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Six1 and Irx1 have reciprocal interactions during cranial placode and otic vesicle formation

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ABSTRACT

The specialized sensory organs of the vertebrate head are derived from thickened patches of cells in the ectoderm called cranial sensory placodes. The developmental program that generates these placodes and the genes that are expressed during the process have been studied extensively in a number of animals, yet very little is known about how these genes regulate one another. We previously found via a microarray screen that Six1, a known transcriptional regulator of cranial placode fate, up-regulates *Irx1* in ectodermal explants. In this study, we investigated the transcriptional relationship between Six1 and Irx1 and found that they reciprocally regulate each other throughout cranial placode and otic vesicle formation. Although *Irx1* expression precedes that of *Six1* in the neural border zone, its continued and appropriately patterned expression in the pre-placodal region (PPR) and otic vesicle requires Six1. At early PPR stages, Six1 expands the *Irx1* domain, but this activity subsides over time and changes to a predominantly repressive effect. Likewise, Irx1 initially expands *Six1* expression in the PPR, but later represses it. We also found that Irx1 and Sox11, a known direct target of Six1, reciprocally affect each other. This work demonstrates that the interactions between Six1 and Irx1 are continuous during PPR and placode development and their transcriptional effects on one another change over developmental time.

1. Introduction

The specialized sensory organs of the vertebrate head, which include the olfactory epithelium, lens, cranial nerve sensory ganglia, and the auditory, vestibular and lateral line organs, are derived from thickened patches of cells in the ectoderm called cranial sensory placodes. The developmental program that generates these placodes has been studied extensively in a number of animals (reviewed in Grocott et al., 2012; Moody and LaMantia, 2015; Moody and Saint-Jeannet, 2014; Patthey et al., 2014; Saint-Jeannet and Moody, 2014; Schlosser, 2006, 2010; Streit, 2004, 2007). During gastrulation, interactions between the neural and non-neural ectoderm, combined with signals from the underlying mesoderm and endoderm, cause a border zone (BZ) to form between them. The BZ is further specified by the expression of a number of transcription factors, including members of the Dlx, Msx, Pax and Zic families. Later interactions between these factors and differential input from the FGF, Wnt and BMP signaling pathways separate this progenitor field into two sets of precursors: those giving rise to the neural crest (NC) and those giving rise to the pre-placodal region of the ectoderm (PPR). Under the influence of local signaling and transcription factor interactions, the PPR subsequently segregates into individual placodes that will differentiate into the cranial sensory organs.

Several studies in *Xenopus*, chick, mouse and zebrafish identified two genes that are required for the formation of the PPR as well as individual placode differentiation: the Six1 transcription factor and one of its co-factors, Eya1 (reviewed in Grocott et al., 2012; Moody and LaMantia, 2015; Moody and Saint-Jeannet, 2014; Saint-Jeannet and Moody, 2014). These two factors up-regulate genes that are expressed in the PPR and maintain the boundaries of the PPR by down-regulating genes that are required to form the adjacent NC and epidermis (Brugmann et al., 2004; Groves and LaBonne, 2014; Hong and Saint-Jeannet, 2007; Matsuo-Takasaki et al., 2005). Over the years, a large number of genes have been catalogued that are expressed during placode development (Grocott et al., 2012; Hintze et al., 2017; Moody and LaMantia, 2015; Moody and Saint-Jeannet, 2014; Riddiford and Schlosser, 2016; Schlosser and Ahrens, 2004; Trevers et al., 2018). However, until recently, very little was known about how these genes regulate one another.

There have been several screens to identify the downstream targets of Six1 and its *Drosophila* homologue, Sine oculis [So]. For example,

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hundreds of targets have been reported in the context of fly eye development (Anderson et al., 2014; Jusiak et al., 2014; Yan et al., 2003; Zhang et al., 2006), as well as mouse kidney, muscle and gonad development (Ando et al., 2005; Buckingham and Rigby, 2014; Chai et al., 2006; Fujimoto et al., 2013). Recently a large number of downstream targets involved in vertebrate placode development have been identified (Hintze et al., 2017; Riddiford and Schlosser, 2016; Plouhinec et al., 2017; Maharana and Schlosser, 2018). Using a microarray approach to identify potential Six1 targets, we identified over 100 genes that were differentially expressed in Six1-expressing ectodermal explants compared to control explants (Yan et al., 2015). Although the majority of potential Six1 targets were of unknown function, this analysis validated a few genes already reported to be expressed in the PPR and/or placodes, including the PDGF receptor, the p75 neurotropin receptor, and the transcription factors Pax6 and Irx1.

Iroquois homeodomain proteins, named Irx1-6 in vertebrates, are involved in numerous developmental processes across the animal kingdom (Gómez-Skarmeta and Modolell, 2002; Kerner et al., 2009). During the development of frog, fish, chick and mouse, *Irx1* is expressed in several organs, quite prominently in the neural plate/tube, the early stage PPR, and the otic vesicle, which gives rise to the inner ear and the auditory/vestibular sensory ganglia (Bosse et al., 1997; Cheng et al., 2001; Gómez-Skarmeta et al., 1998; Li et al., 2010; Schlosser and Ahrens, 2004). Previous work in *Xenopus* showed that *Irx1* is expressed throughout the BZ in response to the interactions between the neural plate and epidermis (Glavic et al., 2004). These authors showed that *Irx1* expression temporally precedes that of *Six1* in the BZ, is then down-regulated in the more medial NC domain and the most anterior *Six1*-expressing PPR domain, and it can up-regulate *Six1* expression via transcriptional repression. In light of our microarray finding that Six1 also up-regulates *Irx1* and the observation that *Six1* and *Irx1* expression domains overlap in the otic placode, we further investigated the transcriptional relationship between Six1 and Irx1. We find that Six1 is required to maintain *Irx1* expression in the PPR and to initiate its expression in the otic vesicle. Irx1 feeds back to down-regulate *Six1* (and *Eya1*), and this activity appears to require Fgf8 signaling. We also demonstrate that *Sox11*, a direct target of Six1 (Brugmann et al., 2004), can independently modulate *Irx1* expression. Together, these findings indicate that feed-forward and feed-back interactions between Irx1 and Six1, as well as Irx1 and Sox11, are important for the proper development of the cranial placodes, particularly the precursors of the inner ear.

2. Methods

2.1. Cloning

A 3′-Flag tagged version of *Xenopus laevis* Six1 (Pandur and Moody, 2000) that contains the native 5′ UTR (*5′UTR-Six1-3′Flag*) was constructed in *pCS2+* by adding the native 5′-UTR to the previously described 3′-Flag tagged Six1 construct (Neilson et al., 2017).

2.2. Obtaining embryos and microinjections

Fertilized *Xenopus laevis* eggs were obtained by gonadotropin-induced natural mating of wild type, outbred adult frogs as previously described (Moody, 2000). Embryos were selected at the 2-cell stage if the first cleavage furrow bisected the lightly pigmented region of the animal hemisphere to accurately identify the dorsal-ventral axis (Klein, 1987; Miyata et al., 1987). When these selected embryos reached the 16-cell stage, one animal blastomere (Fig. 1) was microinjected with 1 nl of either mRNAs or antisense morpholino oligonucleotides (MOs) according to standard methods (Moody, 1999, 2000).

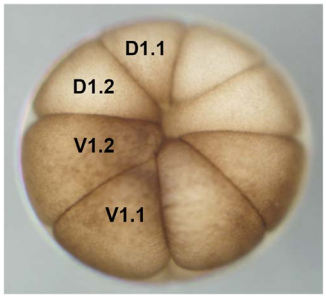

Fig. 1. Animal pole view of a 16-cell stage embryo with dorsal to the top. Nomenclature on the left side indicates blastomere names according to Hirose and Jacobson (1979).

2.3. In vitro synthesis of mRNAs and antisense RNA probes

mRNAs encoding *Xenopus Six1* (Brugmann et al., 2004), *Six1-hGR* (Schlosser et al., 2008), *Six1-VP16* (Brugmann et al., 2004), *Six1-EnR* (Brugmann et al., 2004), *5′UTR-Six1-3′Flag*, *MT-Six1-rescue* (Brugmann et al., 2004), *5′Flag-Six1* (Neilson et al., 2017), *Six1-3′Flag* (Neilson et al., 2017)), *Eya1* (David et al., 2001), *Sox11-Δ3′UTR* (Yan et al., 2009), *Irx1* (Glavic et al., 2004), *Irx1-EnR-hGR* (Glavic et al., 2004), *Irx1-HD-hGR* (Glavic et al., 2004), *cFGFR1* (Neilson and Friesel, 1996) and a nuclear-localized *β-galactosidase* (*nβgal*) were synthesized in vitro (mMessage mMachine kit, Ambion). Antisense RNA probes for in situ hybridization (ISH) were synthesized in vitro (MEGAscript kit; Ambion) as previously described (Sullivan et al., 2001; Yan et al., 2009).

