GR, Eya1-GR or Six1-GR+Eya1-GR) fold change (FC) values in response to DEX treatment. The Pearson correlation was high for each of the treatment groups (>0.9 for all pairwise comparisons), indicating the similarity of expression profiles between independently treated samples.

Re-analysis of differential expression for combined treatment groups

As a second approach to finding genes that showed differential expression in response to DEX treatment, RNA-Seq data of several treatment groups were merged to add statistical power to the analysis. In one analysis, all replicates from our three different treatment groups were considered as equivalent to focus on genes with similar differential expression profiles across all treatment groups (comprising the Six1+Eya1$_m$ dataset with six replicates). In another analysis, all treatment groups involving *Six1* overexpression (i.e. injection of Six1-GR alone or Six1-GR+Eya1-GR: Six1$_m$ with 4 replicates) were treated as equivalent as were all treatment groups involving *Eya1* overexpression (Eya1-GR, Six1-GR+Eya1-GR: Eya1$_m$ with 4 replicates). This allowed us to focus on genes whose activation was limited by either Six1 or Eya1 levels. We considered a gene to be significantly differentially expressed if it passed Cuffdiff's statistical test (q < 0.05) in addition to meeting the criteria outlined above.

Gene set enrichment analysis (GSEA) and Gene Ontology

Xenopus annotations were converted to their human orthologs from the Human Uniprot database, and functionally annotated using the online tools 'PantherDB' (*Mi et al., 2013*; http://pantherdb.org) and 'DAVID' (*Huang et al., 2009*; https://david.ncifcrf.gov). For GSEA of placodal transcriptomes after injection of Six1 and/or Eya1, the placodal transcriptome of un-injected, CHX treated placodal explants was specified as a background set, whereas GSEA of the transcriptome of untreated explants was conducted using the default 'human dataset' in DAVID as background. The enrichment score (E) for each group is reported as the geometric mean of the EASE scores (a

modified Fisher's exact score) that are associated with the enriched annotation terms belonging to that group (*Huang et al., 2007*).

cDNA synthesis and qPCR

RNA was extracted from explants after CHX or CHX+DEX treatment as detailed above. cDNA was synthesised using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), using 500 ng total RNA according to the manufacturer's protocol. qPCR was performed using Taqman reagents on a StepOne Plus machine (Applied Biosystems, Foster City, California), using *Smn2* as a reference (*Dhorne-Pollet et al., 2013*; *Supplementary file 4*). qPCR was performed in triplicate and the entire experiment was repeated three times from independently prepared RNA. Relative Quantification (RQ) values and $\log_2$ fold change (FC) were averaged across biological replicates.

Subcloning and gene synthesis

The full coding region of *Hes8, Crem, FosB, Tbx15, Atoh1* and *Isl2* was synthesised from transcript models from RNA-Seq data (KT722743; KT722744; KT722745; KT722746; KT722747; KT722748) by Genescript into the cloning vector pUC57 and subsequently sub-cloned into the expression vector pCS2$^+$ using the following restriction sites: *Hes8* and *Crem:* ClaI/EcoRI; *Atoh1:* XbaI; *Tbx15* and *FosB:* BamHI/EcoRI; *Isl2:* EcoRI/StuI. Primers with added ClaI and EcoRI sites (to the forward and reverse primers, respectively) were designed (*Supplementary file 4*) to amplify the entire coding region of *Tbx6*, which was then subcloned into pCS2$^+$ between the ClaI/EcoRI sites. *Znf214, Mab21l2-b* and *Pou3f2b* were ordered (pCMV-SPORT6, Fisher Scientific, Waltham, Massachusetts; Clone IDS: 5512398, 5515985 and 4203106).

Hes9 (pCR4-TOPO) was ordered from Source Bioscience (Clone accession: BC169570) and was subcloned into the EcoRI site of pCS2$^+$.

