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EYA1 and SIX1 drive the neuronal developmental program in cooperation with the SWI/SNF chromatin-remodeling complex and SOX2 in the mammalian inner ear

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SUMMARY

Inner ear neurogenesis depends upon the function of the proneural basic helix-loop-helix (bHLH) transcription factors NEUROG1 and NEUROD1. However, the transcriptional regulation of these factors is unknown. Here, using loss- and gain-of-function models, we show that EYA1 and SIX1 are crucial otic neuronal determination factors upstream of NEUROG1 and NEUROD1. Overexpression of both *Eya1* and *Six1* is sufficient to convert non-neuronal epithelial cells within the otocyst and cochlea as well as the 3T3 fibroblast cells into neurons. Strikingly, all the ectopic neurons express not only *Neurog1* and *Neurod1* but also mature neuronal markers such as neurofilament, indicating that *Eya1* and *Six1* function upstream of, and in the same pathway as, *Neurog1* and *Neurod1* to not only induce neuronal fate but also regulate their differentiation. We demonstrate that EYA1 and SIX1 interact directly with the SWI/SNF chromatin-remodeling subunits BRG1 and BAF170 to drive neurogenesis cooperatively in 3T3 cells and cochlear nonsensory epithelial cells, and that SOX2 cooperates with these factors to mediate neuronal differentiation. Importantly, we show that the ATPase BRG1 activity is required for not only EYA1- and SIX1-induced ectopic neurogenesis but also normal neurogenesis in the otocyst. These findings indicate that EYA1 and SIX1 are key transcription factors in initiating the neuronal developmental program, probably by recruiting and interacting with the SWI/SNF chromatin-remodeling complex to specifically mediate *Neurog1* and *Neurod1* transcription.

KEY WORDS: EYA1, SIX1, Neurogenesis, SWI/SNF complex, SOX2, Reprogramming, Inner ear, Mouse

INTRODUCTION

Inner ear neurogenesis initiates at approximately embryonic day (E) 9.0 when a subset of otic ectodermal cells that express neural fate markers delaminate from the ectoderm and form the vestibulocochlear (VIIIth) ganglion with neural crest-derived glial precursors (D'Amico-Martel and Noden, 1983). Otic neurogenesis depends on the proneural bHLH factor NEUROG1, which is necessary for the commitment of ectodermal cells to a neural fate; *Neurog1*$^{-/-}$ embryos lack the VIIIth ganglion and fail to express neural fate markers in the otocyst (Ma et al., 2000; Ma et al., 1998). The neuronal differentiation factor NEUROD1 has been suggested to function downstream of NEUROG1 (Ma et al., 1998), and deletion of *Neurod1* also leads to the absence of vestibulocochlear neurons (Kim et al., 2001). Previous gain-of-function studies in *Xenopus* have demonstrated that ectopic expression of *Neurod1* can induce ectopic neurogenesis (Lee et al., 1995). Similarly, ectopic expression of *Neurog1* is sufficient to induce ectopic neurogenesis and *Neurod1* expression (Ma et al., 1996), further indicating that *Neurog1* functions upstream of and in the same pathway as *Neurod1*. By contrast, a recent study has suggested that *Neurog1* and *Neurod1* might not function in the same signaling pathway to regulate neurogenesis in the cochlea, as *Neurog1* can induce neuronal phenotype but fails to induce *Neurod1* expression in the ectopic neurons in cochlear nonsensory epithelial cells (Puligilla et al., 2010). In addition, neither *Neurog1* and *Neurod1* nor combination of both with *Sox2* is sufficient to induce neurofilament (NF)-positive neurons in the cochlea (Puligilla et al., 2010), indicating that other factors are required for neuronal maturation. Thus, it is unclear whether *Neurog1* and *Neurod1* function in the same transcriptional cascade during inner ear neurogenesis. Furthermore, the transcriptional regulation of *Neurog1*- or *Neurod1*-controlled neurogenesis and the factors that are sufficient to induce neuronal differentiation remain poorly understood.