2.4. Antisense oligonucleotides

To knock-down endogenous levels of proteins, two translation-blocking antisense morpholino oligonucleotides that bind to *Xenopus laevis Six1* mRNA (Six1-MOs) or two that bind to *Sox11* mRNAs (Sox11-MOs) were synthesized (Gene Tools, LLC), and co-injected at equimolar concentrations (9 ng). Their specificity and efficacy were validated previously in vivo (Brugmann et al., 2004; Chen et al., 2016). Herein, we also show biochemically that the two Six1-MOs can block translation of endogenous, wild type *Six1* mRNA and the Flag-tagged mRNA containing the endogenous 5′UTR (*5′UTR-Six1-3′Flag*), but not the *MT-Six1-rescue* mRNA (Supplemental Fig. 1A, B). For Western blot analysis, *Xenopus laevis* oocytes were first injected with 9 ng of Six1-MO1+MO2 and then injected with 2 ng of *5′UTR-Six1-3′Flag* mRNA (MO sensitive) or *MT-Six1-rescue* mRNA (MO insensitive); *MT-Six1-rescue* mRNA is insensitive to both MO1 and MO2 due to: 1) the removal of the endogenous 5′UTR of *Six1*; 2) the initiator ATG in the Myc-tag being greater than 30 bp away from the MO binding site in the *Six1* open reading frame (ORF); and 3) mismatches in the ORF sequence (Supplemental Fig. 1A). Other oocytes were injected only with 2 ng of *5′UTR-Six1-3′Flag* or *MT-Six1-rescue* mRNAs. The oocytes were cultured overnight at 18 °C. Lysates were prepared and Western blotting performed as previously described (Neilson et al., 2017) using an anti-Six1 antibody (D5S2S, Cell Signaling Technology). In addition, the specificity of Six1 - MO1 + MO2 effects on *Irx1* expression was shown by: 1) co-injecting *MT-Six1-rescue* mRNA (Supplemental Fig. 1A); and 2) assaying for *Irx1* expression in Six1 F0 CRISPR mutants (Supplemental Fig. 1C, D).

2.5. Hormone-inducible constructs

For those embryos injected with hormone-inducible (h-GR) constructs, dexamethasone (Dex), a synthetic hormone that activates hGR-fusion proteins (Kolm and Sive, 1995; Mattioni et al., 1994), was added to the culture medium (10 μM final concentration) at indicated stages. Dex-treated uninjected embryos and hGR-injected embryos that were not exposed to Dex were included as controls, as previously described

(Zaghloul and Moody, 2007). To assay whether Irx1 directly represses *Sox11*, embryos injected with *Irx1-EnR-hGR* mRNA were treated with cycloheximide (Chx, 10 μg/ml) 40 min prior to Dex treatment, as previously described (Kurth et al., 2005; Yan et al., 2009).

2.6. Fixation, histochemistry and in situ hybridization (ISH)

Embryos were cultured to neural plate (st 16–18) or neural tube (st 24–28) stages (Nieuwkoop and Faber, 1994), fixed in 4% paraformaldehyde (in 0.1 M MOPS, 2 mM EGTA Magnesium, 1 mM $MgSO_4$, pH 7.4), stained for β-Gal histochemistry if injected with mRNAs, and processed for in situ hybridization (ISH) as previously described (Yan et al., 2009). Each experiment was repeated in 2–5 independent trials with different sets of parents. Embryos were scored for gene expression changes independently by at least two of the authors, and the values reported are means of their independent scores. Where noted, differences in frequency were tested for significance ($p < 0.05$) by the Chi-square test.

3. Results

3.1. Six1 is required for Irx1 expression in the late PPR and otic placode

Previous work showed that Six1 is required for the formation of the PPR. When its expression is knocked down in vivo by microinjection of a combination of two antisense oligonucleotides (Six1 - MO1 + MO2; Supplemental Fig. 1A), the expression domains of two early PPR genes, *Eya1* and *Sox11*, were dramatically reduced (Brugmann et al., 2004). We confirmed the efficacy and specificity of Six1-MOs by Western blot analyses and confirmed the phenotype in F0 CRISPR mutants (Supplemental Fig. 1B-D). We found that Six1 knock-down in the major precursors of the BZ (blastomeres D1.2 and V1.2; Fig. 1; Moody, 1987) caused a loss of *Irx1* expression in the PPR (Fig. 2A) and early formed placodes (Fig. 2B). When Six1 mRNA that is insensitive to MO

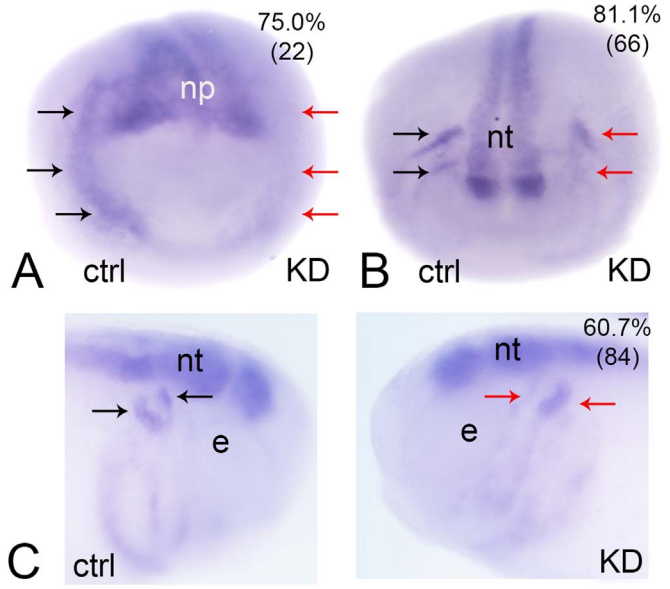

Fig. 2. Six1 is required for *Irx1* expression in the PPR and otic vesicle. (**A**) *Irx1* expression in the PPR (black arrows) is distinct on the control side (ctrl) but not detected on the MO-mediated knock-down (KD) side (red arrows). np, neural plate. (**B**) *Irx1* expression in two placodes is smaller and fainter on the KD side (red arrows) compared to the control side (black arrows). nt, neural tube. (**C**) *Irx1* expression in the control otic vesicle (left image) forms two distinct patches (black arrows), whereas the anterior patch often is undetectable on the KD side (red arrows). e, eye. A, B, anterior views; C, side views, all with dorsal to the top. Percentages are the frequencies of the phenotypes; numbers in parentheses are the sample sizes.

binding (*5′Myc-Six1-rescue*; Supplemental Fig. 1A) was co-injected, the reduced *Irx1* expression caused by the MOs was observed significantly less frequently (33.3%, n = 33; $p < 0.00001$), demonstrating the specificity of the knock-down in vivo. *Irx1* expression also was reduced in the otic vesicle at neural tube stages, particularly in the anterior region (Fig. 2C). Thus, while *Irx1* expression appears to precede that of *Six1* in the BZ (Glavic et al., 2004), its continued and appropriately patterned expression in the PPR, early placodes and otic vesicle requires Six1.

3.2. Six1 gain-of-function down-regulates Irx1

Previous work demonstrated that Six1 and Eya1 maintain the cranial placodes in an undifferentiated state, and that their levels need to be reduced in order for bHLH-regulated neurogenesis to commence (Schlosser et al., 2008). Because Irx1 plays an important role in neurogenesis in the neural plate by promoting the formation of bHLH-expressing neural progenitors (de la Calle-Mustienes et al., 2002; Gómez-Skarmeta et al., 1998, 2001), we tested whether increased levels of Six1 or Six1 +Eya1 affected *Irx1* expression. Six1 gain-of-function (gof) down-regulated the PPR expression of *Irx1* at each dose (200 pg, 400 pg, 800 pg); the frequency of embryos showing down-regulation was significantly greater at 800 pg (Fig. 3B; $p < 0.05$). The effect of Six1 gof was not significantly modulated by the addition of *Eya1* mRNA (Fig. 3A, B; $p > 0.05$). Since the Six1-Eya1 complex is thought to act as a transcriptional activator (Brugmann et al., 2004; Ikeda et al., 2002; Silver et al., 2003), these results suggest that Six1 either activates a repressor of *Irx1*, or directly represses *Irx1*. To address this, we expressed repressive and activating Six1 constructs. We found that the Six1 repressive construct (*Six1EnR*) down-regulated *Irx1* at about the same frequency as wild-type Six1 (Fig. 3C; 73.8%, n = 42, $p > 0.05$), whereas a Six1 activating construct (*Six1VP16*) broadened the *Irx1* expression domain (Fig. 3D; 68.1%; n = 47). Thus, although Six1 can activate *Irx1*, when its expression is increased over endogenous levels, Six1 represses *Irx1*, even in the presence of exogenous Eya1.

Expression of Six1, Six1 + Eya1 or Six1EnR also reduced *Irx1* in the otic vesicle (Fig. 3E) with no significant differences between the groups (Fig. 3F; $p > 0.05$). To determine if the otic effect was specific to *Irx1* or to otic development in general, we also tested the effect of Six1 gof on other otic genes; *Sox9* and *Pax2* also were reduced (Fig. 3G). These results suggest that otic vesicle molecular differentiation in general is delayed in the presence of elevated levels of Six1.

To determine whether Six1 affects *Irx1* expression after the establishment of the PPR, mRNA encoding a hormone-inducible Six1 (Six1-hGR) was injected into the PPR lineage and the resulting protein was activated by Dex treatment at different stages. When Dex was added to the culture medium at the early stage of *Irx1* expression (st 14; Glavic et al., 2004), about a third of the embryos showed broader *Irx1* expression in the PPR (Fig. 4). When Dex was added at st 16, when the PPR is well established (Schlosser and Ahrens, 2004), the ability to broaden the *Irx1* domain was greatly reduced and, in most embryos, the Irx1 domain was reduced (Fig. 4). When Dex was added after the placodes begin to segregate (st 18), *Irx1* otic vesicle expression was reduced at a high frequency and never was broader (Fig. 4). Each experimental group was significantly different from the no Dex control group, and each was significantly different from each of the other experimental groups ($p < 0.05$). Thus, even after the initiation of *Irx1* expression in the PPR, Six1 continues to affect its expression. At early PPR stages, Six1 can expand the *Irx1* domain, consistent with the knock-down data presented above that Six1 is required, but over time this activity subsides and changes to predominantly reduce it.