In-situ-hybridisation

Embryos injected with *myc-GFP* were sorted under a fluorescent microscope and fixed using a standard protocol (*Sive et al., 2000*). *LacZ*-injected embryos were fixed and then stained with X-gal solution to reveal lacZ. Wholemount in-situ-hybridisation was carried out under high stringency conditions at 60°C as previously described (*Harland, 1991*) using digoxigenin-labelled antisense probes. Probes for *Six1* (*Pandur and Moody, 2000*), *N-tubulin* (*Oschwald et al., 1991 Sox2* (*De Robertis et al., 1997*), *Sox3* (*Penzel et al., 1997*), *Ripply3* (*Janesick et al., 2012*), *Hes2* (*Sölter et al., 2006*), *Sim1* (*Martin et al., 2007*), *Gbx2* (*von Bubnoff et al., 1996*), *Lhx5* (*Bachy et al., 2001*), *Sox21* (*Cunningham et al., 2008*), *Emx1.2* (*Green and Vetter, 2011*), *Pou4f1.2* (*Hutcheson and Vetter, 2001*), and *Tlx1* (*Patterson and Krieg, 1999*) were synthesised as previously described. Primers were designed with promoter sites added (forward, T7; reverse, SP6) for *Hes8, Hes9, Gfi1a, Tbx15, Ngn1, Pou4f1.2* and *Isl2* and were used to amplify a ~800 bp fragment from plasmid DNA (*Supplementary file 4*) which was then used as a template for probe synthesis using T7 RNA polymerase to make an antisense probe. pCMV-SPORT6 with *Znf214, Mab21l2-b* and *Pou3f2b* were linearised with SalI and antisense probes synthesised with T7. pCS2$^+$ vectors containing *Tbx6, FosB* and *Crem* were linearised with BamHI and transcribed with T7. pCS2$^+$ with *Atoh1* was linearised with NotI and transcribed with SP6.

Vibratome sections and immunohistochemistry

In order to analyse the distribution of gene expression domains in finer detail, serial 40–50 μM vibratome sections were cut from selected embryos after wholemount in-situ hybridisation. Where staining with X-gal was insufficient to reveal the injected site, lacZ distribution was revealed immunohistochemically using a polyclonal rabbit anti-LacZ (MP Biomedicals Cappel, Santa Ana, California; Cat.: 55976; 1:1000) and an Alexa594-conjugated anti-rabbit antibody (1:1000).

Availability of data and material

All sequencing data have been deposited in the NCBI BioProject database under BioProject PRJNA317049. All scripts used in analysis are available at https://github.com/nriddiford/Six1-Eya1-RNA-Seq.git.

Acknowledgements

Additional information

Author contributions

NR, Carried out the experimental work and bioinformatic analysis, Wrote the manuscript; GS, Conceived the project, Assisted with several experiments, Wrote the manuscript

Author ORCIDs

Nick Riddiford, http://orcid.org/0000-0002-4739-4233
Gerhard Schlosser, http://orcid.org/0000-0002-1300-1331

Ethics

Animal experimentation: All animal experiments were performed in full accordance with Irish and European legislation, were approved by the NUI Galway Animal Care Research Ethics Committee (ACREC, 003/10) and were covered under the animal license (Cruelty to Animals Act, 1876) B100/4291 to G. Schlosser.

Additional files

Supplementary files

• Supplementary file 1. Top 1000 expressed genes (by FPKM) in the placodal transcriptome.
DOI: 10.7554/eLife.17666.024

• Supplementary file 2. Tables of differentially expressed genes after overexpression of Six1-GR, Eya1-GR or Six1-GR+Eya1-GR: Individual treatment groups. Table 1: Genes with at least two-fold up-regulation after injection of Six1-GR and treatment with CHX + DEX. Table 2: Genes with at least two-fold up-regulation after injection of Eya1-GR and treatment with CHX + DEX. Table 3: Genes with at least two-fold up-regulation after injection of Six1-GR+Eya1-GR and treatment with CHX + DEX. Table 4: Genes with at least two-fold down-regulation after injection of Six1-GR and treatment with CHX + DEX. Table 5: Genes with at least two-fold down-regulation after injection of Eya1-GR and treatment with CHX + DEX. Table 6: Genes with at least two-fold down-regulation after injection of Six1-GR+Eya1-GR and treatment with CHX + DEX.
DOI: 10.7554/eLife.17666.025

• Supplementary file 3. Tables of differentially expressed genes after overexpression of Six1-GR, Eya1-GR or Six1-GR+Eya1-GR: Merged treatment groups. Table 1: Genes with at least two-fold up-regulation after injection of Six1-GR or Six1-GR+Eya1-GR and treatment with CHX + DEX. Table 2: Genes with at least two-fold up-regulation after injection of Eya1-GR or Six1-GR+Eya1-GR and treatment with CHX + DEX. Table 3: Genes with at least two-fold up-regulation after injection of Six1-GR or Eya1-GR or Six1-GR+Eya1-GR and treatment with CHX + DEX. Table 4: Genes with at least two-fold down-regulation after injection of Six1-GR or Six1-GR+Eya1-GR and treatment with CHX + DEX. Table 5: Genes with at least two-fold down-regulation after injection of Eya1-GR or Six1-GR+Eya1-GR and treatment with CHX + DEX. Table 6: Genes with at least two-fold down-regulation after injection of Six1-GR or Eya1-GR or Six1-GR+Eya1-GR and treatment with CHX + DEX.

• Supplementary file 4. Primer sequences with modifications.