The regulation of chromatin structure is known to be an important level of transcriptional control during neural development (Hong et al., 2005; Hsieh and Gage, 2005; Wu et al., 2007). Studies in *Xenopus* indicated that the SWI/SNF ATP-dependent chromatin-remodeling protein BRG1 is required for neuronal differentiation by mediating the transcriptional activities of NEUROG and NEUROD proteins (Seo et al., 2005). In mammals, the ATPase subunit of the SWI/SNF complex is encoded by two homologs, *Brm* (Brahma; *Smarca2* – Mouse Genome Informatics) and *Brg1* (Brahma-related gene 1; *Smarca4* – Mouse Genome Informatics). The BRG1/BRM-associated-factor (BAF) complex, consisting of 12 protein subunits, is a major type of ATP-dependent chromatin-remodeling complex in vertebrates and is essential for many aspects of mammalian development, including neural development, proliferation, differentiation and tumorigenesis (Ho and Crabtree, 2010). Studies of BAF complexes in mammals indicate that these complexes undergo progressive changes in subunits during transition from a pluripotent stem cell to a multipotent neuronal progenitor to a committed neuron (Ho et al., 2009), and have suggested that BAF subunits might have nonredundant and dosage-sensitive roles in neural development (Bultman et al., 2000; Kim et al., 2001). Several transcription factors have been shown to interact with SWI/SNF complexes and recruit the complexes to specific genes (Chi, 2004; Kadam and Emerson, 2002; Peterson and Workman, 2000).

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Moreover, many genes have been shown to require SWI/SNF complexes for activation in yeast, fruit flies and mammals (Armstrong et al., 2002; Krebs et al., 2000; Liu et al., 2001; Ng et al., 2002; Sudarsanam et al., 2000). Recently, the ATP-dependent chromatin-remodeling enzyme CHD7 has been shown to regulate neurogenesis in the inner ear (Hurd et al., 2010). However, whether the SWI/SNF complexes play a role in mammalian inner ear neurogenesis and whether they interact with other transcription factors to regulate the transcriptional activities of *Neurog1* and *Neurod1* are not understood.

The murine eyes absent (Eya) and homeobox Six gene families, homologous to *Drosophila eyes absent* and *sine oculis*, respectively, play essential roles for inner ear development. Haploinsufficiency for human *EYA1* or *SIX1* leads to branchio-oto-renal syndrome (Abdelhak et al., 1997; Ruf et al., 2004), and genetic deletion of either gene in mice results in early arrest of inner ear development at the otocyst stage (Xu et al., 1999; Zheng et al., 2003; Zou et al., 2006). We have shown that *Six1* functions downstream of and interacts genetically with *Eya1* during inner ear development (Xu et al., 1999; Zheng et al., 2003; Zou et al., 2006), and their gene products participate in protein-protein interaction (Buller et al., 2001). In *Eya1*- or *Six1*-null mutants, neurogenesis is initiated normally but the neuroblast cells fail to form a morphologically detectable ganglion owing to abnormal apoptosis (Zou et al., 2004), indicating that both genes are necessary for sensory neuron maintenance. These observations raise several possibilities. First, *Eya1* and *Six1* might be dispensable for neuroblast specification within the otocyst. Second, both genes might function in parallel to regulate neuronal development in the inner ear.

In this study, we tested these possibilities and the possible involvement of EYA1 and SIX1 together with the SWI/SNF chromatin-remodeling complexes in activating *Neurog1* and *Neurod1* transcription. Our results from loss- and gain-of-function analyses indicate that EYA1 and SIX1 are crucial neuronal determination factors for activating neuronal developmental program in the inner ear, probably by recruiting and interacting with the SWI/SNF chromatin-remodeling complex to mediate specifically the transcriptional activities of *Neurog1* and *Neurod1*.