3.3. Reciprocal Irx1-Six1 interactions

Previous work showed that Irx1 is required for *Six1* expression in the PPR (Glavic et al., 2004); we confirmed this result by expressing a

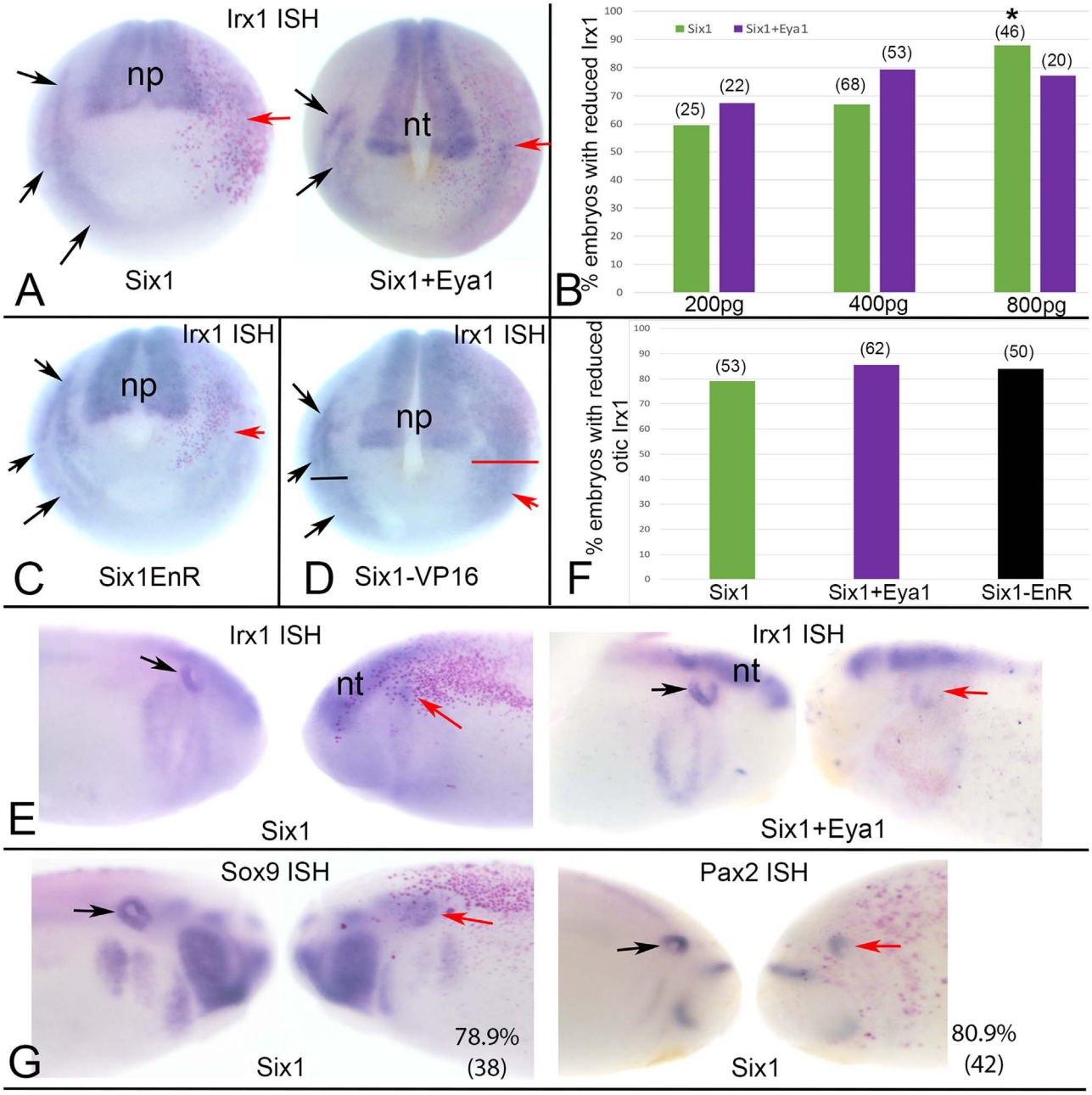

Fig. 3. Increasing Six1 levels represses *Irx1* expression. (**A**) Six1 or Six1+Eya1 mRNAs (right sides) reduce the size of the *Irx1* expression domain in the PPR or placodes. Black arrows denote *Irx1* PPR/placode expression on the control side; red arrows denote the same on the injected side, which also is indicated by the pink βGal lineage tracer. np, neural plate; nt, neural tube. (**B**) The percentage of embryos showing the same *Irx1* phenotypes as in (A). *, indicates a significant increase ($p < 0.05$) in frequency comparing 800 pg to either 200 pg or 400 pg of Six1 mRNA. Co-expressing *Six1 +Eya1* mRNAs does not significantly alter the frequency of the phenotype ($p > 0.05$). There were no significant differences between *Six1* or *Six1 +Eya1* mRNAs. Numbers above bars indicate the sample size. (**C**) The Six1 repressive construct (Six1EnR, 400 pg) also reduces *Irx1* PPR expression (red arrow). (**D**) The Six1 activating construct (Six1VP16, 400 pg) broadens the *Irx1* PPR domain (red arrow), as indicated by the red bar (compare to control width, black bar). (**E**) *Six1* and *Six1 +Eya1* mRNAs reduce *Irx1* expression in the otic vesicle. Black arrows denote otic vesicle on control side, and red arrows denote it on injected side of same embryo. (**F**) The percentage of embryos that show the same phenotype as in (E) when injected with *Six1*, *Six1 +Eya1* or *Six1EnR* mRNA. Numbers above bars indicate the sample size. There are no significant differences ($p > 0.05$) across the groups. (**G**) Six1 also reduces *Sox9* and *Pax2* expression in the otic vesicle. Black arrows denote otic vesicle on control side, and red arrows denote it on injected side of same embryo. A, C, D are anterior views, E, G are side views, all with dorsal to the top.

dominant-negative Irx1 construct that reduced *Six1* in every embryo (Fig. 5A). Expression of this construct also showed that Irx1 is required for the otic expression of *Six1* and *Pax2* (Fig. 5C, D). Thus, there is a reciprocal dependency between Irx1 and Six1 in both the PPR and otic vesicle.

We previously showed that increased levels of Six1 and Eya1 promote immature neural precursors in cranial placodes and repress neural progenitor differentiation (Schlosser et al., 2008). Because other studies demonstrated that Irx1 is required for the formation of neural progenitors (de la Calle-Mustienes et al., 2002; Gómez-Skarmeta et al., 1998, 2001), we hypothesized that Irx1 would down-regulate *Six1* and *Eya1* after PPR stages to allow neural progenitor differentiation to proceed. Indeed, we found that increasing Irx1 in the PPR lineage down-regulated both PPR genes (Fig. 6A, B); the only significant difference in mRNA dose was between the effects of 200 pg and 800 pg of Irx1 on *Six1* expression (Fig. 6C). At later stages, we found that increased Irx1 also reduced the expression of *Sox9* and *Pax2* in the otic vesicle (Fig. 6D). Since previous studies indicate that Irx1 acts as a transcriptional

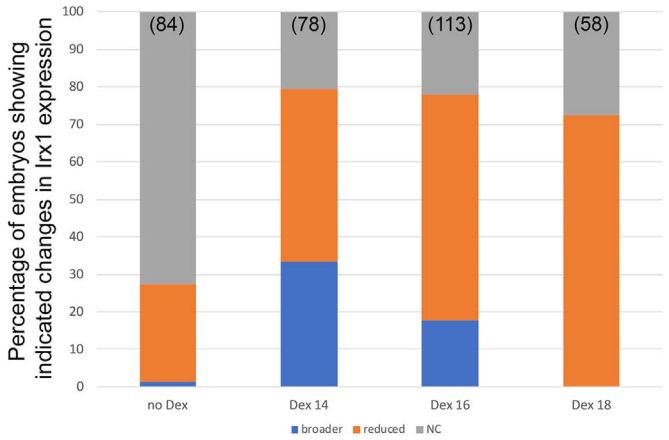

Fig. 4. Early activation of Six1 broadens *Irx1* PPR expression, whereas later activation reduces it. Embryos were injected with *Six1-hGR* mRNA (400 pg) and treated with Dexamethasone (Dex) at indicated stages. Those treated at st 14 or 16 were fixed at st 18–19 to analyze *Irx1* PPR/placode expression. Those treated at st 18 were fixed at st 24–28 to analyze *Irx1* otic expression. Each bar indicates the percentage of embryos in which *Irx1* expression was either broader compared to control side (blue), reduced compared to control side (orange), or the same as control side (no change, NC, grey) of the same embryo. The data for injected embryos that were not exposed to Dex and fixed at st 18/19 are shown in the left-most bar (no Dex) to indicate the background leakiness of the construct. Each experimental group was significantly different from the no Dex group, and significantly different from each other ($p < 0.05$). Control, uninjected embryos treated with Dex rarely showed an asymmetry in *Irx1* expression (Dex st 14: 7.7%, n = 52; Dex st 16: 5.3%, n = 19; Dex st 18: 4.8%, n = 21).

repressor (Glavic et al., 2001; Gómez-Skarmeta et al., 2001), we tested whether it activates or represses *Six1* by injecting mRNA encoding a hormone-inducible Irx1-EnR fusion protein previously shown to enlarge the Six1 PPR domain when activated at st 12 (Glavic et al., 2004). When we activated Irx1-EnR at st 14 it also predominantly expanded the *Six1* domain, but when activated at st 16, Irx1-EnR predominantly reduced the *Six1* domain (Fig. 6E). Activation of Irx1-EnR at st 18 reduced *Six1* otic expression at a high frequency that was not different from wild type Irx1 ($p > 0.05$). Each experimental group was significantly different from the no Dex group, and significantly different from each other ($p < 0.05$). Thus, the timing of Irx1 repressive activity differentially affects *Six1* expression. At first it expands *Six1* expression in the PPR, and later reduces it, even in the otic vesicle.