Major datasets

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset URL	Database, license, and accessibility information
Riddiford N, Schlosser G	2016	PRJNA317049	http://www.ncbi.nlm.nih.gov/bioproject?LinkName=biosample_bioproject&from_uid=4640079	Publicly available at the NCBI BioProject database (accession no: PRJNA317049)

References

Abdolazimi Y, Stojanova Z, Segil N. 2016. Selection of cell fate in the organ of Corti involves the integration of Hes/Hey signaling at the *Atoh1* promoter. *Development* **143**:841–850. doi: 10.1242/dev.129320

Ahmed M, Wong EY, Sun J, Xu J, Wang F, Xu PX. 2012a. Eya1-Six1 interaction is sufficient to induce hair cell fate in the cochlea by activating *Atoh1* expression in cooperation with Sox2. *Developmental Cell* **22**:377–390. doi: 10.1016/j.devcel.2011.12.006

Ahmed M, Xu J, Xu PX. 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. doi: 10.1242/dev.071670

Ahrens K, Schlosser G. 2005. Tissues and signals involved in the induction of placodal *Six1* expression in *Xenopus laevis*. *Developmental Biology* **288**:40–59. doi: 10.1016/j.ydbio.2005.07.022

Akam M. 1998. Hox genes: from master genes to micromanagers. *Current Biology* **8**:R676–R678. doi: 10.1016/S0960-9822(98)70433-6

Ando Z, Sato S, Ikeda K, Kawakami K. 2005. *Slc12a2* is a direct target of two closely related homeobox proteins, Six1 and Six4. *FEBS Journal* **272**:3026–3041. doi: 10.1111/j.1742-4658.2005.04716.x

Bachy I, Vernier P, Retaux S. 2001. The LIM-homeodomain gene family in the developing *Xenopus* brain: conservation and divergences with the mouse related to the evolution of the forebrain. *Journal of Neuroscience* **21**:7620–7629.

Bergsland M, Ramsköld D, Zaouter C, Klum S, Sandberg R, Muhr J. 2011. Sequentially acting Sox transcription factors in neural lineage development. *Genes & Development* **25**:2453–2464. doi: 10.1101/gad.176008.111

Bermingham NA, Hassan BA, Price SD, Vollrath MA, Ben-Arie N, Eatock RA, Bellen HJ, Lysakowski A, Zoghbi HY. 1999. Math1: an essential gene for the generation of inner ear hair cells. *Science* **284**:1837–1841. doi: 10.1126/science.284.5421.1837

Bhattacharyya S, Bailey AP, Bronner-Fraser M, Streit A. 2004. Segregation of lens and olfactory precursors from a common territory: cell sorting and reciprocity of Dlx5 and Pax6 expression. *Developmental Biology* **271**:403–414. doi: 10.1016/j.ydbio.2004.04.010

Blevins MA, Towers CG, Patrick AN, Zhao R, Ford HL. 2015. The SIX1-EYA transcriptional complex as a therapeutic target in cancer. *Expert Opinion on Therapeutic Targets* **19**:213–225. doi: 10.1517/14728222.2014.978860

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**:2114–2120. doi: 10.1093/bioinformatics/btu170

Bricaud O, Collazo A. 2011. Balancing cell numbers during organogenesis: Six1a differentially affects neurons and sensory hair cells in the inner ear. *Developmental Biology* **357**:191–201. doi: 10.1016/j.ydbio.2011.06.035

Brugmann SA, Pandur PD, Kenyon KL, Pignoni F, Moody SA. 2004. Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a transcriptional activator and repressor. *Development* **131**:5871–5881. doi: 10.1242/dev.01516

Bylund M, Andersson E, Novitch BG, Muhr J. 2003. Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nature Neuroscience* **6**:1162–1168. doi: 10.1038/nn1131

Chai L, Yang J, Di C, Cui W, Kawakami K, Lai R, Ma Y. 2006. Transcriptional activation of the *SALL1* by the human SIX1 homeodomain during kidney development. *Journal of Biological Chemistry* **281**:18918–18926. doi: 10.1074/jbc.M600180200

Cheng L, Arata A, Mizuguchi R, Qian Y, Karunaratne A, Gray PA, Arata S, Shirasawa S, Bouchard M, Luo P, Chen CL, Busslinger M, Goulding M, Onimaru H, Ma Q. 2004. *Tlx3* and *Tlx1* are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. *Nature Neuroscience* **7**:510–517. doi: 10.1038/nn1221

Christophorou NA, Bailey AP, Hanson S, Streit A. 2009. Activation of Six1 target genes is required for sensory placode formation. *Developmental Biology* **336**:327–336. doi: 10.1016/j.ydbio.2009.09.025

