Cellular/Molecular

# Progression of Neurogenesis in the Inner Ear Requires Inhibition of *Sox2* Transcription by Neurogenin1 and Neurod1

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***Sox2* is required for proper neuronal formation in the CNS, but the molecular mechanisms involved are not well characterized. Here, we addressed the role of Sox2 in neurogenesis of the developing chicken inner ear. Overexpressing *Sox2* from a constitutive (β-actin) promoter induces the expression of the proneural gene, *Neurogenin1* (*Ngn1*); however, the expression of a downstream target of Ngn1, *Neurod1,* is unchanged. As a result, there is a reduction of neural precursors to delaminate and populate the developing cochleovestibular ganglion. In contrast, overexpression of either *Ngn1* or *Neurod1* is sufficient to promote the neural fate in this system. These results suggest that high levels of Sox2 inhibit progression of neurogenesis in the developing inner ear. Furthermore, we provide evidence that Ngn1 and Neurod1 inhibit *Sox2* transcription through a phylogenetically conserved *Sox2* enhancer to mediate neurogenesis. We propose that Sox2 confers neural competency by promoting *Ngn1* expression, and that negative feedback inhibition of *Sox2* by Ngn1 is an essential step in the progression from neural precursor to nascent neuron.**

## Introduction

Sry-related HMG-box 2 (Sox2) is a transcription factor that contains a high-mobility-group (HMG) DNA-binding domain (Lefebvre et al., 2007). Its presumed role during vertebrate neural development is to maintain a proliferative neural progenitor pool and confer neural competency (Taranova et al., 2006; Pevny and Nicolis, 2010). The lack of *Sox2* in neural tissues affects progenitor proliferation, expression of proneural genes, and neuronal differentiation (Ferri et al., 2004; Taranova et al., 2006). In addition, Sox2 is required for neural stem cell maintenance *in vivo* and *in vitro*, and neurospheres generated from neural stem cells that express a low level of *Sox2* have reduced capacity to develop into mature neurons (Cavallaro et al., 2008; Favaro et al., 2009). As neural progenitors enter neurogenesis, downregulation of Sox2 is required, which is in part mediated by serine protease(s) (Bani-Yaghoub et al., 2006). Nonetheless, the underlying mechanisms that enable Sox2 to render neural competency, and facilitate initiation of neurogenesis remain largely uncharacterized (Van Raay et al., 2005; Taranova et al., 2006).

Neurons of the cochleovestibular ganglion (CVG, VIIIth cranial ganglion) innervate all sensory organs within the inner ear. Proper CVG formation requires the evolutionarily conserved basic helix-loop-helix genes *Neurogenin1* (*Ngn1*) and *Neurod1* (Liu et al., 2000; Ma et al., 2000; Matei et al., 2005), which in other neural tissues are required for neuronal fate specification, and differentiation, respectively (Ma et al., 1996; Ma et al., 1998, Bertrand et al., 2002; Chae et al., 2004). Beginning at the otic cup stage and continuing until the early stages of otocyst development, a subpopulation of cells in the neural sensory competent domain (NSD) of the otic epithelium sequentially expresses first *Ngn1*, and then *Neurod1*. These *Ngn1*- and *Neurod1*-positive neuroblasts soon exit from the NSD, and coalesce to form neurons of the CVG. After neuroblast delamination is complete, the remaining NSD is thought to gradually split to form the sensory hair cells, and supporting cells that comprise the sensory patches within the inner ear. The expression of *Sox2* changes during this developmental period with high levels in the NSD, and its derived sensory patches but low levels in the delaminated neuroblasts, and sensory hair cells (Neves et al., 2007) (see Results). Mutant mice that have compromised *Sox2* expression in the inner ear show deficits in both neural/neuronal, and sensory components (Kiernan et al., 2005; Puligilla et al., 2010). Similarly, mutations of *SOX2* in humans can lead to sensorineural hearing loss in addition to brain defects, and anophthalmia (Hagstrom et al., 2005; Kelberman et al., 2006). Therefore, Sox2 has an important role in both brain, and sensory organ development but the molecular mechanisms involved are largely uncharacterized. Here we have examined the roles of Sox2 in neurogenesis of the devel-

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oping inner ear using a transient gain-of-function approach. We find that overexpression of *Sox2* readily induces *Ngn1* expression in the otic epithelium, but proper neuroblast delamination requires Ngn1, and Neurod1 to transcriptionally downregulate *Sox2* via interacting with a *cis*-regulatory repressive element within the nasal-otic placode-specific enhancer 1 (*Nop-1*) of *Sox2*.

## Materials and Methods

*Eggs,* in ovo *electroporation, and expression constructs.* Fertilized chicken eggs (B&E) were incubated at 38°C and staged according to Hamburger and Hamilton (1992). Full-length cDNA of chicken *Sox2* was subcloned into the *pMES-IRES-GFP* expression vector, in which cDNA is driven by a chicken $\beta$-actin promoter. Mouse *Ngn1* cDNA was subcloned into *pMES-IRES-GFP* and *pCMV-DsRed-Express* (Clontech) vector. Chicken *Neurod1* cDNA was subcloned into the *pCI-IRES-H2B-RFP* vector. The *Nop-1* and *Nop1-Ebox Mutant* (E-box sequence *CAGGTG* mutated to *AGCTAA*) regulatory elements of *Sox2* were subcloned into an enhanced green fluorescent protein vector, *ptkEGFPv2*. Various plasmids were delivered to the right otic cup between 10 and 17 somite stages (E1.5) by electroporation. This was conducted by filling the right otic cup with plasmids at a concentration of 3–4 $\mu$g/$\mu$l tinted with fast green. Then, a negative platinum electrode was placed above the right otic cup and the positive electrode inserted underneath the embryo at the location of the left otic cup. Two to four pulses at 7 V with 100 ms duration and spacing were applied using a CUY21 electroporator (Bex). Then, the eggs were sealed and returned to the incubator for 6–48 h. For coelectroporation, the respective plasmid constructs were used at ~3 $\mu$g/$\mu$l concentrations each.

In situ *hybridization and immunohistochemistry. In situ* hybridization on cryosections was performed as previously described (Wu and Oh, 1996; Raft et al., 2007). Chicken digoxigenin-labeled $\alpha$-sense RNA probes were generated for *Sox2*, *GFP*, *Ngn1*, *Neurod1*, and *Lunatic fringe* (*Lfng*). Similar cryosections for *in situ* hybridization were used for immunostaining. The primary antibodies used were rabbit polyclonal $\alpha$-Sox2 (1:4000 Millipore Bioscience Research Reagents), goat polyclonal $\alpha$-GFP-FITC-conjugated (1:400, GeneTex), rabbit polyclonal $\alpha$-DsRed-Express (1:100, Clontech), and mouse monoclonal $\alpha$-neuron-specific $\beta$-III Tubulin-Northern Lights 557 conjugated (1:25; TuJ-1-NL557, R&D Systems). The secondary antibodies used were goat $\alpha$-rabbit Alexa Fluor 568 and 488 (1:250, Invitrogen). Antibody labeling was performed according to standard protocol (Raft et al., 2007), except the sections were subjected to antigen retrieval by citrate boiling for 5 min before immunostaining for $\alpha$-Sox2.

*Double-labeling of tissue sections with* in situ *and immunohistochemistry.* To analyze gene expression at a cellular level, ear sections were first probed for RNA transcripts (e.g., *Ngn1*), followed by labeling with rabbit polyclonal $\alpha$-GFP antibody (1:500 Invitrogen) overnight at 4°C. During the *in situ* procedure, proteinase K exposure was reduced to 1 min and subsequent colorimetric development was carefully monitored and terminated when ectopic *Ngn1* hybridization signals were apparent. The secondary antibody used was goat $\alpha$-rabbit Alexa Fluor 488 (1:250 Invitrogen).

*Ganglion size measurements, cell counts, and statistical analyses.* For analyzing the size of ganglia after electroporation, ear sections were subjected to *Neurod1 in situ* hybridization and photographed using a Zeiss microscope. The *Neurod1*-positive, ganglionic regions were traced with the NIH ImageJ analyzer and computed. The size of otocysts was estimated in a similar manner by tracing the outline of the otocyst in sections and summing their areas. Two-tailed Student's *t* tests were performed between control and treated samples with $\alpha$ levels of 0.05, 0.01, and 0.001.