3.4. Possible role for Fgf signaling

When *Irx1* mRNA was injected into a single blastomere of the 16-cell embryo (Fig. 1), some displayed an open blastopore phenotype (Fig. 7A). The frequency of this effect differed depending on which animal blastomere was injected; it rarely occurred when *Irx1* was expressed in the ventral-most blastomere and occurred with increasing frequency when injected into more dorsal cells (Fig. 7B). Because previous work showed that *Six1* expression in the PPR is dependent on Fgf8 (Ahrens and Schlosser, 2005; Litsiou et al., 2005), and this Irx1 phenotype resembled the defect observed in embryos in which FGF signaling was reduced by expressing a dominant-negative FGF receptor (Amaya et al., 1991), we investigated whether FGF signaling might mediate the Irx1 effects on *Six1*. In concordance, we found that increased *Irx1* reduced *Fgf8* expression in the PPR (Fig. 7C). Conversely, the frequency of the open blastopore phenotype was reduced significantly in the D1.1 lineage by co-injecting mRNA encoding a constitutively active FGF receptor (38.9%, n = 59; $p < 0.00001$). Co-injection of *cfgfr1* mRNA also significantly decreased the frequency of the Irx1-mediated reduction in *Six1* (Fig. 7D; 23.3% reduced, n = 60; $p < 0.00001$). These results indicate that one likely mechanism by which Irx1 reduces *Six1* is by down-regulating Fgf8 signaling in the PPR.

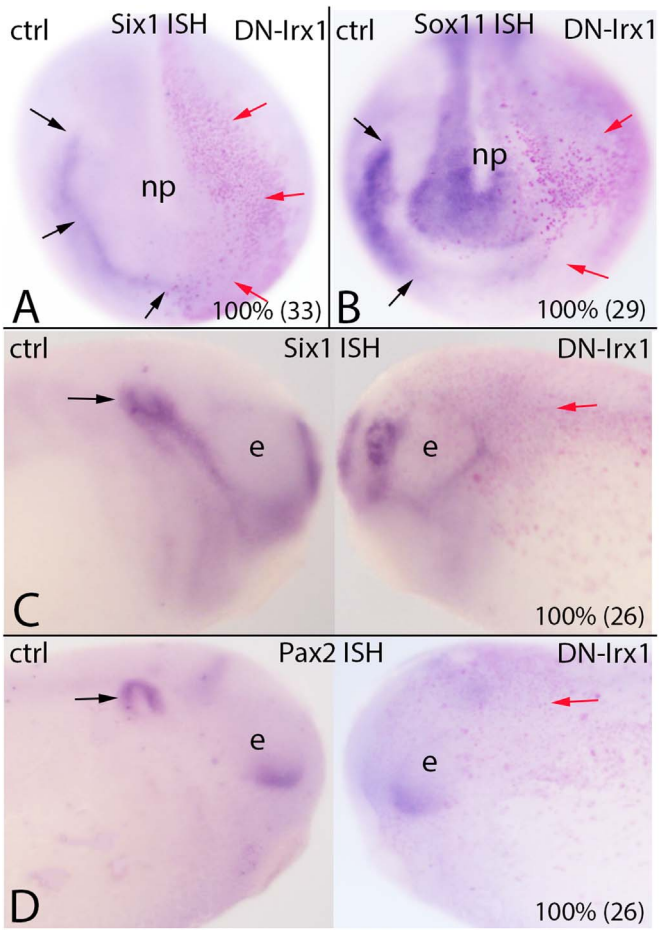

Fig. 5. Irx1 is required for PPR and otic gene expression (A) Injecting a dominant-negative Irx1 construct (DN-Irx1) results in loss of *Six1* PPR expression (red arrows) in every embryo. Black arrows denote normal expression on control (ctrl) side. (B) Injecting DN-Irx1 results in loss of *Sox11* PPR expression (red arrows) in every embryo. Black arrows denote normal expression on control side. (C) Injecting DN-Irx1 results in loss of *Six1* otic expression (red arrow) in every embryo. Black arrow denotes normal expression on control side. (D) Injecting DN-Irx1 results in loss of *Pax2* otic expression (red arrow) in every embryo. Black arrow denotes normal expression on control side. A, B are anterior views; C, D are side views, all with dorsal to the top. np, neural plate; e, eye.

3.5. Irx1 differentially affects border zone and neural crest genes

Because both the PPR and cranial NC are derived from a common BZ precursor field (Saint-Jeannet and Moody, 2014; Schlosser, 2006, 2010; Streit, 2004, 2007), and *Irx1* is initially expressed broadly in this zone (Glavic et al., 2004), we asked whether increased Irx1 alters the expression of BZ/NC genes. Increasing Irx1 expanded the expression domains of *Pax3*, *Zic1*, *Zic2* and *Tfap2α* (Fig. 8A-D). The effects on *Foxd3* expression, however, was mixed; in individual embryos it was either expanded or reduced (Fig. 8E). The frequencies of these two phenotypes were not significantly different between 200 pg and 400 pg (Fig. 8F), but 800 pg caused a significantly higher frequency of repression ($p < 0.05$), similar to the Irx1 effects on *Six1* (Fig. 6C). The NC domain of *Sox9* always was primarily reduced, regardless of mRNA dose (Fig. 8G, H); there were no significant differences in *Sox9* NC phenotype frequencies between the three doses ($p > 0.05$). These results suggest that Irx1 primarily expands genes expressed in the BZ and early NC (e.g., *Pax3*, *Zic1/2*, *Tfap2α*), and primarily reduces later expressed NC genes, such as *Foxd3* and *Sox9*. This is consistent with the observations that at first *Irx1* is expressed throughout the BZ but later is expressed only in the PPR and not in the NC domains (Glavic et al., 2004).