Cimadamore F, Fishwick K, Giusto E, Gnedeva K, Cattarossi G, Miller A, Pluchino S, Brill LM, Bronner-Fraser M, Terskikh AV. 2011. Human ESC-derived neural crest model reveals a key role for SOX2 in sensory neurogenesis. *Cell Stem Cell* **8**:538–551. doi: 10.1016/j.stem.2011.03.011

Coletta RD, Christensen K, Reichenberger KJ, Lamb J, Micomonaco D, Huang L, Wolf DM, Müller-Tidow C, Golub TR, Kawakami K, Ford HL. 2004. The Six1 homeoprotein stimulates tumorigenesis by reactivation of cyclin A1. *PNAS* **101**:6478–6483. doi: 10.1073/pnas.0401139101

Coolen M, Sii-Felice K, Bronchain O, Mazabraud A, Bourrat F, Rétaux S, Felder-Schmittbuhl MP, Mazan S, Plouhinec JL. 2005. Phylogenomic analysis and expression patterns of large *Maf* genes in *Xenopus tropicalis* provide new insights into the functional evolution of the gene family in osteichthyans. *Development Genes and Evolution* **215**:327–339. doi: 10.1007/s00427-005-0476-y

Cunningham DD, Meng Z, Fritzsch B, Casey ES. 2008. Cloning and developmental expression of the soxB2 genes, sox14 and sox21, during Xenopus laevis embryogenesis. *International Journal of Developmental Biology* **52**:999–1004. doi: 10.1387/ijdb.082586dc

Davis SW, Ellsworth BS, Peréz Millan MI, Gergics P, Schade V, Foyouzi N, Brinkmeier ML, Mortensen AH, Camper SA. 2013. Pituitary gland development and disease: from stem cell to hormone production. *Current Topics in Developmental Biology* **106**:1–47. doi: 10.1016/B978-0-12-416021-7.00001-8

De Robertis EM, Kim S, Leyns L, Piccolo S, Bachiller D, Agius E, Belo JA, Yamamoto A, Hainski-Brousseau A, Brizuela B, Wessely O, Lu B, Bouwmeester T. 1997. Patterning by genes expressed in Spemann's organizer. *Cold Spring Harbor Symposia on Quantitative Biology* **62**:169–175. doi: 10.1101/SQB.1997.062.01.022

Dhorne-Pollet S, Thélie A, Pollet N. 2013. Validation of novel reference genes for RT-qPCR studies of gene expression in *Xenopus tropicalis* during embryonic and post-embryonic development. *Developmental Dynamics* **242**:709–717. doi: 10.1002/dvdy.23972

Dykes IM, Tempest L, Lee SI, Turner EE. 2011. Brn3a and Islet1 act epistatically to regulate the gene expression program of sensory differentiation. *Journal of Neuroscience* **31**:9789–9799. doi: 10.1523/JNEUROSCI.0901-11.2011

Eng SR, Lanier J, Fedtsova N, Turner EE. 2004. Coordinated regulation of gene expression by Brn3a in developing sensory ganglia. *Development* **131**:3859–3870. doi: 10.1242/dev.01260

Evsen L, Sugahara S, Uchikawa M, Kondoh H, Wu DK. 2013. Progression of neurogenesis in the inner ear requires inhibition of *Sox2* transcription by neurogenin1 and neurod1. *Journal of Neuroscience* **33**:3879–3890. doi: 10.1523/JNEUROSCI.4030-12.2013

Ghanbari H, Seo HC, Fjose A, Brändli AW. 2001. Molecular cloning and embryonic expression of *Xenopus Six* homeobox genes. *Mechanisms of Development* **101**:271–277. doi: 10.1016/S0925-4773(00)00572-4

Gowan K, Helms AW, Hunsaker TL, Collisson T, Ebert PJ, Odom R, Johnson JE. 2001. Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* **31**:219–232. doi: 10.1016/S0896-6273(01)00367-1

Graham V, Khudyakov J, Ellis P, Pevny L. 2003. SOX2 functions to maintain neural progenitor identity. *Neuron* **39**:749–765. doi: 10.1016/S0896-6273(03)00497-5

Green YS, Vetter ML. 2011. EBF factors drive expression of multiple classes of target genes governing neuronal development. *Neural Development* **6**:19. doi: 10.1186/1749-8104-6-19

Grifone R, Demignon J, Giordani J, Niro C, Souil E, Bertin F, Laclef C, Xu PX, Maire P. 2007. Eya1 and Eya2 proteins are required for hypaxial somitic myogenesis in the mouse embryo. *Developmental Biology* **302**:602–616. doi: 10.1016/j.ydbio.2006.08.059

Grocott T, Tambalo M, Streit A. 2012. The peripheral sensory nervous system in the vertebrate head: a gene regulatory perspective. *Developmental Biology* **370**:3–23. doi: 10.1016/j.ydbio.2012.06.028

Harland RM. 1991. In situ hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods in Cell Biology* **36**:685–695. doi: 10.1016/S0091-679X(08)60307-6

Helms AW, Abney AL, Ben-Arie N, Zoghbi HY, Johnson JE. 2000. Autoregulation and multiple enhancers control *Math1* expression in the developing nervous system. *Development* **127**:1185–1196.