Analyses of Nop-1 activity were conducted by coelectroporating *Nop1-GFP* or *Nop1-EboxMut-GFP* with various plasmids *Ngn1-DsRed*, *DsRed*, *Neurod1-RFP*, or *RFP* as indicated. Ear sections were subjected to double-antibody labeling with $\alpha$-GFP-FITC and $\alpha$-DsRed-Express, counterstained with DAPI, and photographed using a Zeiss microscope. The images were merged in Adobe Photoshop and the total number of cells that coexpressed both GFP and DsRed per ear were counted. In some specimens, adjacent immunolabeled sections were probed for *Ngn1* transcripts using *in situ* hybridization for identifying the NSD. Two-tailed Student's *t* tests were performed on the total number of co-expressing cells with $\alpha$ levels of 0.05 and 0.001, or a two-tailed $\chi^2$ test in a $2 \times 2$ contingency table was used for quantification as indicated.

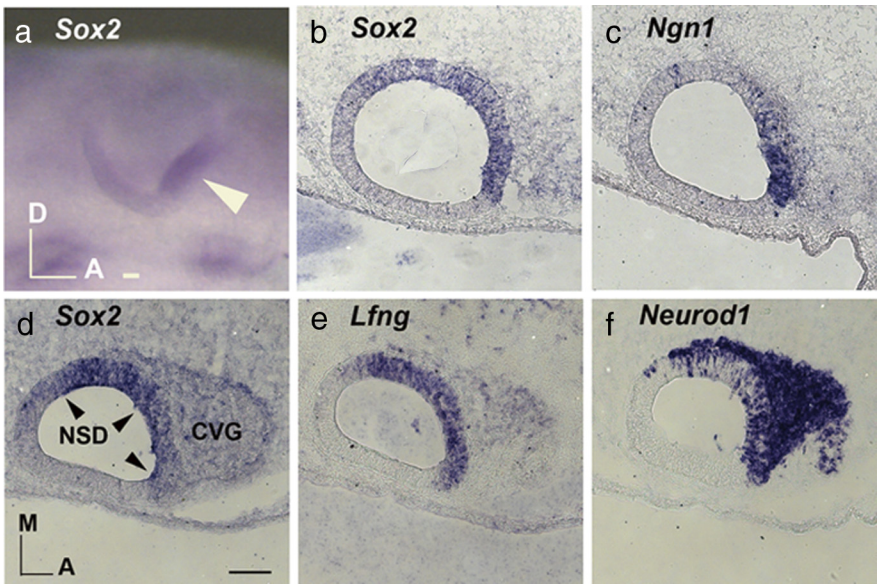


**Figure 1.** Endogenous expression of *Sox2* in the developing chicken inner ear. ***a***, Whole mount expression of *Sox2* in the NSD of the otic cup (arrowhead). ***b*** and ***c*** and ***d–f***, are 12 $\mu$m adjacent sections of otocysts at embryonic day 3 showing overlapping expression of *Sox2* (***b***, ***d***), *Lfng* (***e***), *Ngn1* (***c***), and *Neurod1* (***f***) in the NSD. Only *Neurod1* (***f***) is strongly expressed in the neuroblasts of the CVG. A, Anterior; D, dorsal; M, medial. Scale bars, 100 $\mu$m.

## Results

### *Sox2* expression is strong in the NSD but weak in the CVG

The NSD of the developing inner ear is defined by the overlapping expression domains of a number of genes such as *Lfng*, *fibroblast growth factor 10* (*Fgf10*), and *Sox2* (Cole et al., 2000; Alsina et al., 2004; Neves et al., 2007). Figure 1 illustrates the expression of *Sox2* in the *Lfng*-positive NSD located primarily in the anteroventral region of the otic cup and otocyst (Fig. 1*a*,*b*,*d*,*e*). A subpopulation of cells in the NSD expresses *Ngn1* and *Neurod1*, and these neuroblasts delaminate from the otic epithelium and coalesce to form the CVG. In the delaminated neuroblasts, *Neurod1* but not *Ngn1* transcripts are strongly expressed (Fig. 1*c*,*f*). Both *Sox2* and *Lfng* transcripts are also much reduced in the CVG compared to the NSD (Fig. 1*b*,*d*,*e*). This reduction of *Sox2* expression in the CVG suggests that *Sox2* downregulation is required before neuronal differentiation, similar to its postulated role in other neural tissues.

### *Ectopic Sox2* causes a decrease in the size of CVG but an upregulation of *Ngn1*

Based on the gene expression patterns and the postulated role of Sox2 during neurogenesis, we hypothesized that overexpression of *Sox2* would inhibit neurogenesis in the inner ear.

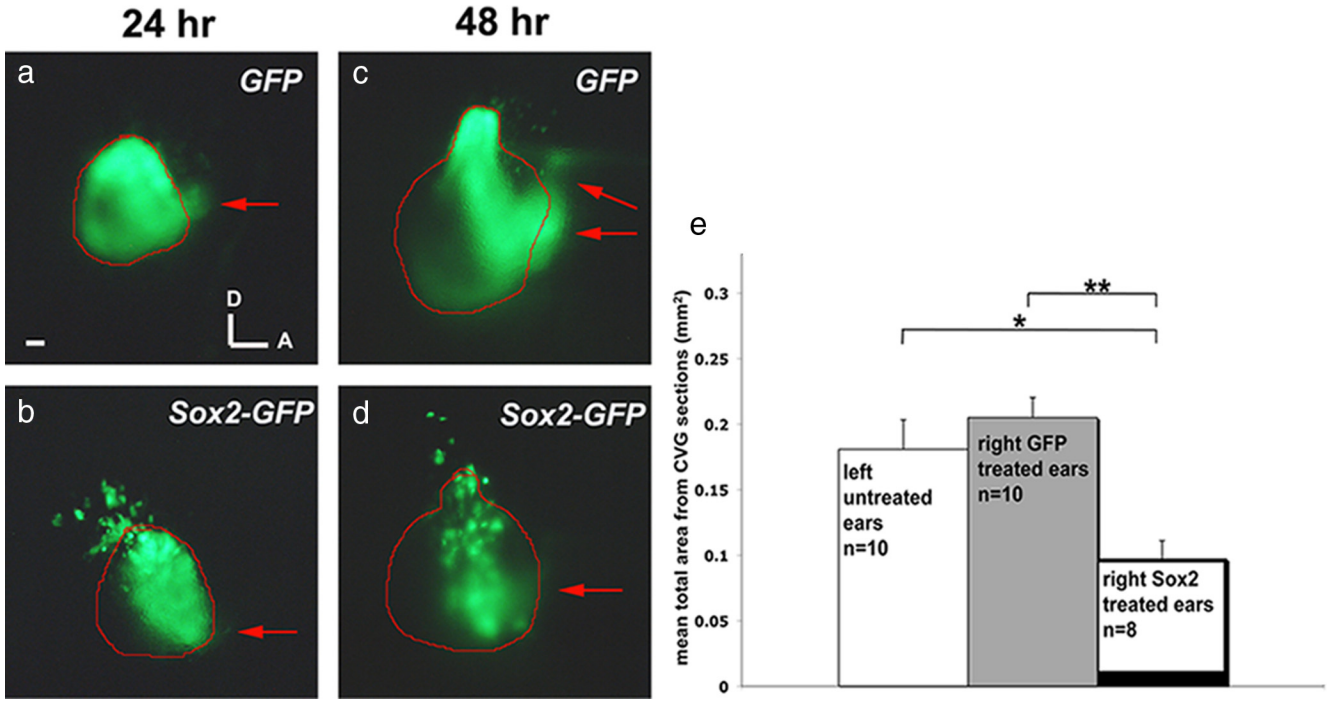


**Figure 2.** Overexpression of *Sox2* leads to a decrease in the size of CVG. ***a–d***, Otocysts at 24 h (***a***, ***b***) and 48 h (***c***, ***d***) after electroporation with *GFP* (***a***, ***c***) or *Sox2-GFP* (***b***, ***d***) between 10 and 17 somites stage. Brightfield images were used to outline the otocysts, shown in red, and red arrows point to the delaminated neuroblasts. There are more delaminated neuroblasts in *GFP* controls than *Sox2-GFP* ears at both time points. ***e***, Quantitative analysis of the size of CVG (see Materials and Methods) indicates that the average size of CVG in *GFP*-treated ears is not significantly different from nonelectroporated control ears, whereas CVGs of *Sox2*-treated ears are ~50% smaller than nonelectroporated and *GFP*-treated ears. Student's *t* test, $*p = 0.0068$; $**p = 0.000084$; error bars represent SEM. Scale bar, 100 $\mu$m.

We tested this hypothesis by electroporating *Sox2-IRES-GFP* (referred as *Sox2-GFP*) or control *IRES-GFP* (referred as *GFP*) plasmid into the otic cup and analyzing the ears 24 and 48 h later. *Sox2-GFP*-treated ears appear to have a smaller CVG (Fig. 2*b*,*d*). Some of the 48 h specimens were sectioned, processed for *in situ* hybridization of *Neurod1* transcripts, and used to quantify the size of the otocyst and ganglion (see Materials and Methods). Although there is no difference in the size of either the otocyst or the CVG between *GFP* electroporated and nonelectroporated ears, electroporation with *Sox2-GFP* resulted in ~50% reduction in the size of CVG, compared to controls (Fig. 2*e*). These results support the hypothesis that overexpression of *Sox2* inhibits neuronal formation.