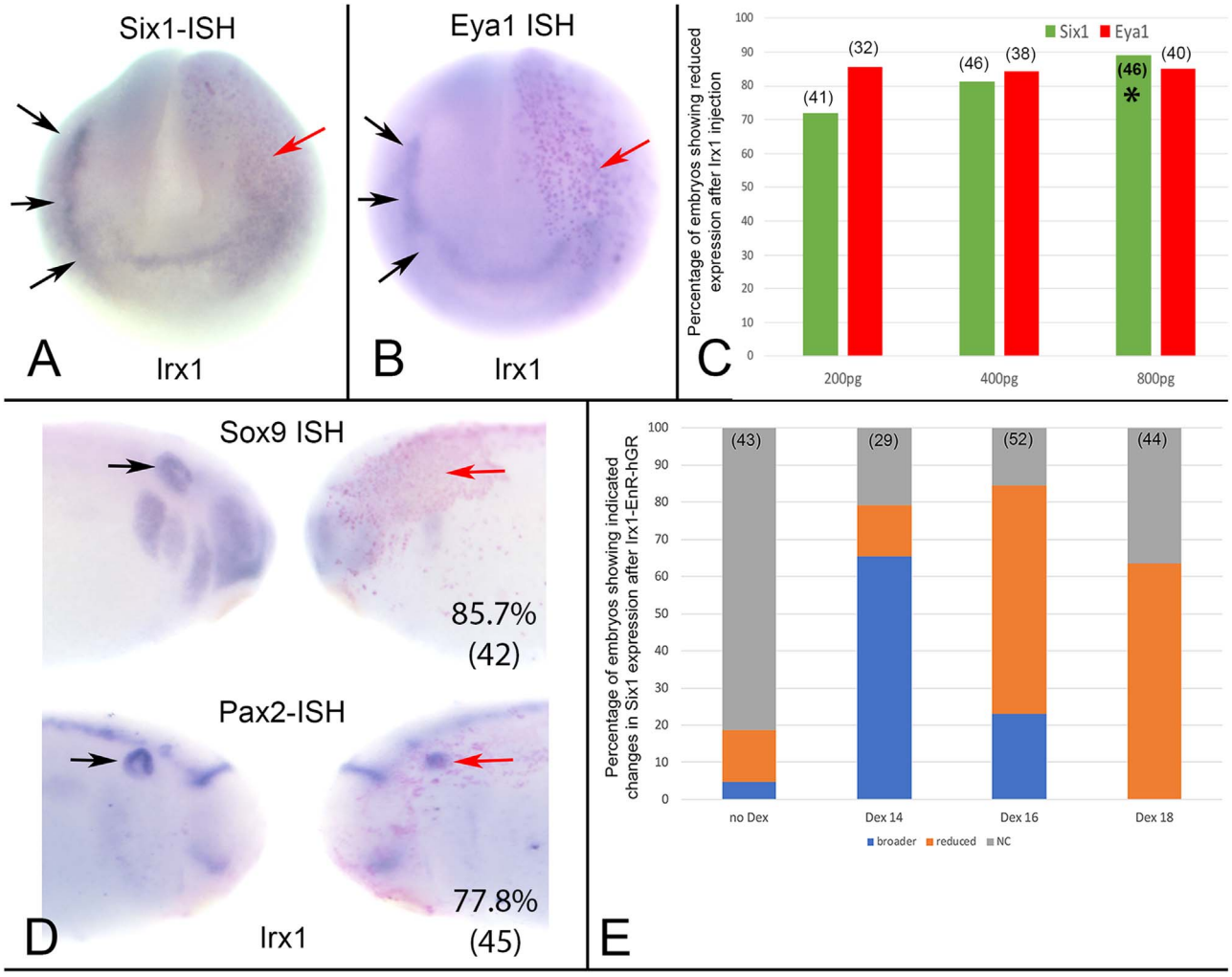

Fig. 6. Increased Irx1 reduces PPR and otic gene expression. (**A**) Increasing Irx1 by mRNA injection (right side) reduced the size of the *Six1* expression domain in the PPR. Black arrows denote *Six1* expression on the control side; red arrow denotes the reduced domain on the injected side. (**B**) Increased Irx1 reduced the size of the *Eya1* expression domain in the PPR. (**C**) The percentage of embryos that showed the same phenotype as in A and B when injected with *Irx1* mRNA. Irx1–800 pg caused the *Six1* phenotype significantly more frequently (*, $p < 0.05$) than Irx1–200 pg. There were no significant differences across *Irx1* mRNA doses for *Eya1* ($p > 0.05$). Numbers above the bars indicate sample size. (**D**) Increased Irx1 reduced the size of the *Sox9* and *Pax2* domains in the otic vesicle (red arrows) compared to control side of same embryo (black arrows). (**E**) Embryos were injected with Irx1-EnR-hGR mRNA (400 pg) and treated with Dexamethasone (Dex) at indicated stages. Analyses of *Six1* expression were performed as described in Fig. 4. Each experimental group was significantly different from the no Dex group, and significantly different from each other ($p < 0.05$). Control, uninjected embryos treated with Dex did not show an asymmetry in *Six1* expression (Dex st 14: 0%, n = 25; Dex st 16: 0%, n = 15; Dex st 18: 0%, n = 25). A, B are anterior views; D are side views, all with dorsal to the top.

3.6. *Irx1 differentially affects PPR/placode genes downstream of Six1*

Increasing Irx1 in the BZ also altered the expression domains of placode genes expressed downstream of Six1. *Sox9*, which is expressed in the early otic placode (Park and Saint-Jeannet, 2010), was reduced at high frequency at all doses (Fig. 8G, I); there were no significant differences in phenotype frequencies between the three doses ($p > 0.05$). *Sox11*, which similar to *Irx1* is expressed early throughout all but the most anterior PPR (Brugmann et al., 2004; Glavic et al., 2004), requires Irx1 (Fig. 5B). It was differentially affected by Irx1 gof (Fig. 9A–C). At 200 pg, Irx1 mostly expanded *Sox11* PPR expression, but at 400 pg and 800 pg it mostly reduced it; these frequencies were significantly different (Fig. 9B). Interestingly, at 800 pg Irx1 caused ectopic *Sox11* expression in the lateral ectoderm in nearly every embryo (Fig. 9C). This phenotype is consistent with the known role of Irx1 in ectopically inducing neural progenitor markers in the lateral ectoderm, presumably by interfering with BMP4 signaling (Gómez-Skarmeta et al., 1998, 2001). To determine if the reduced *Sox11* expression at moderate Irx1 levels (400 pg) might be due to transcriptional repression, we activated Irx1-EnR-hGR at either st 14 or st 16; both treatments predominantly reduced *Sox11* (Fig. 9D); each experimental group was significantly different from the no Dex group ($p < 0.05$) but not significantly different from each other ($p > 0.05$). To test whether the Irx1 effects on *Sox11* are direct, we activated Irx1-EnR-hGR 40 min after adding Chx to the culture medium, a protein synthesis inhibitor that will block indirect transcriptional effects (Kurth et al., 2005; Yan et al., 2009). Under this condition, *Sox11* PPR expression also was significantly reduced (Fig. 9E; 93.3%, n = 45), indicating direct transcriptional repression. Interestingly, *Sox11* ectopic expression in the lateral ectoderm was markedly expanded (Fig. 9E; 81%, n = 42), indicating direct transcriptional activation in this population of cells. This latter result is in concordance with previous reports of Irx1 gain-of-function (Gómez-Skarmeta et al., 1998, 2001).

Previous work indicated that Six1 acts upstream of *Sox11* to expand its placode domain (Brugmann et al., 2004). Since both Six1 and Irx1 directly affect *Sox11*, we tested whether their influence is epistatic. First, we replicated the previous observation that Six1 (200 pg) predominantly expands the *Sox11* placode domain (Fig. 9F), but also found that increasing Six1 to 800 pg predominantly reduces *Sox11* (Fig. 9F, G). The phenotype frequencies between the 200 pg and 800 pg groups were significantly different ($p < 0.05$), similar to the effect of Irx1 on *Sox11*. Next, we found that activation of Six1-hGR at either st

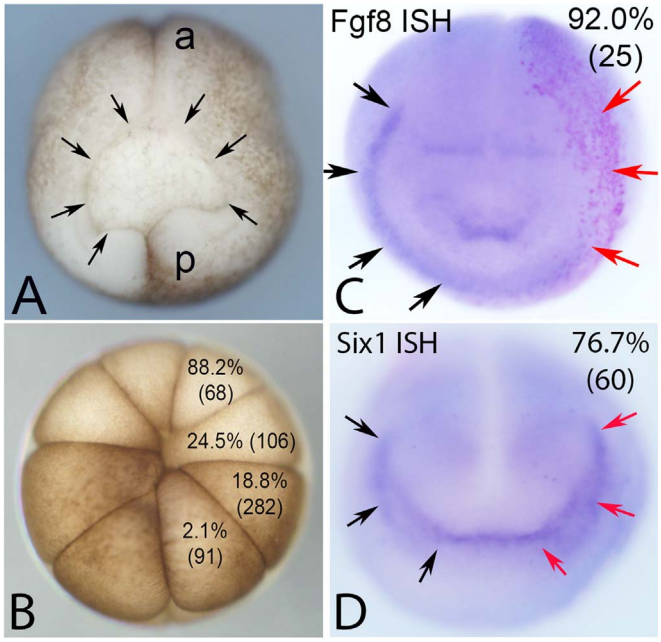

Fig. 7. Irx1 may affect PPR gene expression by downregulating *Fgf*. (A) When Irx1 mRNA (400 pg) was injected into animal blastomeres, an open blastopore phenotype (arrows) was observed at closed neural tube stages. Dorsal view, anterior (a) to the top, posterior (p) to the bottom. (B) The frequency of this phenotype depended upon the cell injected (see Fig. 1 for blastomere nomenclature). (C) Irx1 mRNA (400 pg) injection reduced the size of the *Fgf8* expression domain in the PPR. Black arrows denote *Fgf8* expression in the PPR on the control side; red arrows denote the reduced domain on the injected side. (D) In the majority of embryos co-injected with *Irx1* plus *cFgfr1* mRNAs, *Six1* expression was restored (76.7%, n = 60). Black arrows denote control side; red arrows denote injected side. C, D are anterior views, dorsal to the top.

14 or st 16 caused the majority of embryos to have a broader *Sox11* domain (Fig. 9H); each experimental group was significantly different from the no Dex group ($p < 0.05$), but not significantly different from each other ($p > 0.05$). Thus, while later activation of Irx-EnR-hGR down-regulates *Sox11*, later activation of Six1 up-regulates *Sox11*. This suggests that the Six1 effects and the Irx1 effects on *Sox11* are independent.

We next evaluated the interactions between Irx1 and Sox11. First, when Irx1 activity was reduced by expressing a dominant negative construct, *Sox11* PPR expression was reduced in every embryo (Fig. 5B). Reciprocally, we found that Sox11 knock-down caused *Irx1* placode expression to be reduced and smaller in area at a moderate frequency (Fig. 9I). Second, Sox11 gof caused *Irx1* expression to appear broad and faint rather than resolved into distinct placodes (Fig. 9J), resembling a more immature PPR state. Increased Sox11 also reduced *Irx1* otic expression (Fig. 9K). These results indicate that Irx1 and Sox11 are mutually required to maintain their expression, whereas gof mutually causes down-regulation. Since both Six1 and Sox11 are required for Irx1 expression and Sox11 acts downstream of Six1, we tested whether *Sox11* could rescue the loss of *Irx1* expression in Six1 morphants. It does not; *Irx1* expression remained reduced (68.6%, n = 35; $p > 0.05$). Thus, the effects of Six1 and Sox11 on maintaining Irx1 placode expression are independent.