MATERIALS AND METHODS

Animals and genotyping

Genotyping of *Eya1*- and *Six1*-null and *Eya1*^{lacZ} knock-in mice was performed as described (Xu et al., 1999; Xu et al., 2002; Zou et al., 2008).

Histology, X-gal staining, in situ hybridization (ISH) and immunohistochemistry (IHC)

Histological examination, X-gal staining and whole-mount and section ISH were carried out according to standard procedures with digoxigenin-labeled *Neurog1*, *Neurod1*, *Baf170* and *Brg1* riboprobes. We used six embryos for each genotype at each stage for each probe and the result was consistent in each embryo.

Antibodies for IHC were as follows: anti-NEUROG1 (1:500; Affinity BioReagents), anti-NEUROD1 (1:500; Abcam), anti-MAP2 (1:200; Sigma), anti-TUJ1 (1:200; Sigma), anti-GFP (1:250; Novus), 2H3 (1:500; Hybridoma) and anti-MYO7A (1:1000; Proteus). E9.0-9.25 embryos or cochleae were sectioned at 10 μm.

DNA constructs and yeast two-hybrid screen

Eya1.EGFP or *Six1.EGFP* expression plasmid was described (Ahmed et al., 2012). Dr Jane Johnson (UT Southwestern Medical Center, Texas, USA) kindly provided the *Neurog1* expression plasmid and others were purchased: *Baf170-Flag* (Addgene plasmid 19142) (Xi et al., 2008), *Brg1-Flag* (Addgene plasmid 19148) (Xi et al., 2008), *Baf155-Flag* (Addgene plasmid 24562) (Ho et al., 2009), *Brg1K798R* (Addgene plasmid 1960), scramble shRNA in pLKO.1 lentiviral vector (Addgene plasmid 1864) (Sarbassov et al., 2005), mU6-Brg/Brm shRNA (pRVGP-BB) (Addgene plasmid 15380) (Ramirez-Carrozzi et al., 2006), *Sox2* shRNA (Addgene plasmid 26353) (Bass et al., 2009) and *Neurod1-I-nGFP* (Addgene plasmid 19414). The full-length cDNA of *Six1* or the Eya domain of *Eya1* was cloned into the pGBKT7 vector and used as bait constructs.

For the yeast two-hybrid screen, the MATCHMAKER system (Clontech) was used following manufacturer's instructions. The clones were isolated from a pre-transformed mouse E11 embryonic cDNA library (Clontech, ML4012AH).

Electroporation of electroporation of embryos and cochlear explant cultures, and quantitative real-time PCR (qRT-PCR)

DNA was injected into the lumen of spinal cord or otocyst of mouse embryos and electroporated using a square-wave electroporator (ECM830, BTX) with the following parameters: 10 volts, 50 msecond duration and two to three pulses. After 24-36 hours in culture, the samples were processed for ISH or IHC. E13.5 cochleae were electroporated as described previously (Jones et al., 2006), maintained in culture for 3-4 days in vitro (DIV) and then processed for ISH or IHC.

For qRT-PCR, total RNAs were extracted from otocyst regions using Trizol reagent (Invitrogen). First-strand cDNA was synthesized from 500 ng total RNA using Superscript III (Qiagen) with random primers and the resulting cDNA product was used for real-time PCR using SYBR Green Master Mix (Roche). Primers for *Neurod1* used in RT-PCR were 5′-GCTTGACTATCACATACAA-3′ (forward) and 5′-CTAATTATGAAT-TCGATGGT-3′ (reverse) and reactions were carried out in a LightCycler 480 (Roche). For each relative quantification, three groups of six to ten otocysts generated in three independent experiments were used. Each of these samples was reverse transcribed (RT) three times and RT products were used as a template for each pair of primers in a triplicate PCR reaction. Expression levels were normalized to *Gapdh* (internal control) and the levels in control otocysts were set to 1.

Transient transfection of NIH 3T3 cells, co-immunoprecipitation (coIP), GST pull-down assay and western blot

Transient transfection of 3T3 cells was performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions.