In both chicken neural tube and *Xenopus* retina, overexpression of *Sox2* inhibits neurogenesis, whereas proneural gene expression either remains unchanged or is downregulated in these tissues (Bylund et al., 2003; Van Raay et al., 2005). In contrast, *Sox2-GFP*-treated ears show ectopic *Ngn1* expression 15–20 h after electroporation (Fig. 3*a–d*). Ectopic *Ngn1* expression is observed outside of the NSD as well (Fig. 3*d*, white bracket). Double-labeling of *Sox2-GFP* and control ear sections with anti-GFP immunostaining and *Ngn1 in situ* hybridization indicates that there is a good correlation between GFP expression and *Ngn1* upregulation in *Sox2-GFP* samples (Fig. 3*k–l′*, arrowheads), whereas no upregulation of *Ngn1* is observed in GFP controls (Fig. 3*i–j′*, arrowheads). These results suggest a possible cell-autonomous induction of *Ngn1* by Sox2. Furthermore, *Ngn1* induction is rapid and is detectable within 6–7 h after *Sox2* electroporation (Fig. 3*m–p*), consistent with the notion that Sox2 induces *Ngn1* in a cell-autonomous fashion. Notably, despite the upregulation of *Ngn1*, there is no concomitant upregulation of *Neurod1* in the *Sox2*-treated ears at 15–20 h or later time-points after electroporation (Fig. 3*e–h*; data not shown), suggesting that neurogenesis does not progress in these cells. This result is consistent with the observation of reduced CVG size (Fig. 2).

### Ngn1 is sufficient to promote neurogenesis and upregulate *Neurod1*

Despite the observed upregulation of *Ngn1* in Sox2-treated ears, neurogenesis fails to proceed. These results seem contradictory, because Ngn is a potent inducer of neurogenesis in other neural systems (Ma et al., 1998; Bylund et al., 2003). Therefore, we investigated whether *Ngn1* is equally potent in inducing neurogenesis in ear tissues. Electroporation experiments conducted with *Ngn1-IRES-GFP* (referred as *Ngn1-GFP*) show broad ectopic cell delamination from the entire otocyst within 24 h (Fig. 4*c*), compared to controls (Fig. 4*a*,*b*). Delamination is more evident at 48 h, to the extent that the otocysts are malformed and smaller in size (Fig. 4*d*, white arrows, *g–h″*, white arrowheads). Similar to control neuroblasts (Fig. 4*e–f′*, red arrowhead), these delaminated cells express *Neurod1* (Fig. 4*g–h′*, white arrowheads). The ectopic expression of *Neurod1* and the subsequent delamination of these cells could be a result of an expanded NSD. However, there is no obvious expansion of the *Lfng* expression domain (Fig. 4*g″*,*h″*) ($n = 15$) compared with controls (Fig. 4*e″*,*f″*) as would be expected if this were the case. Because a majority of the GFP-positive cells in the *Ngn1-GFP* specimens appear to have delaminated from the otic epithelium already by 48 h (Fig. 4*g*,*h*), we harvested some specimens 15 h after electroporation and confirmed the presence of ectopic *Neurod1*-positive cells within the otic epithelium at this earlier time point (Fig. 4*i–j′*). This ectopic *Neurod1* expression pattern was not observed in *Sox2-GFP* specimens (Fig. 3*e–h*). Together, these results indicate that unlike

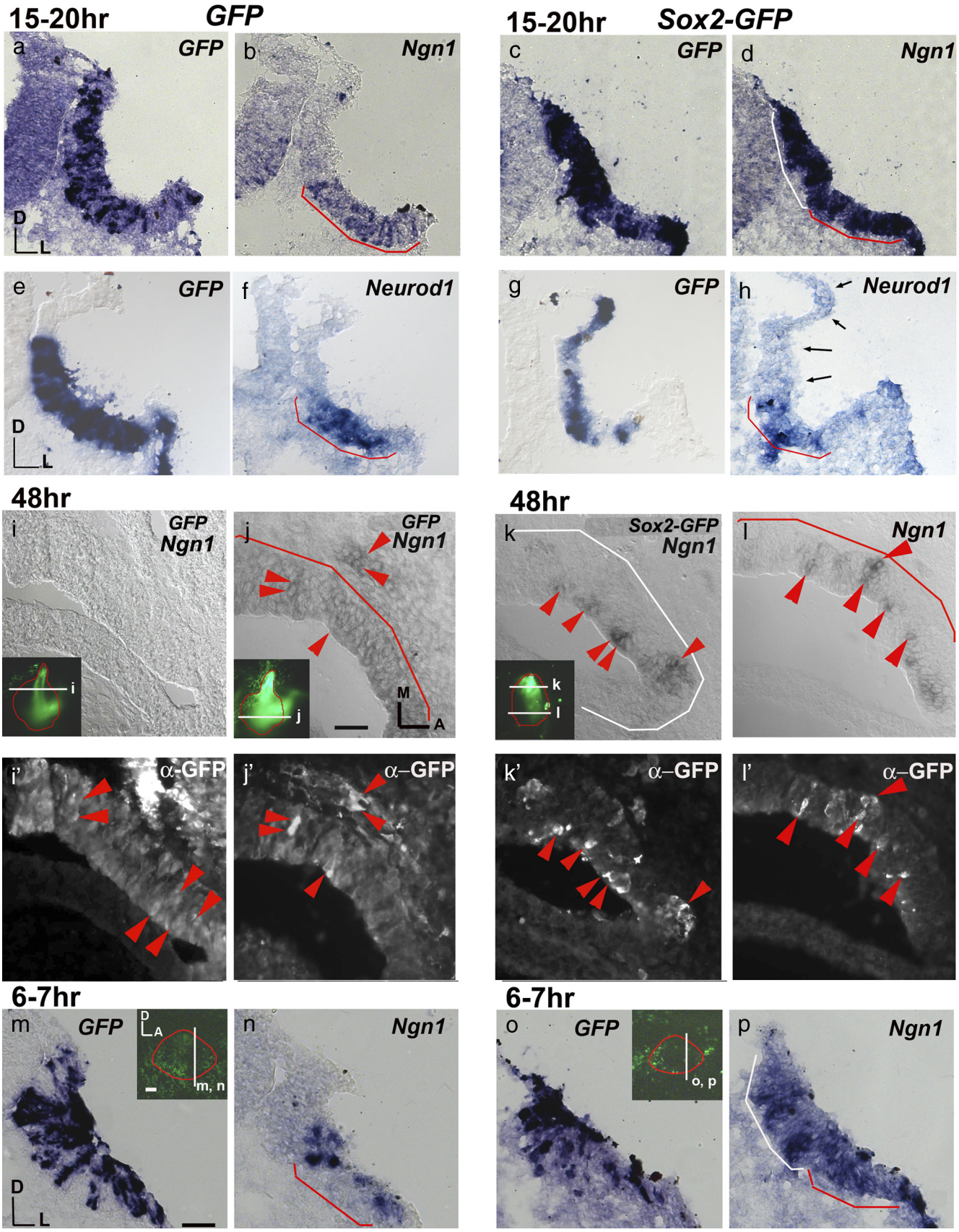


**Figure 3.** *Sox2* upregulates *Ngn1* expression but not *Neurod1*. ***a–h***, *GFP* control (***a***, ***b***, ***e***, ***f***) and *Sox2-GFP*-treated (***c***, ***d***, ***g***, ***h***) otocysts 15–20 h after electroporation probed for *GFP* (***a***, ***c***, ***e***, ***g***), *Ngn1* (***b***, ***d***) and *Neurod1* (***f***, ***h***) transcripts. In the *GFP* control ears, *Ngn1* (***b***) and *Neurod1* (***f***) are expressed only in the NSD (red bracket) but not in the region dorsal to the NSD ($n = 7/7$). ***c***, ***d***, ***g***, ***h***, In *Sox2-GFP*-treated ears, *Ngn1* is strongly expressed in both the NSD (***d***, red bracket) and in a region of ectopic expression dorsal to the NSD (white bracket; $n = 6/6$), but no such upregulation is observed for *Neurod1* (***h***, arrows; $n = 7/7$). ***i–l'***, Sections of *GFP* controls (***i–j'***) and *Sox2-GFP* (***k–l'***)-treated ears double-labeled for ectopic *Ngn1* transcripts and anti-GFP (*Figure legend continues.*)

*Sox2-GFP*, overexpressing *Ngn1* is sufficient to upregulate *Neurod1* and induce ectopic neurogenesis in the inner ear.