Hertzano R, Montcouquiol M, Rashi-Elkeles S, Elkon R, Yücel R, Frankel WN, Rechavi G, Möröy T, Friedman TB, Kelley MW, Avraham KB. 2004. Transcription profiling of inner ears from Pou4f3[(ddl/ddl)] identifies Gfi1 as a target of the Pou4f3 deafness gene. *Human Molecular Genetics* **13**:2143–2153. doi: 10.1093/hmg/ddh218

Hopwood ND, Pluck A, Gurdon JB, Gurdon JB. 1989. MyoD expression in the forming somites is an early response to mesoderm induction in Xenopus embryos. *The EMBO Journal* **8**:3409–3417.

Huang DW, Sherman BT, Tan Q, Collins JR, Alvord WG, Roayaei J, Stephens R, Baseler MW, Lane HC, Lempicki RA. 2007. The DAVID gene functional classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. *Genome Biology* **8**:R183. doi: 10.1186/gb-2007-8-9-r183

Huang da W, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* **4**:44–57. doi: 10.1038/nprot.2008.211

Hudson C, Clements D, Friday RV, Stott D, Woodland HR. 1997. Xsox17α and -β mediate endoderm formation in *Xenopus*. *Cell* **91**:397–405. doi: 10.1016/S0092-8674(00)80423-7

Hutcheson DA, Vetter ML. 2001. The bHLH factors Xath5 and XNeuroD can upregulate the expression of XBrn3d, a POU-homeodomain transcription factor. *Developmental Biology* **232**:327–338. doi: 10.1006/dbio.2001.0178

Janesick A, Shiotsugu J, Taketani M, Blumberg B. 2012. RIPPLY3 is a retinoic acid-inducible repressor required for setting the borders of the pre-placodal ectoderm. *Development* **139**:1213–1224. doi: 10.1242/dev.071456

Jin M, Aibar S, Ge Z, Chen R, Aerts S, Mardon G. 2016. Identification of novel direct targets of Drosophila Sine oculis and Eyes absent by integration of genome-wide data sets. *Developmental Biology* **415**:157–167. doi: 10.1016/j.ydbio.2016.05.007

Jusiak B, Karandikar UC, Kwak SJ, Wang F, Wang H, Chen R, Mardon G. 2014. Regulation of Drosophila eye development by the transcription factor Sine oculis. *PloS One* **9**:e89695. doi: 10.1371/journal.pone.0089695

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology* **14**:R36. doi: 10. 1186/gb-2013-14-4-r36

Kobayashi T, Kageyama R. 2014. Expression dynamics and functions of Hes factors in development and diseases. *Current Topics in Developmental Biology* **110**:263–283. doi: 10.1016/B978-0-12-405943-6.00007-5

Kochhar A, Fischer SM, Kimberling WJ, Smith RJ. 2007. Branchio-oto-renal syndrome. *American Journal of Medical Genetics Part A* **143A**:1671–1678. doi: 10.1002/ajmg.a.31561

Kolm PJ, Sive HL. 1995. Efficient hormone-inducible protein function in *Xenopus laevis*. *Developmental Biology* **171**:267–272. doi: 10.1006/dbio.1995.1279

Kozlowski DJ, Whitfield TT, Hukriede NA, Lam WK, Weinberg ES. 2005. The zebrafish *dog-eared* mutation disrupts *eya1*, a gene required for cell survival and differentiation in the inner ear and lateral line. *Developmental Biology* **277**:27–41. doi: 10.1016/j.ydbio.2004.08.033

Kumar JP. 2009. The sine oculis homeobox (SIX) family of transcription factors as regulators of development and disease. *Cellular and Molecular Life Sciences* **66**:565–583. doi: 10.1007/s00018-008-8335-4

Laclef C, Souil E, Demignon J, Maire P. 2003. Thymus, kidney and craniofacial abnormalities in Six 1 deficient mice. *Mechanisms of Development* **120**:669–679. doi: 10.1016/S0925-4773(03)00065-0

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**:357–359. doi: 10.1038/nmeth.1923

Lanier J, Dykes IM, Nissen S, Eng SR, Turner EE. 2009. Brn3a regulates the transition from neurogenesis to terminal differentiation and represses non-neural gene expression in the trigeminal ganglion. *Developmental Dynamics* **238**:3065–3079. doi: 10.1002/dvdy.22145

Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H. 1995. Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* **268**:836–844. doi: 10.1126/science.7754368

Li X, Oghi KA, Zhang J, Krones A, Bush KT, Glass CK, Nigam SK, Aggarwal AK, Maas R, Rose DW, Rosenfeld MG. 2003. Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. *Nature* **426**:247–254. doi: 10.1038/nature02083

Li Z, Tian T, Lv F, Chang Y, Wang X, Zhang L, Li X, Li L, Ma W, Wu J, Zhang M. 2013. Six1 promotes proliferation of pancreatic cancer cells via upregulation of cyclin D1 expression. *PLoS One* **8**:e59203. doi: 10.1371/journal.pone.0059203

Liu Y, Chakroun I, Yang D, Horner E, Liang J, Aziz A, Chu A, De Repentigny Y, Dilworth FJ, Kothary R, Blais A. 2013. Six1 Regulates MyoD Expression in Adult Muscle Progenitor Cells. *PLoS One* **8**:e67762. doi: 10.1371/journal.pone.0067762

Liu Y, Han N, Zhou S, Zhou R, Yuan X, Xu H, Zhang C, Yin T, Wu K. 2016. The DACH/EYA/SIX gene network and its role in tumor initiation and progression. *International Journal of Cancer* **138**:1067–1075. doi: 10.1002/ijc.29560

Ma Q, Chen Z, del Barco Barrantes I, de la Pompa JL, Anderson DJ. 1998. neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* **20**:469–482. doi: 10.1016/S0896-6273(00)80988-5

Ma Q, Kintner C, Anderson DJ. 1996. Identification of *neurogenin*, a vertebrate neuronal determination gene. *Cell* **87**:43–52. doi: 10.1016/S0092-8674(00)81321-5

Maier EC, Saxena A, Alsina B, Bronner ME, Whitfield TT. 2014. Sensational placodes: neurogenesis in the otic and olfactory systems. *Developmental Biology* **389**:50–67. doi: 10.1016/j.ydbio.2014.01.023

Martin BL, Peyrot SM, Harland RM. 2007. Hedgehog signaling regulates the amount of hypaxial muscle development during *Xenopus* myogenesis. *Developmental Biology* **304**:722–734. doi: 10.1016/j.ydbio.2007.01.022

McCabe KL, Manzo A, Gammill LS, Bronner-Fraser M. 2004. Discovery of genes implicated in placode formation. *Developmental Biology* **274**:462–477. doi: 10.1016/j.ydbio.2004.07.012

Mi H, Muruganujan A, Thomas PD. 2013. PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. *Nucleic Acids Research* **41**:D377–D386. doi: 10.1093/nar/gks1118

Millimaki BB, Sweet EM, Dhason MS, Riley BB. 2007. Zebrafish *atoh1* genes: classic proneural activity in the inner ear and regulation by Fgf and Notch. *Development* **134**:295–305. doi: 10.1242/dev.02734

Mizuseki K, Kishi M, Matsui M, Nakanishi S, Sasai Y. 1998. *Xenopus* Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* **125**:579–587.

Neves J, Uchikawa M, Bigas A, Giraldez F. 2012. The prosensory function of Sox2 in the chicken inner ear relies on the direct regulation of *Atoh1*. *PloS One* **7**:e30871. doi: 10.1371/journal.pone.0030871

Nieber F, Pieler T, Henningfeld KA. 2009. Comparative expression analysis of the neurogenins in Xenopus tropicalis and Xenopus laevis. *Developmental Dynamics* **238**:451–458. doi: 10.1002/dvdy.21845

Nieuwkoop PD, Faber J. 1967. *Normal Table of Development of Xenopus Laevis (Daudin)*. New York: Garland.

Nitta KR, Takahashi S, Haramoto Y, Fukuda M, Onuma Y, Asashima M. 2006. Expression of *Sox1* during *Xenopus* early embryogenesis. *Biochemical and Biophysical Research Communications* **351**:287–293. doi: 10.1016/j.bbrc.2006.10.040

Northcutt RG, Gans C. 1983. The genesis of neural crest and epidermal placodes: a reinterpretation of vertebrate origins. *The Quarterly Review of Biology* **58**:1–28. doi: 10.1086/413055

Ohto H, Kamada S, Tago K, Tominaga SI, Ozaki H, Sato S, Kawakami K. 1999. Cooperation of six and eya in activation of their target genes through nuclear translocation of Eya. *Molecular and Cellular Biology* **19**:6815–6824. doi: 10.1128/MCB.19.10.6815

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. *International Journal of Developmental Biology* **35**:399–405.