For coIP analysis, transfected 3T3 cells or dissected E13.5 cochleae were homogenized and lysed in RIPA buffer (20 mM Tris HCl, pH 7.6, 100 mM NaCl, 0.2% Triton X-100, 0.2% NP-40, 0.2% sodium deoxycholate, 1 mM DTT, 2 mM β-mercaptoethanol, protease inhibitors), then spun and the supernatant was used for IP. Lysates of 3T3 cells or ~2 mg of cochlea lysates were pre-cleared with protein A/G agarose beads and processed for IP analysis as previously described (Bultman et al., 2005) using goat anti-SIX1 (Santa Cruz), rabbit anti-SOX2 (Chemicon) or mouse anti-HA7 (Sigma) antibodies. Cochlear cell lysates used for input was one tenth of the amount used for co-IP. Lysates of 3T3 cells used for input was one quarter of the amount used for coIP.

The GST pull-down assay was performed as described previously (Buller et al., 2001) using 20 μl GST alone, GST-EYA1 (EYA domain) or GST-SIX1 fusion protein was mixed with 5 μl in vitro translated FLAG-BAF170, FLAG-BRG1, NEUROG1 or NEUROD1 protein made using TNT-coupled rabbit reticulocyte lysate (Promega).

Antibodies for western blot were: anti-FLAGM2 (Sigma), anti-NEUROG1 (Affinity BioReagents; ~35 kDa), anti-NEUROD1 (Abcam; ~37-40 kDa), anti-BRG1 (H-88, Santa Cruz; ~200 kDa), anti-BAF170 (E-6, Santa Cruz; ~170 kDa), anti-E2F1 (C-20, Santa Cruz; ~60 kDa), anti-BRN3A (Abcam; ~43 kDa), anti-SIX1 (Santa Cruz; ~32.2 kDa) and anti-SOX2 (Millipore; ~40 kDa). Anti-FLAGM2 antibody detects FLAG-BAF170 (~155 kDa), FLAG-BRG1 (~185 kDa) or HA-FLAG-EYA1 (~70 kDa).

RESULTS

Otocyst-derived neuroblasts are not specified in mice lacking both *Eya1* and *Six1*

As neuroblast precursors are initially specified in *Eya1* or *Six1* mutants (Zou et al., 2004), and EYA1 and SIX1 participate in protein-protein interaction (Buller et al., 2001; Ruf et al., 2004), we

hypothesized that these two genes might act cooperatively to induce neurogenesis. To test this, we first examined the expression of *Neurog1* and *Neurod1* in the *Eya1;Six1* double mutant. At ~E9.0-9.25, *Neurog1* is expressed in neuroblasts within the anteroventral domain of the otic cup (Fig. 1A). Consistent with our previous observation (Zou et al., 2004), *Neurog1*$^{+}$ cells were clearly detectable in each single mutant embryos at ~E9.25, but the number was largely reduced (Fig. 1B,C), and became undetectable by E10.5 owing to abnormal apoptosis (Zou et al., 2004). By contrast, *Neurog1* expression was not observed in *Eya1*$^{-/-}$;*Six1*$^{-/-}$ mutant embryos at E8.75-10.0 (Fig. 1D; data not shown). We also analyzed the expression of the neuronal differentiation marker *Neurod1* (Lee et al., 1995; Ma et al., 1998). In normal E9.25-9.5 embryos, *Neurod1* is strongly expressed in differentiating neuronal progenitors within the otocyst and in migratory cells that will form sensory neurons of the VIIIth ganglion (Fig. 1E,I). In *Eya1*$^{-/-}$, *Six1*$^{-/-}$ or *Eya1*$^{+/-}$;*Six1*$^{-/-}$ mutant embryos, *Neurod1*$^{+}$ cells were also present in the otocyst but were reduced (Fig. 1F,G,J) compared with wild-type controls (Fig. 1E,I). The degree of reduction varied between embryos (Fig. 1F,G,J), similar to observations for *Neurog1* (data not shown). However, *Neurod1* expression was undetectable in the double mutant (*n*=6; Fig. 1H,K,L). In addition, *Neurog1* and *Neurod1* expression in other cranial ganglia was also affected in the double or single mutants (Fig. 1D,F-H,J-L; data not shown). The lack of *Neurog1* and *Neurod1* expression in the double mutant otocysts was further confirmed by section ISH (data not shown).