### Overexpressing *Ngn1* leads to downregulation of endogenous Sox2

In the chicken neural tube, overexpression of *Ngn2* leads to the downregulation of *Sox2* (Bylund et al., 2003). To determine whether Ngn1 inhibits Sox2 expression in the inner ear, we analyzed immunostaining of Sox2 in *Ngn1-GFP*-treated ears 15 h after electroporation. Sox2 immunostaining is significantly reduced in *Ngn1-GFP* ears (Fig. 5*c–d′*, asterisk) compared to *GFP* control ears (Fig. 5*a*,*b′*). Thus, whereas ectopic *Ngn1* drives neuroblast formation, Sox2 expression is concomitantly downregulated. These results are consistent with the observation that the endogenous *Sox2* transcripts are reduced in the CVG (Fig. 1*b*,*d*) and suggest that *Sox2* downregulation by Ngn1 is required for neurogenesis to proceed normally.

### *Nop-1* regulatory element of *Sox2* is inhibited by Ngn1

The tissue-specific expression of *Sox2* is mediated by many *cis*-regulatory elements including *Nop-1*, which confers expression in the developing chicken inner ear (Uchikawa et al., 2003). This *Nop-1* enhancer has a repressive domain (Sugahara et al., unpublished results). This repressive domain contains an E-box (*CANNTG*) consensus sequence motif (Blackwell et al., 1993; Bertrand et al., 2002; Seo et al., 2007), which can be bound by E-proteins in complex with proneural bHLH transcription factors, such as Ngn1 or Neurod1 (Markus et al., 2002). To test whether Ngn1 can directly interact with *Nop-1* to inhibit *Sox2* transcription, a *Nop-1* enhancer driven *EGFP* (*Nop1-GFP*) was electroporated into the otic cup in the presence of *Ngn1-DsRed* or *DsRed* alone and analyzed 15 h later (Fig. 6). Based on the total number of GFP and DsRed coexpressing cells, the *Nop1-GFP* enhancer activity is significantly reduced in the presence of *Ngn1-DsRed* compared to *DsRed* controls (Fig. 6*a–c*). In contrast, the number of double-labeled cells is not significantly decreased when a plasmid with a mutated E-box in *Nop-1* (*Nop1-EboxMut-GFP*) was used (Fig. 6*d–f*). These findings indicate that Ngn1 inhibits *Nop-1* activity and it may inhibit *Sox2* transcription *in vivo* by binding to the E-box of *Nop-1*.

### Neurod1 is sufficient to promote neurogenesis and inhibit *Nop-1* activity

*Neurod1*, which is activated by Ngn1 (Ma et al., 1996), is required for the formation of the CVG (Liu et al., 2000). To test whether *Neurod1* induces neurogenesis similar to *Ngn1*, *Neurod1-IRES-RFP* (*Neurod1-RFP*), or *IRES-RFP* (*RFP*) were electroporated at the otic cup stage and harvested 48 h later (Fig. 7*a–f″*). *Neurod1-RFP* ears show ectopic neuroblast delamination that is positive for a neuronal marker, TuJ1 (Fig. 7*e*,*e′*,*f*,*f′*) compared with *RFP* controls (Fig. 7*b–c″*). Therefore, expression of *Neurod1* is also sufficient to mediate neurogenesis in the developing inner ear.

In addition, we tested whether Neurod1 is also capable of inhibiting *Nop-1* activity. *Nop1-GFP* and *Nop1-EboxMut-GFP* plasmids were coelectroporated with *Neurod1-RFP* (Fig. 7*g–l*). The *Nop1-GFP* enhancer activity is significantly reduced in the presence of *Neurod1-RFP* compared with controls (Fig. 7*i*) based on the reduced number of colabeled cells. However, the number of colabeled cells is not significantly different between *Neurod1-RFP* and *RFP* controls when *Nop1-EboxMut-GFP* was used (Fig. 7*l*). These findings suggest that Neurod1 is capable of inhibiting *Nop-1* activity in a similar manner as Ngn1.

### *Nop-1* activity is inhibited within the NSD

Next, to determine whether inhibition of *Sox2* transcription through *Nop-1* is biologically relevant, we tested whether the presence of endogenous proneural bHLH factors within the NSD could alter *Nop1-GFP* expression. We electroporated specimens with *Nop1-GFP* and *RFP* and adjacent sections were processed for immunostaining and *Ngn1 in situ* hybridization. Using the *Ngn1* expression domain to demarcate the NSD, colabeled cells inside and outside of the NSD were counted (Fig. 8). The number of colabeled cells in the NSD (Fig. 8*b″*, arrows, *i*) was significantly reduced compared with colabeled cells outside of the NSD (Fig. 8*b′*,*d″*, arrowheads), suggesting that the *Nop1-GFP* enhancer activity is inhibited in the NSD. This reduction in the number of colabeled cells was not observed when the *Nop1-EboxMut-GFP* plasmid was used (Fig. 8*e–h″*, arrowheads). Consistently, the proportion of double-labeled cells outside the NSD is not significantly different between *Nop1-GFP* and *Nop1-EboxMut-GFP* specimens (Fig. 8*j*). Together, these results suggest that *Nop-1* activity can be inhibited by some endogenous factors within the NSD. Although these endogenous factors have not been identified, taking into consideration the known functions of Ngn1 and Neurod1 in the CNS, they are the most likely candidates expressed in the NSD that interact with the E-box binding site in *Nop-1*.

## Discussion

### The role of Sox2 in mediating neural competence

Increasing evidence suggests that neural development from progenitor state to the formation of neuronal subtypes is a tightly regulated cascade of molecular events. The factors that define neural competency and the roles of Sox2 in this process are not clear. Sox2 may mediate neural competency by facilitating the initiation of neurogenesis. Evidence for Sox2's role in inner ear neurogenesis first came from analyses of the mouse mutants, *ysb* and *lcc*, in which *Sox2* expression in the inner ear is reduced or absent, respectively (Kiernan et al., 2005; Puligilla et al., 2010). Neuronal formation is affected in the *lcc* mutants even though the underlying molecular mechanisms have not been thoroughly investigated (Puligilla et al., 2010). Ectopic neuronal formation in cochlear explants by *Sox2* is additional supporting evidence for a role of Sox2 in inner ear neurogenesis (Puligilla et al., 2010). Our results demonstrate a rapid induction of *Ngn1* by *Sox2*, suggesting a direct, cell-autonomous action via transcriptional regulation. In support of this notion, Sox2 has been shown to upregulate *Ngn1* expression in a cochlear cell line and recently in chicken otocysts (Jeon et al., 2011; Neves et al., 2012), and a Sox2 binding site has been identified in the 5′ promoter region of *Ngn1* (Jeon et al., 2011). Similarly, a Sox2 binding site has also been identified in the *cis*-regulatory element of *Ngn1* in zebrafish embryos using chromatin immunoprecipitation (Okuda et al.,

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(*Figure legend continued.*) antibody 48 h after electroporation. *GFP* control ears show GFP immunostaining in the NSD with no ectopic *Ngn1* expression (***j***,***j′***, red arrowheads). No *Ngn1* transcript labeling is evident in dorsal sections of *GFP* controls (***i***), despite the presence of GFP immunostaining (***i′***). In *Sox2-GFP* ears, double-labeled cells (red arrowheads) are present in the dorsal non-NSD (***k***, ***k′***) and ventral NSD region (***l***, ***l′***) ($n = 4/4$). ***m–p***, Otic cups electroporated with *GFP* control (***m***, ***n***) or *Sox2-GFP* (***o***, ***p***) and harvested after 6 to7 h. Adjacent sections were probed for *GFP* (***m***, ***o***) and *Ngn1* (***n***, ***p***). *Ngn1* expression is upregulated in the NSD (***p***, red bracket) as well as in regions outside the NSD (***p***, white bracket) of *Sox2*-treated ears ($n = 7/7$), compared with *GFP* control (***n***, NSD red bracket) ($n = 7/7$). Ears are outlined in red, and the level of section is indicated with white lines (insets). Scale bars, 100 $\mu$m.