Pandur PD, Moody SA. 2000. *Xenopus Six1* gene is expressed in neurogenic cranial placodes and maintained in the differentiating lateral lines. *Mechanisms of Development* **96**:253–257. doi: 10.1016/S0925-4773(00)00396-8

Patterson KD, Krieg PA. 1999. *Hox11*-family genes *XHox11* and *XHox11L2* in *xenopus*: *XHox11L2* expression is restricted to a subset of the primary sensory neurons. *Developmental Dynamics* **214**:34–43. doi: 10.1002/(SICI)1097-0177(199901)214:1<34::AID-DVDY4>3.0.CO;2-R

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*. *International Journal of Developmental Biology* **41**:667–677.

Petit C, Levilliers J, Hardelin JP. 2001. Molecular genetics of hearing loss. *Annual Review of Genetics* **35**:589–645. doi: 10.1146/annurev.genet.35.102401.091224

Pevny L, Placzek M. 2005. *SOX* genes and neural progenitor identity. *Current Opinion in Neurobiology* **15**:7–13. doi: 10.1016/j.conb.2005.01.016

Pieper M, Eagleson GW, Wosniok W, Schlosser G. 2011. Origin and segregation of cranial placodes in *Xenopus laevis*. *Developmental Biology* **360**:257–275. doi: 10.1016/j.ydbio.2011.09.024

Rezsohazy R, Saurin AJ, Maurel-Zaffran C, Graba Y. 2015. Cellular and molecular insights into Hox protein action. *Development* **142**:1212–1227. doi: 10.1242/dev.109785

Ruf RG, Xu P-X, Silvius D, Otto EA, Beekmann F, Muerb UT, Kumar S, Neuhaus TJ, Kemper MJ, Raymond RM, Brophy PD, Berkman J, Gattas M, Hyland V, Ruf E-M, Schwartz C, Chang EH, Smith RJH, Stratakis CA, Weil D, et al. 2004. SIX1 mutations cause branchio-oto-renal syndrome by disruption of EYA1-SIX1-DNA complexes. *PNAS* **101**:8090–8095. doi: 10.1073/pnas.0308475101

Saint-Jeannet JP, Moody SA. 2014. Establishing the pre-placodal region and breaking it into placodes with distinct identities. *Developmental Biology* **389**:13–27. doi: 10.1016/j.ydbio.2014.02.011

Salanga MC, Meadows SM, Myers CT, Krieg PA. 2010. ETS family protein ETV2 is required for initiation of the endothelial lineage but not the hematopoietic lineage in the Xenopus embryo. *Developmental Dynamics* **239**:1178–1187. doi: 10.1002/dvdy.22277

Sato S, Ikeda K, Shioi G, Nakao K, Yajima H, Kawakami K. 2012. Regulation of Six1 expression by evolutionarily conserved enhancers in tetrapods. *Developmental Biology* **368**:95–108. doi: 10.1016/j.ydbio.2012.05.023

Schlosser G, Ahrens K. 2004. Molecular anatomy of placode development in *Xenopus laevis*. *Developmental Biology* **271**:439–466. doi: 10.1016/j.ydbio.2004.04.013

Schlosser G, Awtry T, Brugmann SA, Jensen ED, Neilson K, Ruan G, Stammler A, Voelker D, Yan B, Zhang C, Klymkowsky MW, Moody SA. 2008. *Eya1* and *Six1* promote neurogenesis in the cranial placodes in a *SoxB1*-dependent fashion. *Developmental Biology* **320**:199–214. doi: 10.1016/j.ydbio.2008.05.523

Schlosser G, Northcutt RG. 2000. Development of neurogenic placodes in *Xenopus laevis*. *Journal of Comparative Neurology* **418**:121–146. doi: 10.1002/(SICI)1096-9861(20000306)418:2<121::AID-CNE1>3.0.CO;2-M

Schlosser G. 2006. Induction and specification of cranial placodes. *Developmental Biology* **294**:303–351. doi: 10.1016/j.ydbio.2006.03.009

Schlosser G. 2010. Chapter Four – Making Senses: Development of Vertebrate Cranial Placodes. *International Review of Cell and Molecular Biology* **283**:129–234. doi: 10.1016/S1937-6448(10)83004-7

Schlosser G. 2015. Vertebrate cranial placodes as evolutionary innovations–the ancestor's tale. *Current Topics in Developmental Biology* **111**:235–300. doi: 10.1016/bs.ctdb.2014.11.008

Seo S, Lim JW, Yellajoshyula D, Chang LW, Kroll KL. 2007. Neurogenin and NeuroD direct transcriptional targets and their regulatory enhancers. *The EMBO Journal* **26**:5093–5108. doi: 10.1038/sj.emboj.7601923

Simões-Costa M, Tan-Cabugao J, Antoshechkin I, Sauka-Spengler T, Bronner ME. 2014. Transcriptome analysis reveals novel players in the cranial neural crest gene regulatory network. *Genome Research* **24**:281–290. doi: 10.1101/gr.161182.113

Sive HL, Grainger RM, Harland RM. 2000. *Early Development of Xenopus Laevis: A Laboratory Manual*. Cold Spring Harbor Laboratory Press.