3.7. Irx1 represses later placode genes associated with neural differentiation

Finally, we tested the role of Irx1 in placode neurogenesis. Sox2 and Sox3 are reported to hold neural precursors in both the neural plate and the placodes in a stem-like state prior to the expression of bHLH neurogenic factors (Chen et al., 2016; Ellis et al., 2004; Schlosser et al., 2008). Because Irx1 is required for the transition of neural plate stem cells to neural progenitors (de la Calle-Mustienes et al., 2002; Gómez-Skarmeta et al., 1998, 2001), we predicted that increased Irx1 would reduce the placodal expression *Sox2* and *Sox3* to accomplish this transition. Indeed, both were reduced in the majority of cases (Fig. 10A, B). However, Irx1 gof expanded their expression along the border of the neural plate in a minority of cases (Fig. 10A, B). Consistent with its reported ability to ectopically induce neural tissue in the lateral ectoderm (Gómez-Skarmeta et al., 1998, 2001; de la Calle-Mustienes et al., 2002), we also observed ectopic lateral ectoderm expression (*Sox2*, 74.6%; *Sox3*, 54.1%), similar to Irx1 gof effects on Sox11 (Fig. 9C). We also found that increased Irx1 reduced the expression of genes required for neural differentiation in the placodes, including *p27*, *NeuroD* and *Tubb2* (Fig. 10C). Since high levels of Six1 also reduce these neurogenic genes (Schlosser et al., 2008), we tested whether this reduction requires Six1 activity by expressing Irx1 in Six1 morphants (Fig. 10D); in no case did the loss of Six1 interfere with the Irx1 effects on these three genes. These results indicate that Irx1 regulates Sox genes in the cranial placodes differently than reported for the neural plate, but regulates neurogenic genes in a similar fashion to that reported for the neural ectoderm. Surprisingly, this activity is independent of Six1.

It is interesting to note that the effects of Six1 reported herein appear to be independent from other closely related family members. Increasing Six2 did not affect *Irx1* PPR expression (0%, n = 11), nor did increased Irx1 affect *Six2* (0%, n = 24) or Six 4.1 (0%, n = 23). Increased Six1 also had no notable effect on the PPR or otic expression of *Six2* (0%, n = 84) or *Six4* (0%, n = 92). Thus, these other family members appear to have non-overlapping roles in *Xenopus* cranial placode development.

4. Discussion

It has long been known that Six1 and Eya1 are required for the formation of the PPR and for maintaining cells in an undifferentiated state as the neurogenic cranial sensory placodes form (reviewed in Moody and LaMantia, 2015; Saint-Jeannet and Moody, 2014). Although a large number of genes have been identified as expressed in the developing PPR and placodes (Grocott et al., 2012; Hintze et al., 2017; Riddiford and Schlosser, 2016; Schlosser and Ahrens, 2004; Trevers et al., 2018), how they interact during placode formation and differentiation has only started to be explored in molecular detail. Understanding the relationships between the players in the transcriptional network that regulates the transition from immature PPR to placode neural progenitors is particularly important for assuring that some of these cells acquire a neuronal identity. The experiments presented herein shed light on the relationships between a few of the key transcription factors that are expressed at the early stages of cranial sensory organ development: the early formation of the PPR and its transition into placodes with neurogenic/otic potential.

4.1. Irx1-Six1 interactions during PPR formation

Previous work demonstrated that *Irx1* is expressed throughout the BZ domain, which gives rise to both NC and PPR, and then becomes confined to the posterior PPR (Glavic et al., 2004). Irx1 up-regulates *Six1* in the early *Xenopus* PPR (Glavic et al., 2004), and *Irx2* is induced by head mesoderm prior to *Six1* in chick (Hintze et al., 2017). Therefore, we were surprised when our microarray screen identified *Irx1* as a Six1 downstream target (Yan et al., 2015). To better understand the relationship between these two transcription factors, we tested their reciprocal interactions and found that in fact they differentially regulate each other at different developmental time points, from border zone to otic formation.

The fact that *Irx1* is first expressed throughout the BZ and then is excluded from the NC domain and maintained in the PPR domain suggests an early function in regulating BZ gene expression and the decision to form NC versus PPR domains. This is supported by the

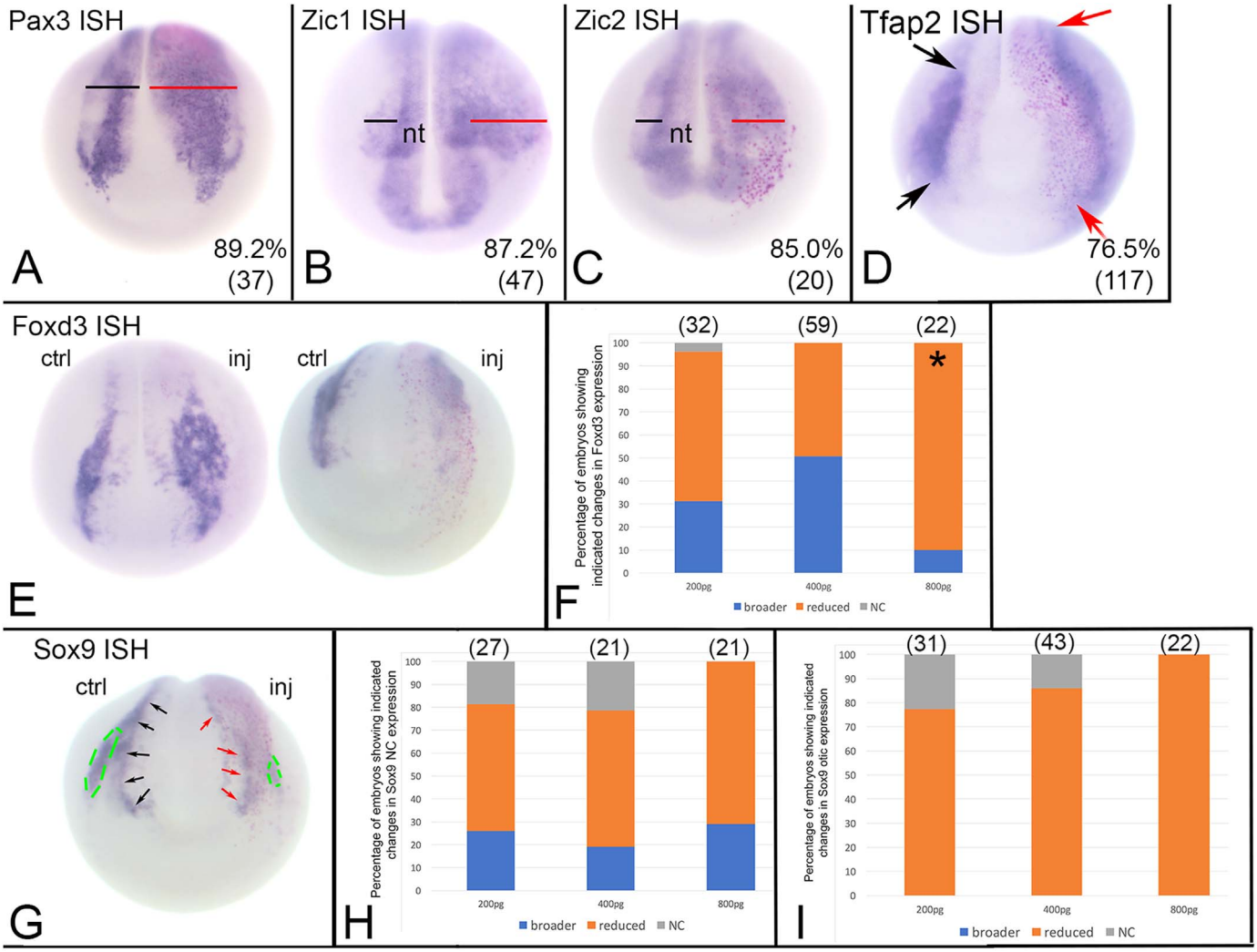

Fig. 8. Increased Irx1 alters BZ and NC gene expression. (**A**) Increased Irx1 expands the width of the *Pax3* domain (red bar) compared to control side (black bar). (**B**) Increased Irx1 expands the width of the *Zic1* neural crest domain (red bar) compared to control side (black bar). (**C**) Increased Irx1 expands the width of the *Zic2* neural crest domain (red bar) compared to control side (black bar). (**D**) Increased Irx1 extends the anterior-posterior extent of the *Tfap2α* neural crest domain (between red arrows) compared to control side (between black arrows). (**E**) Increased Irx1 either expands (left embryo) or reduces (right embryo) the *Foxd3* domain. inj, injected side; ctrl, control side. (**F**) Percentages of embryos showing reduced (orange), expanded (blue) or no change (NC, grey) of *Foxd3* domains after injection of different doses of *Irx1* mRNA. The frequencies of the *Foxd3* phenotypes were not significantly different between 200 pg and 400 pg, but 800 pg caused reduction of *Foxd3* at a significantly higher frequency (*, $p < 0.05$). (**G**) Increased Irx1 reduced the *Sox9* neural crest domain (red arrows) compared to control side (black arrows). It also reduced the size of the *Sox9*-expressing otic placode (outlined in green). (**H**) Percentages of embryos showing reduced (orange), expanded (blue) or no change (NC, grey) in *Sox9* neural crest (NC) domains after injection of different doses of *Irx1* mRNA. These phenotypes were not significantly different across the doses ($p > 0.05$). (**I**) Percentages of embryos showing reduced (orange), expanded (blue) or no change (NC, grey) in *Sox9* otic placode expression after injection of different doses of *Irx1* mRNA. These phenotypes were not significantly different across the doses ($p > 0.05$). All embryos are anterior views with dorsal to the top. Numbers in brackets above bars are sample sizes.

observation that Irx1 upregulates *Six1* (Glavic et al., 2004) and the data herein that show it also expands the expression of some BZ genes (*Pax3*, *Zic1/2*, *Tfap2α*) that are reported to be required for NC and/or PPR formation, including *Six1* (reviewed in Moody and LaMantia, 2015). Since increased Six1 also expands these gene domains (Maharana and Schlosser, 2018), it remains to be tested whether the Irx1 effects are mediated through Six1. Consistent with these experimental data, recent transcriptomic analyses of PPR development link expression of Six1 and Irx1 (and the closely related Irx2) to early stages (Hintze et al., 2017; Trevers et al., 2018). The challenge now is to decipher how the other classically described BZ genes (Maharana and Schlosser, 2018) and the newly identified ones (Hintze et al., 2017; Trevers et al., 2018) interact with Irx1 to form the BZ and its derivatives.