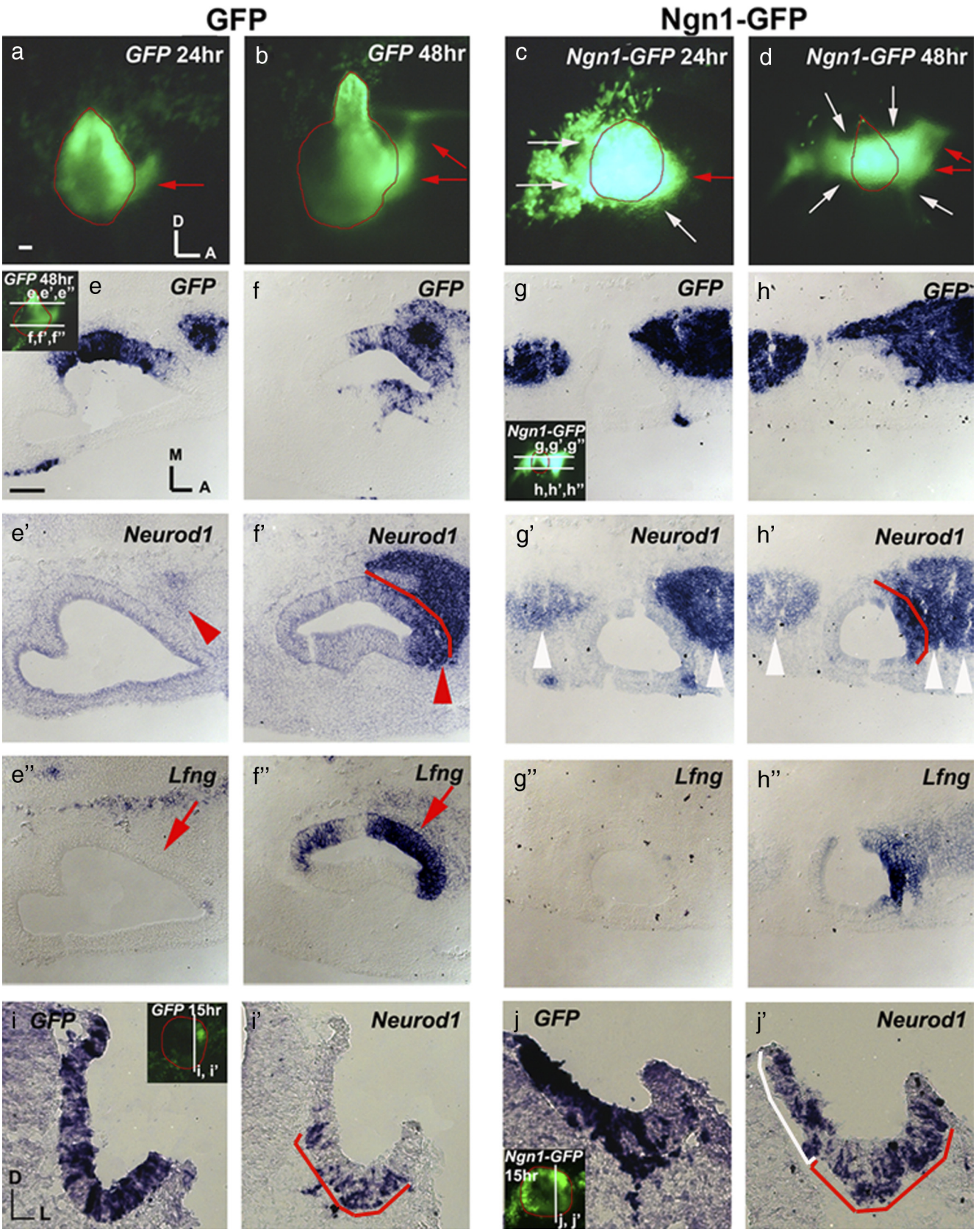



**Figure 4.** Ngn1 is sufficient to promote neurogenesis and upregulate *Neurod1*. ***a–d***, Otic cup electroporated with *GFP* (***a***, ***b***) or *Ngn1-GFP* (***c***, ***d***) and harvested after 24 h (***a***, ***c***) and 48 h (***b***, ***d***). The outline of the otocyst is indicated in red. Red arrows point to the presumably delaminated neuroblasts. Neuroblasts delaminate from the NSD (***c***, ***d***, red arrows) as well as ectopically from other regions of the otocyst in *Ngn1-GFP* ears (***c***, ***d***, white arrows) compared with *GFP* ears (***a***, ***b***). ***e–h***″, Adjacent tissue sections probed for *GFP*, *Neurod1*, and *Lfng* transcripts in *GFP* control (***e–f***″) and *Ngn1-GFP* (***g–h***″)-treated ears 48 h after electroporation. In the *GFP* control ears, *Neurod1* is expressed only in the CVG located anteromedial to the otocyst (***e***′, ***f***′, red arrowheads) and the *Lfng*-positive NSD in the ventral otocyst (***f***′, ***f***″, arrow), but not dorsal to the NSD (***e***′, ***e***″, arrow), which is *Lfng* negative ($n = 10/10$). In *Ngn1-GFP* ears (***g***, ***h***″), (*Figure legend continues*.)

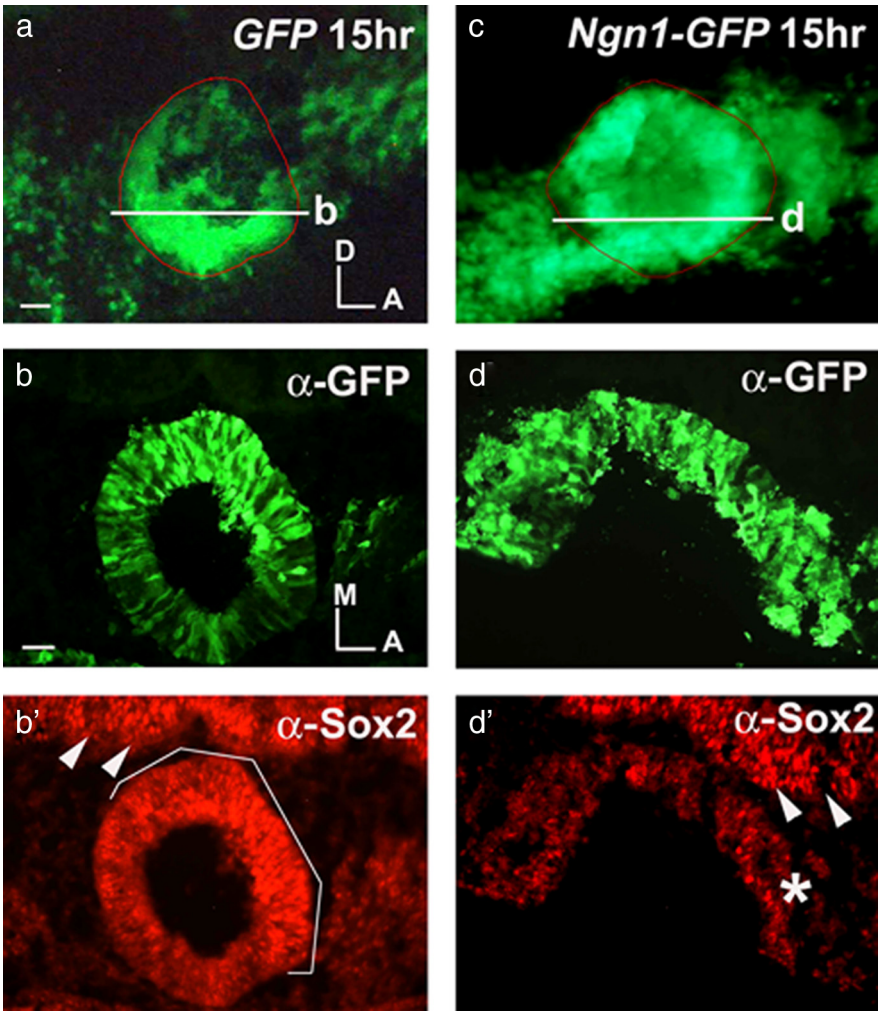



**Figure 5.** Overexpressing *Ngn1* leads to downregulation of Sox2. ***a***, ***c***, Otic cups were electroporated with *GFP* (***a***) and *Ngn1-GFP* (***c***) and harvested at 15 h. Ears shown in ***a*** and ***c*** were sectioned (***b*** and ***d***, respectively) and double-labeled with anti-GFP (***b***, ***d***) and anti-Sox2 (***b***, ***d′***) antibodies. ***b***, ***b′***, Sox2 immunostaining in the NSD of *GFP*-treated ears (***b′***, white bracket) ($n = 4/4$) indicates that some of the cells are positive for both Sox2 and GFP. ***d′***, The GFP-positive region in *Ngn1-GFP* ears shows reduced Sox2 immunostaining (asterisk) ($n = 4/5$). In contrast, staining in the neural tube is similar in-treated and control samples (***b′***, ***d′***, white arrowheads). *Ngn1-GFP* electroporated ears often show a delay in otic cup closure (***c–d′***). The ears are outlined in red (***a***, ***c***), and the white lines in ***a*** and ***c*** indicate the level of section in ***b*** and ***d***, respectively. Scale bars, 100 $\mu$m.

2010). Therefore, the binding of Sox2 to a regulatory element of the *Ngn1* promoter may be one of the requirements for acquiring neural competence.