As the otocyst was smaller in the double mutant (Zheng et al., 2003), we sought to clarify the specificity of the observation described above by marker gene expression analysis from otic placode stage. *Dlx5* is expressed very early in the otic placode and its expression is restricted to the dorsal region of the otocyst in normal embryos (Fig. 1M,N). In *Eya1*$^{-/-}$;*Six1*$^{-/-}$ mutant embryos, *Dlx5* expression was detected in the otic placode at ~E8.5 (data not shown) and in the otocyst at E9.5 (Fig. 1O,P), but its expression domain was shifted to the ventral region of the double mutant otocyst (Fig. 1P), suggesting absence of the ventral portion of the otocyst in the double mutant, which normally gives rise to neuronal and sensory structures of the inner ear. Thus, as otic placode induction and otocyst formation appear to occur in the double mutant embryos and as *Neurog1* and *Neurod1* expression is reduced or absent in the double mutant (unlike the *Eya1* or *Six1* single mutants, which initially express *Neurog1* and *Neurod1*) it may be concluded that the combined action of *Eya1* and *Six1* is necessary for the induction of neuronal fate in the otocyst.

Eya1 and *Six1* are co-expressed in neuroblasts and spiral neurons

We performed expression and colocalization studies from the initiation of neurogenesis in the otic ectoderm. As the inserted *lacZ* transgene for *Eya1*$^{lacZ/+}$ or *Six1*$^{lacZ/+}$ displayed an expression pattern identical to their mRNA distribution obtained by ISH (Zheng et al., 2003; Zou et al., 2008), we analyzed their expression by staining for β-galactosidase activity of *Eya1*$^{lacZ/+}$ or *Six1*$^{lacZ/+}$ heterozygotes. The strongest *Eya1* expression domain in the otic cup at ~E8.75-9.0 is located in the anteroventral region (Fig. 2A), which appears to mark the neuroblasts (data not shown). From ~E9.25, *Eya1* expression expands to the entire ventral half of the otic cup and vesicle and colocalizes with NEUROG1^{+}NEUROD1^{+} cells (Zou et al., 2006). A similar observation was made for *Six1* expression (Fig. 2D) (Zheng et al., 2003). Both genes are also expressed in differentiating vestibulocochlear neurons as well as the spiral ganglion and colocalize with NEUROD1 (Fig. 2B,C,E,F). These observations suggest that these genes might be essential for neuronal fate induction and their differentiation and/or maturation.

Ectopic expression of both *Eya1* and *Six1* is sufficient to induce ectopic activation of *Neurog1* or *Neurod1* and neurogenesis in mouse embryos and cochleae

The absence of *Neurog1* and *Neurod1* expression in the *Eya1;Six1* double mutant suggests that EYA1 and SIX1 might activate their expression to initiate neuronal development. This