Although *Irx1* expression precedes *Six1* expression in the BZ (Glavic et al., 2004), we show that its continued expression in the PPR requires Six1; thus, there is a reciprocal positive up-regulation. Six1 and Irx1 mutually enhance each other's expression in the PPR and they both downregulate some NC genes, in particular the NC-specifier *Foxd3* (Brugmann et al., 2004; Maharana and Schlosser, 2018). This is consistent with the idea that NC and PPR genes mutually inhibit each other resulting in the separation of the two domains from the BZ. The transcriptional regulation of this process has recently been experimentally demonstrated for a large number of the known genes (Maharana and Schlosser, 2018), but Irx1 was not included in this analysis. From our data, we predict that *Irx1* expression becomes confined to the PPR via positive feedback from Six1, and perhaps from negative feedback from some NC genes. Because Irx1 upregulates several BZ genes that later are confined to NC (e.g., *Pax3*, *Zic1/2*, *Tfap2α*), we predict that *Irx1* may be downregulated by these genes as the NC domain forms. It will be important to include Irx1 in the recently predicted GRNs (Grocott et al., 2012; Hintze et al., 2017; Maharana and Schlosser, 2018) to resolve the transcriptional dynamics between all of these players during the transition from BZ to NC/PPR. This will improve

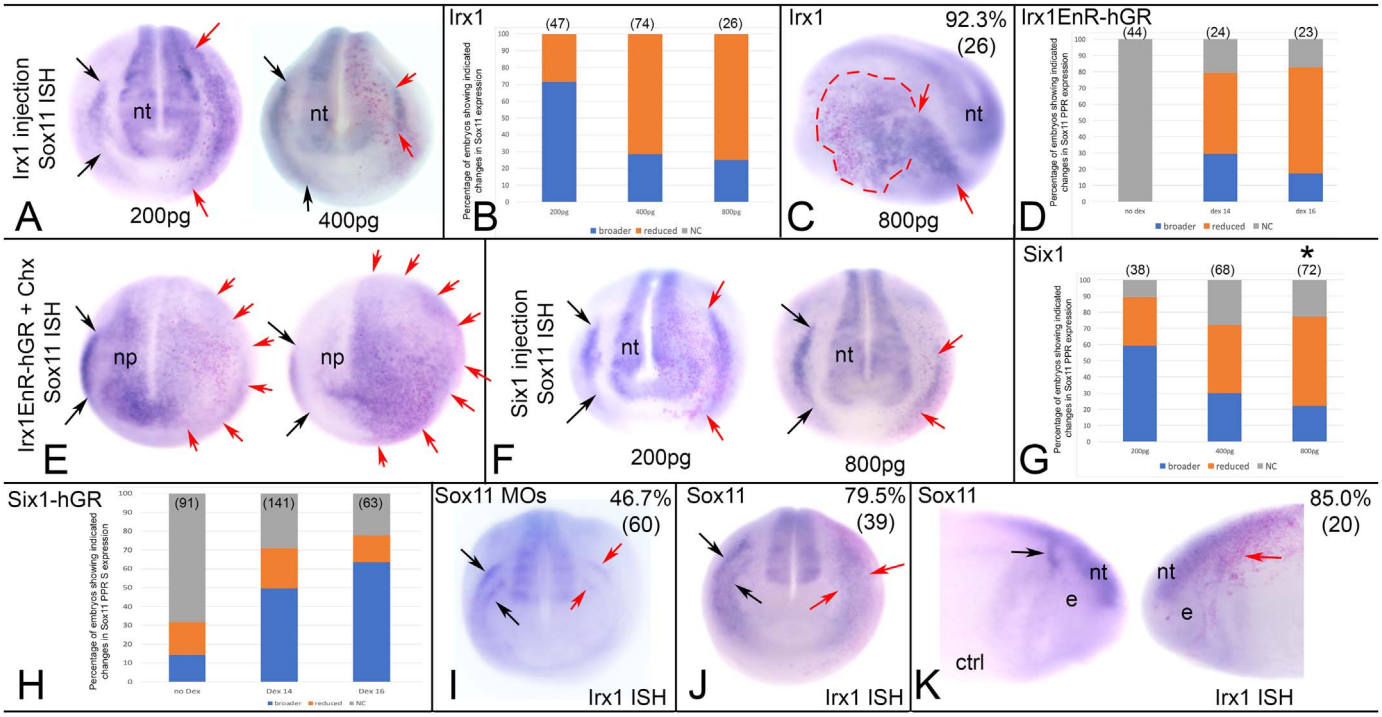

Fig. 9. Increased Irx1 alters placode gene expression. (**A**) A low dose (200 pg) of Irx1 mRNA expanded the *Sox11* PPR domain (between the red arrows) compared to control side (between the black arrows); in contrast, a higher dose (400 pg) reduced it. nt, neural tube. (**B**) Percentages of embryos showing reduced (orange), expanded (blue) or no change (NC, grey) in *Sox11* PPR domain after injection of different doses of *Irx1* mRNA. There was no difference between 400 pg and 800 pg ($p > 0.05$). (**C**) 800 pg of Irx1 mRNA expanded the *Sox11* PPR domain (between the red arrows) in a minority of cases (see B), but in a majority of cases it also caused ectopic *Sox11* expression in the lateral ectoderm (outlined by red dashes). (**D**) Percentages of embryos showing reduced (orange), expanded (blue) or no change (NC, grey) in *Sox11* PPR domain after activation of Irx1-EnR-hGR at st 14 or st 16. Analyses were performed as described in Fig. 4. Each experimental group was significantly different from the no Dex group, but not significantly different from each other ($p > 0.05$). Control, uninjected embryos treated with Dex did not show an asymmetry in *Sox11* expression (Dex st 14: 0%, n = 14; Dex st 16: 4.5%, n = 22). (**E**) Two examples showing that treatment of Irx1-EnR-hGR injected embryos with Chx 40 min before st 14 Dex treatment resulted in ectopic *Sox11* expression throughout the lateral ectoderm (red arrows) compared to discrete PPR expression (between black arrows) and neural plate (np) expression on control side. (**F**) A low dose (200 pg) of Six1 mRNA expanded the *Sox11* PPR domain (between red arrows) compared to control side (between the black arrows), whereas a high dose (800 pg) reduced it. (**G**) Percentages of embryos showing reduced (orange), expanded (blue) or no change (NC, grey) in *Sox11* PPR domain after injection of different doses of *Six1* mRNA. 800 pg showed a significantly different frequency compared to 200 pg (*, $p < 0.05$), but not 400 pg. (**H**) Percentages of embryos showing reduced (orange), expanded (blue) or no change (NC, grey) in *Sox11* PPR domain after activation of Six1-hGR at st 14 or st 16. Analyses were performed as described in Fig. 4. Each experimental group was significantly different from the no Dex group, but not significantly different from each other ($p > 0.05$). Control, uninjected embryos treated with Dex did not show an asymmetry in *Sox11* expression (Dex st 14: 0%, n = 14; Dex st 16: 4.5%, n = 22). (**I**) *Irx1* expression in two placodes (black arrows) is distinct on the control side but nearly undetected on the Sox11-MO injected side (red arrows) in nearly half of embryos. (**J**) Increasing Sox11 prevents *Irx1* expression from resolving into distinct placodes, indicated on the control side by black arrows. It remains broad and faint (between red arrows), as is typical of an earlier stage PPR. (**K**) Increasing Sox11 reduces *Irx1* otic expression (red arrow) compared to control side (black arrow) of same embryo. e, eye; nt, neural tube. A, E, F, I, and J are anterior views; C is an anterior-lateral view; K are lateral views; all with dorsal to the top.

our ability to differentiate various kinds of stem cells into neural crest or placode derivatives (e.g., Dincer et al., 2013; Leung et al., 2013; Tchieu et al., 2017).

4.2. Dosage and timing

Because Six1 can act as either a transcriptional activator or repressor, we wondered whether it would have different effects after injecting different amounts of mRNAs, as has been shown for placode neurogenesis genes (Schlosser et al., 2008; Riddiford and Schlosser, 2017). We observed that 800 pg of Six1 reduced *Irx1* at a higher frequency than 200 pg; similarly, 800 pg of Irx1 reduced *Six1* at a higher frequency than 200 pg, but there were not different effects at different doses. Higher amounts of mRNAs are expected to produce higher concentrations of protein, which may cause an increased effect due to more binding sites being occupied and/or low-affinity sites being activated. However, it also is possible that 800 pg results in non-physiological levels of protein that lead to abnormally forced molecular interactions. Regardless, these data demonstrate that the mutual effects of Six1 and Irx1 are not concentration dependent. In contrast, both Irx1 and Six1 cause expansion of *Sox11* at 200 pg but predominantly reduction at 400 pg and 800 pg. This is similar to dose-dependent effects of Six1/Eya1 on neurogenic genes (Schlosser et al., 2008; Riddiford and Schlosser, 2017). In these cases, the different levels of protein may result in differential binding to high versus lower affinity sites and/or may allow binding to different co-factor proteins. These possibilities will need to be tested at the genomic level.