Thus far, there is no direct evidence that Sox2 induces proneural genes in other neural systems. Nevertheless, data from several studies place Sox2 upstream of proneural genes. For example, Sox2 is thought to function downstream from Frizzled 5 but upstream of the proneural gene, *Xash3,* in mediating retina formation in *Xenopus* (Van Raay et al., 2005). In addition, *Sox3*, another SoxB1 group family member, is postulated to be downstream of Lef1 in inducing the proneural gene, *Zash1a*, in the developing zebrafish hypothalamus (Lee et al., 2006). In both cases, overexpression of *Sox2* or *Sox3* rescues the neural phenotype caused by knockdown of *Frizzled 5* and *Lef1*, respectively (Van Raay et al., 2005; Lee et al., 2006). The lack of a direct causal relationship between overexpressing Sox and induction of proneural genes in these systems (in contrast to the inner ear results shown here) could be due to the cellular context of the tissue at the time of the experiment. It is clear that the onset of proneural gene expression is highly regulated by multiple activators and repressors. The presence of repressors may mask the positive effects of Sox2 on proneural gene expression. For example, the Notch signaling pathway, which is thought to be active at the same developmental time as Sox2, has been shown to inhibit *Ngn2* expression in the chicken neural tube by upregulating *Hes1* and *Hes5* (Holmberg et al., 2008). Therefore, Notch and Sox proteins may function in an antagonistic manner to coordinate the timing of neural progression. Furthermore, if proneural genes are already activated *in vivo* at the time of the experiment, overexpression of Sox2 would inhibit rather than promotes expression of proneural/neuronal genes (Agathocleous et al., 2009). These results are also consistent with our finding that neural progression is inhibited by overexpression of *Sox2*.

Although we demonstrate that the cellular context of the otic cup is conducive to *Ngn1* induction by exogenous Sox2, it is unlikely to be the sole factor in inducing *Ngn1 in vivo* (Fig. 9). It has been shown recently that *Ngn1* and *Neurod1* transcripts are downregulated in *Eya1*- or *Six1*-null inner ears, and overexpression of both *Eya1* and *Six1*, but not either factor alone, is able to induce neurogenesis in mouse embryos *in vitro* (Ahmed et al., 2012b). Sox2, which is known to partner with multiple proteins and transcription factors (Wilson and Koopman, 2002; Uchikawa et al., 2011), has also been shown to physically interact with Eya1 in several embryonic cell lines (Zou et al., 2008). Therefore, it is possible that Sox2 interacts with factors such as Eya1 and Six1 in regulating *Ngn1* expression. In support of this notion, Sox2 has been recently shown to interact with Eya1 and Six1 and activate another proneural gene, *Atoh1*, in cochlear cultures (Ahmed et al., 2012a).

### Neurogenesis proceeds only after *Sox2* is downregulated

Overexpression of *Ngn1* is sufficient to initiate neurogenesis. However, despite the fact that overexpressing *Sox2* induces *Ngn1* in the otocyst, *Neurod1* expression and neuroblast delamination from the otic epithelium are inhibited. We attribute the lack of *Neurod1* upregulation in *Sox2*-treated specimens to high levels of *Sox2* driven by the *β-actin* promoter, which cannot be effectively inhibited by Ngn1. This result is also consistent with previous findings that Sox2 downregulation is required for neurogenesis

←

(*Figure legend continued.*) *Neurod1*-positive neuroblasts are detected both anterior and posterior to the otocyst in both dorsal (***g′***) and ventral (***h′***) sections (white arrowheads). However, *Neurod1* expression in the otic epithelium is within the *Lfng*-positive NSD (***h′***, ***h″***) ($n = 4/4$), similar to controls (***f***, ***f′***). ***i–j′***, Adjacent tissue sections probed for *GFP* and *Neurod1* transcripts in *GFP*- (***i***, ***i′***) and *Ngn1-GFP*- (***j***, ***j′***) treated ears 15 h after electroporation. *Neurod1* expression is upregulated in the ear epithelium in *Ngn1-GFP*-treated ears (***j′***, white bracket) ($n = 6/8$) but not in the *GFP* control (***i′***) ($n = 6/6$). Red brackets outline the NSD. Scale bars, 100 $\mu$m.

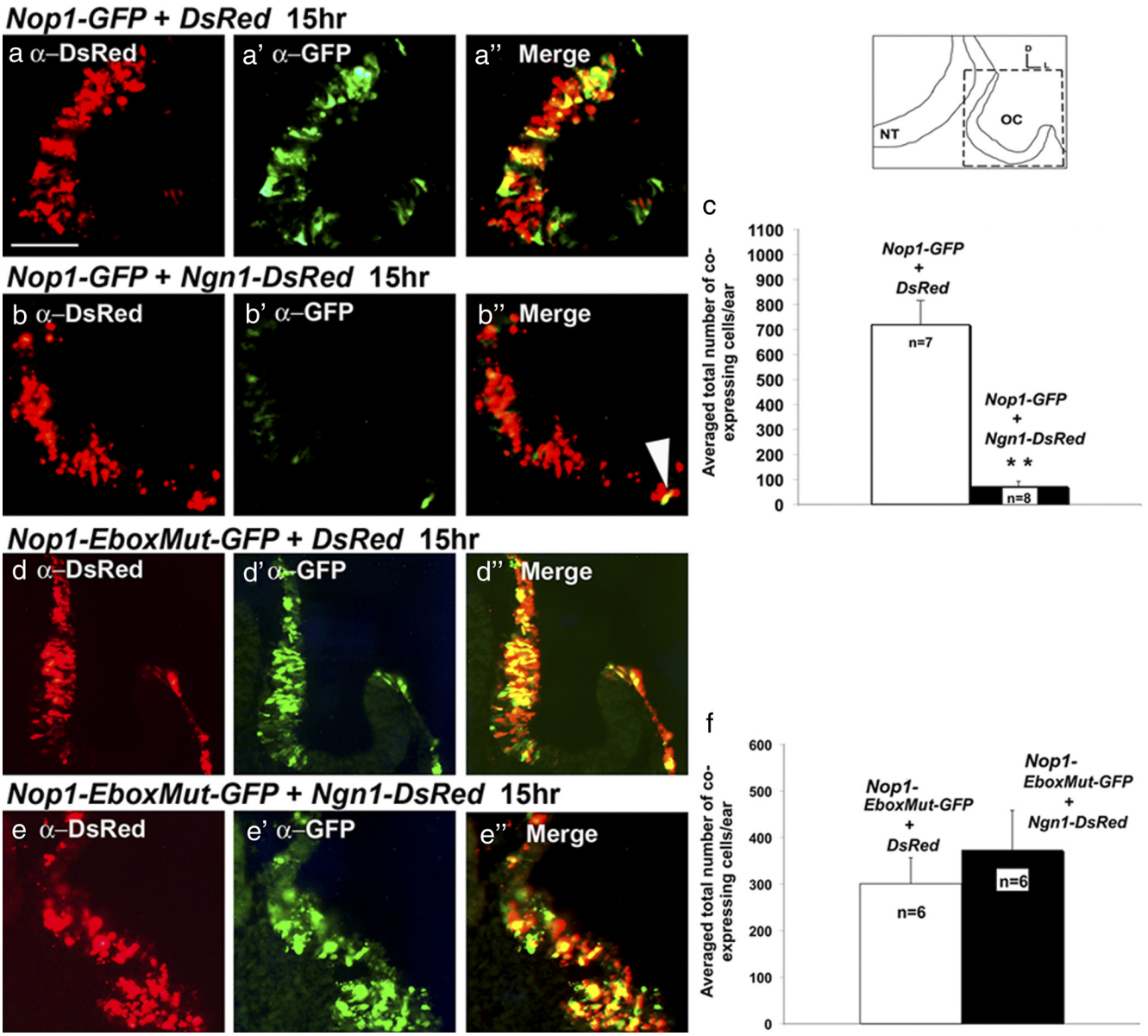



**Figure 6.** Ngn1 inhibits *Nop-1* activity. ***a–b″***, *Nop1-GFP* was co-electroporated with *DsRed* (***a–a″***) or *Ngn1-DsRed* (***b–b″***). Fifteen hours after electroporation, ears were harvested, sectioned, and double-labeled with anti-GFP and anti-DsRed antibodies, and counterstained with DAPI (data not shown). Images were merged. Whereas many cells in the control samples coelectroporated with *DsRed* (***a″***) are double-labeled, few double-labeled cells are present in ears that were coelectroporated with *Ngn1-DsRed* (***b″***, arrowhead). ***c***, Quantification of GFP and DsRed colabeled cells indicates that the number of colabeled cells is significantly reduced in the presence of *Ngn1-DsRed* compared to *DsRed* control ($p = 0.00036$). ***d–e″***, *Nop1-EboxMut-GFP* was coelectroporated with *DsRed* (***d–d″***) or *Ngn1-DsRed* (***e–e″***). In contrast, the number of colabeled cells of *Nop1-EboxMut-GFP* is not significantly affected in the *Ngn1-DsRed* ears compared with control ears (***f***) ($p = 0.49$). Error bars represent SEM. Orientation of sections is indicated in the schematic. NT, Neural tube; OC, otic cup. Scale bar, 100 $\mu$m.

to proceed (Bylund et al., 2003; Bani-Yaghoub et al., 2006). Furthermore, we provide the first evidence that both Ngn1 and Neurod1 can inhibit Sox2 at the transcriptional level. Ngn1 and Neurod1 function via interacting with the *Nop-1* enhancer, which is conserved between chickens and mammals (Uchikawa et al., 2003). Moreover, our results indicate that a key step of neural progression is the induction of *Neurod1*, which requires Ngn1, as well as a reduced level of Sox2. This notion is consistent with the reduced *Sox2* hybridization signals in the CVG compared with the NSD (Fig. 1).