Streit A. 2002. Extensive cell movements accompany formation of the otic placode. *Developmental Biology* **249**:237–254. doi: 10.1006/dbio.2002.0739

Su YX, Hou CC, Yang WX. 2015. Control of hair cell development by molecular pathways involving *Atoh1, Hes1* and *Hes5*. *Gene* **558**:6–24. doi: 10.1016/j.gene.2014.12.054

Sölter M, Locker M, Boy S, Taelman V, Bellefroid EJ, Perron M, Pieler T. 2006. Characterization and function of the bHLH-O protein XHes2: insight into the mechanisms controlling retinal cell fate decision. *Development* **133**:4097–4108. doi: 10.1242/dev.02567

Tadjuidje E, Hegde RS. 2013. The Eyes Absent proteins in development and disease. *Cellular and Molecular Life Sciences* **70**:1897–1913. doi: 10.1007/s00018-012-1144-9

Taverner NV, Kofron M, Shin Y, Kabitschke C, Gilchrist MJ, Wylie C, Cho KW, Heasman J, Smith JC. 2005. Microarray-based identification of VegT targets in *Xenopus*. *Mechanisms of Development* **122**:333–354. doi: 10.1016/j.mod.2004.10.010

Thompson JA, Ziman M. 2011. Pax genes during neural development and their potential role in neuroregeneration. *Progress in Neurobiology* **95**:334–351. doi: 10.1016/j.pneurobio.2011.08.012

Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols* **7**:562–578. doi: 10.1038/nprot.2012.016

von Bubnoff A, Schmidt JE, Kimelman D. 1996. The *Xenopus laevis* homeobox gene *Xgbx-2* is an early marker of anteroposterior patterning in the ectoderm. *Mechanisms of Development* **54**:149–160. doi: 10.1016/0925-4773(95)00468-8

Wallis D, Hamblen M, Zhou Y, Venken KJT, Schumacher A, Grimes HL, Zoghbi HY, Orkin SH, Bellen HJ. 2003. The zinc finger transcription factor *Gfi1*, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. *Development* **130**:221–232. doi: 10.1242/dev.00190

Xiang M, Gao WQ, Hasson T, Shin JJ. 1998. Requirement for Brn-3c in maturation and survival, but not in fate determination of inner ear hair cells. *Development* **125**:3935–3946.

Xu H, Dude CM, Baker CV. 2008. Fine-grained fate maps for the ophthalmic and maxillomandibular trigeminal placodes in the chick embryo. *Developmental Biology* **317**:174–186. doi: 10.1016/j.ydbio.2008.02.012

Xu PX. 2013. The EYA-SO/SIX complex in development and disease. *Pediatric Nephrology* **28**:843–854. doi: 10.1007/s00467-012-2246-1

Xu PX, Adams J, Peters H, Brown MC, Heaney S, Maas R. 1999. *Eya1*-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nature Genetics* **23**:113–117. doi: 10.1038/12722

Yan B, Neilson KM, Ranganathan R, Maynard T, Streit A, Moody SA. 2015. Microarray identification of novel genes downstream of Six1, a critical factor in cranial placode, somite, and kidney development. *Developmental Dynamics* **244**:181–210. doi: 10.1002/dvdy.24229

Yu Y, Davicioni E, Triche TJ, Merlino G. 2006. The homeoprotein six1 transcriptionally activates multiple protumorigenic genes but requires ezrin to promote metastasis. *Cancer Research* **66**:1982–1989. doi: 10.1158/0008-5472.CAN-05-2360

Zheng W, Huang L, Wei ZB, Silvius D, Tang B, Xu PX. 2003. The role of *Six1* in mammalian auditory system development. *Development* **130**:3989–4000. doi: 10.1242/dev.00628

Zou D, Erickson C, Kim EH, Jin D, Fritzsch B, Xu PX. 2008. *Eya1* gene dosage critically affects the development of sensory epithelia in the mammalian inner ear. *Human Molecular Genetics* **17**:3340–3356. doi: 10.1093/hmg/ddn229

Zou D, Silvius D, Fritzsch B, Xu PX. 2004. Eya1 and Six1 are essential for early steps of sensory neurogenesis in mammalian cranial placodes. *Development* **131**:5561–5572. doi: 10.1242/dev.01437