It also is notable that Irx1 and Six1 have different effects when they are activated at different developmental times. When they are activated at early PPR stages, they predominantly cause mutual expansion, yet when activated at late PPR and placode stages, they are mutually repressive. The early mutual expansion may stabilize the PPR region and distinguish those cells from the adjacent NC-forming region. The later mutual repression may come into play at times when Six1 is needed to maintain immature placode cells whereas Irx1 is needed to initiate neurogenesis. While Irx1 is considered to be exclusively a transcriptional repressor (Glavic et al., 2004), herein we show using the Six1-EnR and Six1-VP16 constructs that Six1 can act as either a repressor or activator of *Irx1*. Previous work demonstrates that the type of Six1 activity depends upon the availability of different cofactors (Brugmann et al., 2004). Therefore, at least for Six1, the presence of different cofactors at different developmental times may influence its effects on *Irx1*. Interestingly, when wild-type mRNAs are introduced at cleavage stages, Irx1 and Six1 mutually reduce rather than expand each other. While the underlying cause of this discrepancy needs to be addressed experimentally, one possibility is that there are different

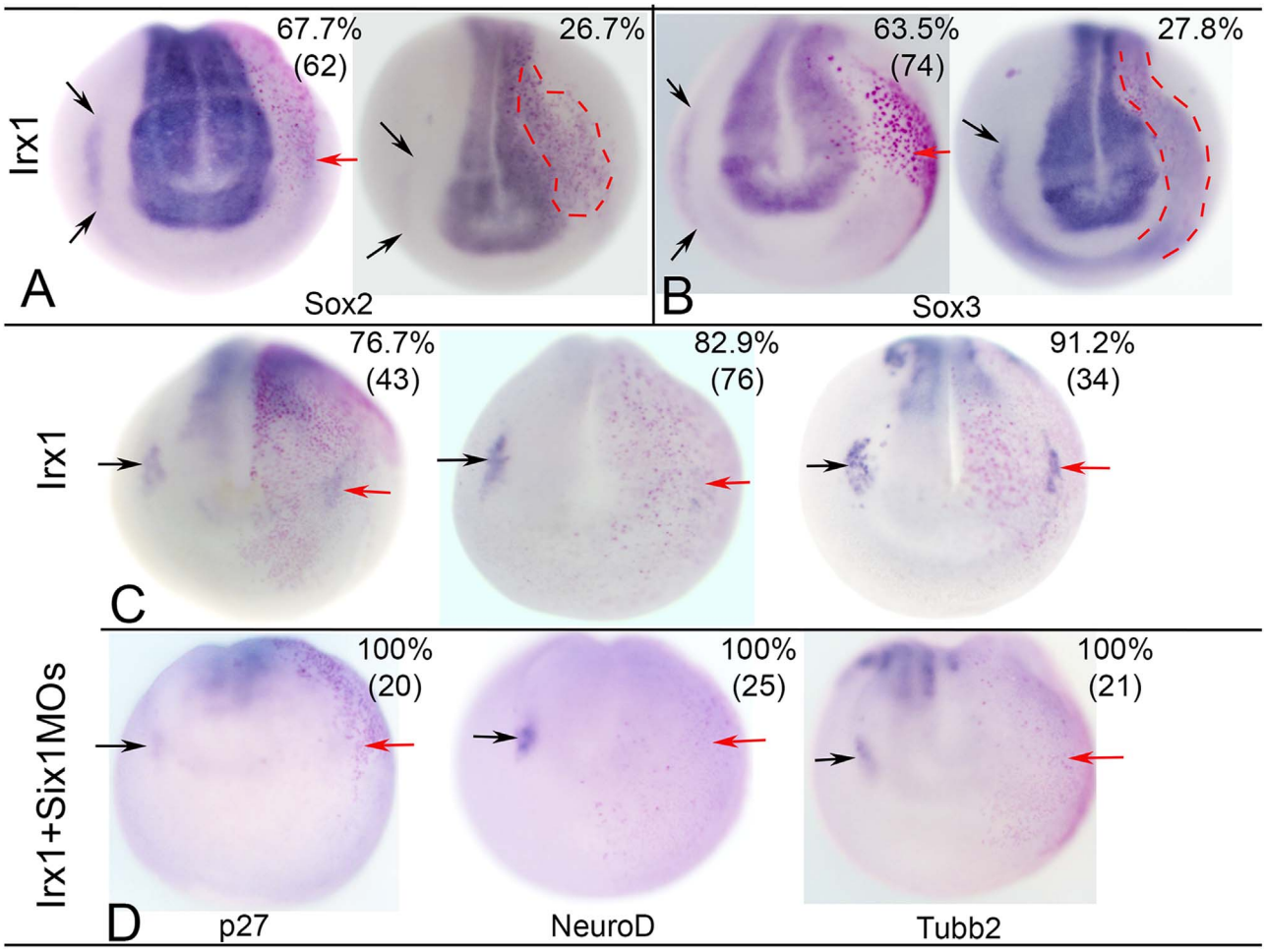

Fig. 10. Increased Irx1 reduces neural stem and neural differentiation genes in placodes. (**A**) Left: increased Irx1 reduced the *Sox2* placode domain (red arrow) compared to control side (between black arrows) in the majority of cases. Right: expression was expanded in the lateral ectoderm along the cranial neural border in some cases. (**B**) Left: increased Irx1 reduced the *Sox3* placode domain (red arrow) compared to control side (between black arrows) in the majority of cases. Right: expression was expanded in the lateral ectoderm along the cranial neural border in some cases. (**C**) Increased Irx1 reduced *p27*, *NeuroD* and *Tubb2* in their placode domains (red arrows) compared to control side (black arrows). (**D**) Increased Irx1 also reduced these three genes in embryos in Six1 morphants. Black arrows denote control sides; red arrows denote injected sides. np, neural plate; nt, neural tube. All embryos are anterior views with dorsal to the top.

genomic architectures at the two stages that affect binding efficiencies and/or proximity of enhancers.

4.3. Irx1-Six1 interactions during cranial placode neurogenesis

As the PPR begins to resolve into individual placodes, Six1 expands *Sox11* and represses *Irx1*. Sox11 appears to play an intermediate role in the placode neurogenic network because it is required for *Irx1* expression, but this activity changes to a predominantly repressive effect over time. Irx1 also reciprocally affects the expression of *Sox11*. At low levels, Irx1 mostly expands *Sox11*, but at moderate levels it mostly reduces it. Although we predicted that Six1′s later repressive effect on *Irx1* would be mediated by expanding *Sox11*, our data indicate that the Six1 and Sox11 effects are independent; Sox11 overexpression does not rescue Six1 knock-down.

Sox2 and Sox3 are considered markers of neural stem cells because they are required for the initiation of neural differentiation, acting upstream of pro-neural genes (Ellis et al., 2004; Schlosser et al., 2008). We and others previously showed that high levels of Six1 activated at neural plate stages promote immature, proliferating neural precursors in the cranial placodes, promoting expression of *Sox2*/*Sox3*, and suppressing pro-neural (*NeuroG*) and neural differentiation (*p27*, *NeuroD*, *Tubb2*) genes; in contrast, low levels promote neurogenesis (Schlosser et al., 2008; Riddiford and Schlosser, 2017). Six1/Eya1 can directly activate *Sox2/Sox3* (Riddiford and Schlosser, 2016), which in turn are required for *NeuroD* activation (Schlosser et al., 2018); this pathway has been shown to be mediated by activating members of the Notch signaling pathway (Riddiford and Schlosser, 2017). Because Irx1 is required for the transition of neural plate stem cells to neural progenitors (de la Calle-Mustienes et al., 2002; Gómez-Skarmeta et al., 1998, 2001), we predicted that increased Irx1 would reduce *Sox2* and *Sox3*. In the majority of cases they were reduced, but expansion of *Sox2/Sox3* domains also were observed, as reported previously (Gómez-Skarmeta et al., 2001). We found the same effects on *Sox11*, which also is required for neural plate neurogenic gene expression (Chen et al., 2016). We also found that Irx1 gain-of-function down-regulated neural differentiation genes, consistent with previous observations in the neural plate (Gómez-Skarmeta et al., 1998, 2001; de la Calle-Mustienes et al., 2002). In contrast to down-regulation of Sox genes in the placodes, Irx1 induced the ectopic expression of *Sox11*, *Sox2* and *Sox3* in the lateral ectoderm adjacent to the neural plate. Others have observed the ectopic induction of neural progenitor markers in the lateral ectoderm under similar experimental conditions (Gómez-Skarmeta et al., 1998, 2001; Schlosser et al., 2008). Whether these Irx1 effects cause ectopic placode expression, as is the case for Six1/Eya1 (Schlosser et al., 2008) or expansion of the neural ectoderm

(Gómez-Skarmeta et al., 2001) still needs to be addressed. Regardless, it is likely this is mediated by the ability of Irx1 to block BMP4 activity (Gómez-Skarmeta et al., 2001; Glavic et al., 2001).

4.4. In summary

There is significant interest in the gene interactions that regulate the formation of the neural border zone and its division into neural crest and sensory placode derivatives, as these are the embryonic precursors of all the cranial sensory structures. Several recent screens have revealed a large number of genes expressed during this developmental window that are being experimentally related to each other. In this work, we demonstrate that two such factors, Irx1 and Six1, have reciprocal interactions, the effects of which vary depending upon the timing of their expression. While they mutually upregulate each other at early stages to expand the PPR, they then mutually downregulate each other during placode neurogenesis and otic vesicle formation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.12.003.

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