Notably, in neural stem cells of the adult mouse hippocampus, Sox2 functions as a repressor of *Neurod1,* and the removal of Sox2 is required before Wnt-signaling can activate *Neurod1* (Kuwabara et al., 2009). Based on these results, we postulate that a key component of neural progression is the transcriptional repression of *Sox2,* which in turn leads to the alleviation of repression on *Neurod1*. Although both Ngn1 and Neurod1 are capable of engaging in an inhibitory feedback loop of *Sox2* transcription, the earlier onset of *Ngn1* expression relative to that of *Neurod1* suggests that Ngn1 is a more important factor in repressing Sox2 activity. This inhibitory action on *Sox2* does not necessarily have to be mediated through binding to *Nop-1* alone.

In addition to the provided evidence that high levels of exogenous Sox2 block progression of neurogenesis cell-autonomously, there is likely to be a non-cell-autonomous contribution to the reduction in the size of the CVG in *Sox2* overexpressed specimens. Ngn1 is thought to positively regulate *Delta1* expression, which activates the Notch signaling pathway in neighboring cells to inhibit

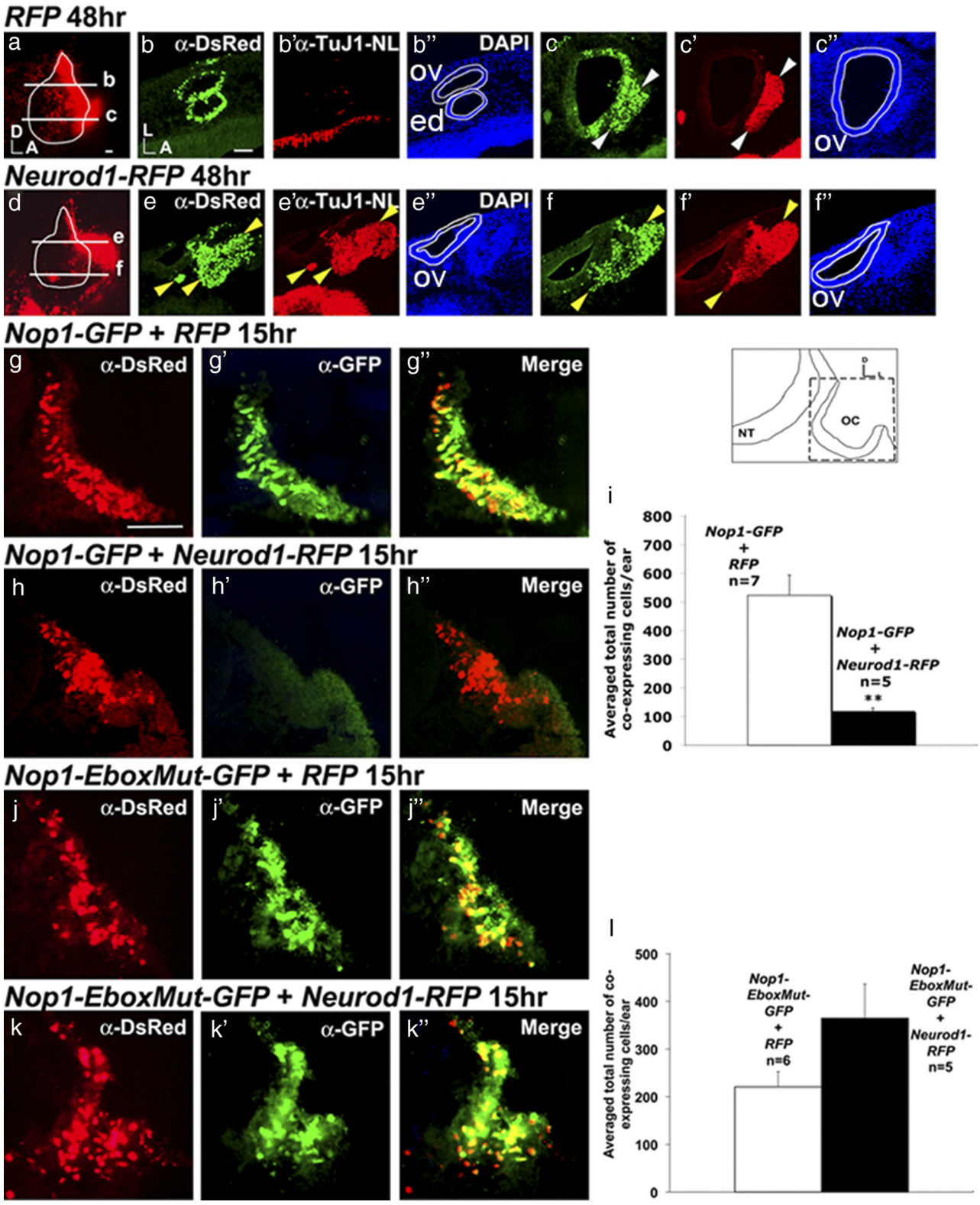
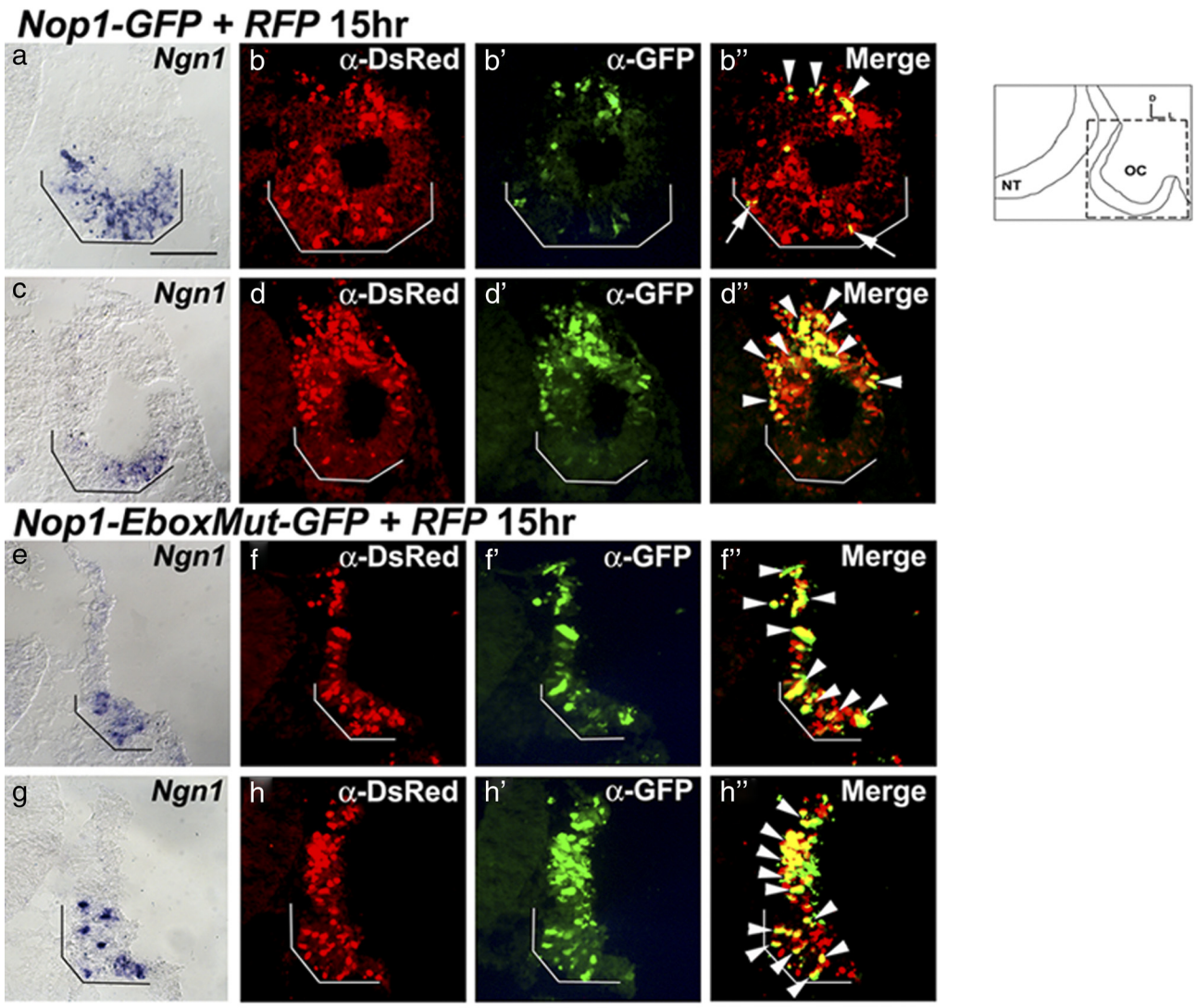



**Figure 7.** Neurod1 is sufficient to promote neurogenesis and inhibits *Nop-1* activity. ***a***, ***d***, Otocysts electroporated with *RFP* (***a***) or *Neurod1-RFP* (***d***) at 48 h after electroporation. Ears are outlined and levels of sections are indicated in white. ***b–c″***, Ear sections were double-labeled with anti-DsRed (***b***, ***c***) and anti-TuJ1 (***b′***, ***c′***, and counterstained with DAPI (***b″***, ***c″***). Only ventral sections of *RFP* controls ($n = 4/4$) show delaminated DsRed-positive and Tuj1-positive neuroblasts (***c–c″***). ***e–f″***, In *Neurod1-RFP*-treated ears, double-labeled anti-DsRed and anti-TuJ1-positive neuroblast delamination is observed in both dorsal (***e***, ***e′***, yellow arrowheads) and ventral sections (***f***, ***f′***, yellow arrowheads) ($n = 3/3$). ***g–h″***, *Nop1-GFP* coelectroporated with *RFP* control (***g–g″***) or *Neurod1-RFP* (***h–h″***). Embryos were harvested 15 h after electroporation, sectioned, double-labeled with anti-GFP and anti-DsRed, and counterstained with DAPI (data not shown). (***g″***) and (***h″***) are merged images. ***i***, Quantification of the number of GFP and DsRed colabeled cells. The number of double-labeled cells is significantly reduced in the presence of *Neurod1-RFP* compared to controls (**$p = 0.0013$). ***j–k″***, *Nop1-EboxMut-GFP* is co-electroporated with RFP (***j***) or *Neurod1-RFP* (***k***). ***l***, The number of colabeled cells is not significantly affected by *Neurod1-RFP* compared with controls ($p = 0.12$). Error bars represent SEM. Orientation of the sections is indicated in the schematic. NT, Neural tube; OC, otic cup; ov, otic vesicle; ed, endolymphatic duct. Scale bars, 100 $\mu$m.




i

| Totals within NSD | Co-expressing cells | Single-labeled cells (GFP or DsRed) | Total # of labeled cells |
|---|---|---|---|
| Nop1GFP + RFP Observed n=5 | 1,548 *** | 10,549 | 12,097 |
| Nop1EboxMutGFP + RFP Expected n=5 | 7,277 | 5,919 | 13,196 |

j

| Totals outside NSD | Co-expressing cells | Single-labeled cells (GFP or DsRed) | Total # of labeled cells |
|---|---|---|---|
| Nop1GFP + RFP Observed n=4 | 5,152 | 4,467 | 9,619 |
| Nop1EboxMutGFP + RFP Expected n=4 | 5,626 | 4,632 | 10,258 |

**Figure 8.** The activity of *Nop-1* is inhibited within the endogenous *Ngn1* and *Neurod1*-positive NSD. ***a–h″***, Ears coelectroporated with *Nop1-GFP* and *RFP* (***a–d″***) or *Nop1-EboxMut-GFP* and *RFP* as controls (***e–h″***) 15 h after electroporation. Adjacent sections probed for either *Ngn1* transcripts (***a***, ***c***, ***e***, ***g***; serving as an indicator for the NSD, black brackets) or double-labeled with anti-DsRed and anti-GFP (***b–b″***, ***d–d″***, ***f–f′***, ***h–h″***) and counterstained with DAPI (data not shown). ***b″***, ***d″***, ***f″***, and ***h″*** are merged images. ***b″***, ***d″***, Doubled-labeled cells are present outside of the NSD (arrowheads), but fewer are present within the NSD (***b″*** white bracket and arrows) of *Nop-1*-treated ears. In contrast, ears electroporated with the *Nop1-EboxMut-GFP* plasmid show double-labeled cells outside (***f–f″***, ***h–h″***, arrowheads) as well as inside the NSD (***f–f″***, ***h–h″***, arrowheads within white brackets). ***i***, ***j***, Quantification of GFP and DsRed double-labeled cells within (***i***) and outside (***j***) the NSD using $\chi^2$ tests. The data indicate that the number of colabeled cells in *Nop1-GFP* ears is significantly reduced within the NSD compared with *Nop1-EboxMut-GFP* ears ($p < 0.0001$; $\chi^2 = 4982.53$), whereas no significant difference was observed between the two treatments outside the NSD ($p = 0.0693$; $\chi^2 = 3.299$). The level of sections is indicated in the schematic. NT, Neural tube; OC, otic cup. Scale bar, 100 $\mu$m.

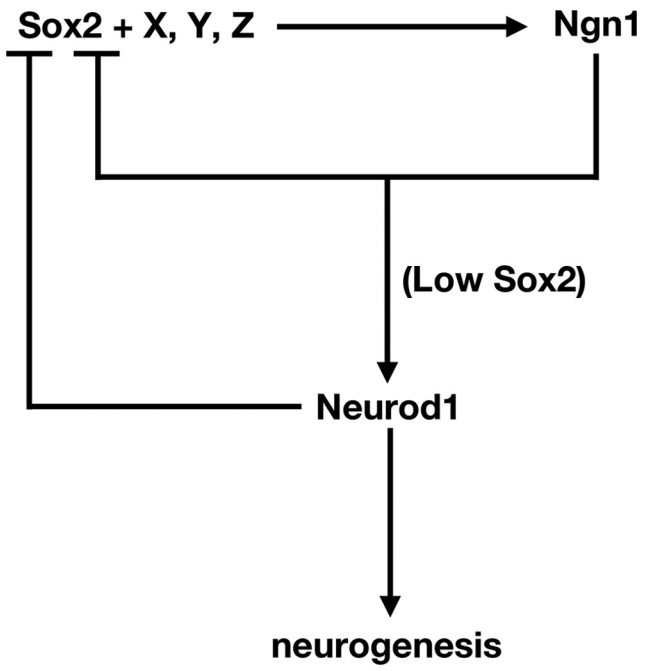



**Figure 9.** A model showing Sox2 and other unknown factors (X–Z) initiate neurogenesis by upregulating *Ngn1*. Ngn1 inhibits *Sox2* transcription to upregulate *Neurod1* and promote progression of neurogenesis. Neurod1 further inhibits *Sox2* transcription and mediates neuronal differentiation.

the neural fate (Ma et al., 1996; Ma et al., 1998, Brooker et al., 2006). Our preliminary results indicate that *Delta1* expression is also upregulated in the ectopic *Sox2* specimens, suggesting that lateral inhibition of the neural fate via Delta-Notch signaling could contribute to the reduced CVG size as well.

### Sox2, Ngn1, and Neurod1 form a regulatory network within the NSD

The ability of Ngn1, Neurod1, and Sox2 (to some extent) to drive neural fates outside the NSD indicates that these genes can override extrinsic signaling by factors, such as Wnts, Sonic hedgehog, and retinoic acid that normally interact to limit the NSD to the anteroventral region of the otic cup (Riccomagno et al., 2005; Bok et al., 2007, 2011). Given the well established relationship between Sox and the Wnt signaling pathway in other systems (Agathocleous et al., 2009; Kormish et al., 2010) and the importance of Wnt signaling in dorsal-ventral patterning of the inner ear (Riccomagno et al., 2005), it is likely that exogenous Sox2 is overriding the Wnt signaling that normally restricts the neural sensory competent region to the ventral otic cup and otocyst. This hypothesis warrants further investigation.

In conclusion, we have identified a molecular mechanism whereby Sox2 mediates neural competency and progression in the inner ear (Fig. 9). Given the similarity in the postulated role of Sox2 between the inner ear and other neural systems (Bylund et al., 2003; Van Raay et al., 2005; Bani-Yaghoub et al., 2006), this mechanism may be generally applicable to other neural tissues. The feedforward and feedback control between Sox2 and proneural genes elucidate a piece of the puzzle in this complex program of neural development.

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