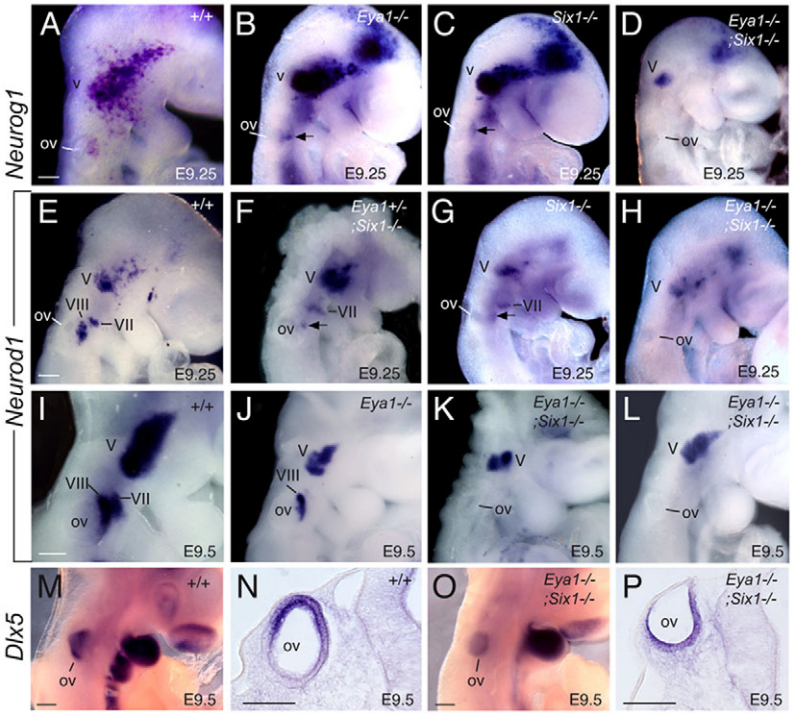

Fig. 1. Otic neurogenesis is blocked in mice lacking *Eya1* and *Six1*. (**A-D**) Whole-mount ISH for *Neurog1* in wild-type (A), *Eya1*$^{-/-}$ (B), *Six1*$^{-/-}$ (C) and *Eya1*$^{-/-}$;*Six1*$^{-/-}$ (D) embryos at E9.25. (**E-L**) Whole-mount ISH for *Neurod1* in wild-type (E,I), *Eya1*$^{+/-}$;*Six1*$^{-/-}$ (F), *Six1*$^{-/-}$ (G), *Eya1*$^{-/-}$ (J) and *Eya1*$^{-/-}$;*Six1*$^{-/-}$ (H,K,L) embryos at E9.25-9.5. (**M-P**) Whole-mount ISH for *Dlx5* in wild-type control (M) and *Eya1;Six1* mutant (O) at E9.5. N and P show sections through otocyst regions of the embryos shown in M and O, respectively. Arrows point to the reduced expression in the otocyst (ov) or VIIIth ganglion. V, trigeminal (Vth) ganglion. Scale bars: 100 μm.

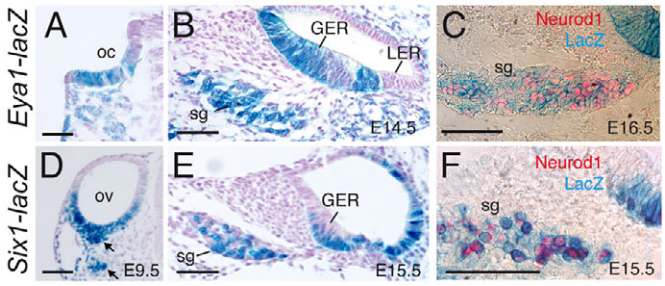

Fig. 2. *Eya1* and *Six1* are expressed in otic neuroblast precursors and spiral ganglion. (**A-F**) Sections of X-gal-stained E8.75 *Eya1*$^{lacZ/+}$ embryo (A), E14.5 *Eya1*$^{lacZ/+}$ cochlear (B), E16.5 *Eya1*$^{lacZ/+}$ cochlear stained with anti-NEUROD1 (red, C), E9.5 *Six1*$^{lacZ/+}$ embryo (arrow points to differentiating neurons within the VIIIth ganglionic anlagen) (D), E15.5 *Six1*$^{lacZ/+}$ cochlear (E), E15.5 *Six1*$^{lacZ/+}$ cochlear stained with anti-NEUROD1 (red, F). GER and LER are indicated. oc, otic cup; sg, spiral ganglion. Scale bars: 50 μm.

led us to investigate whether *Eya1* and *Six1* gain-of-function could induce ectopic expression of *Neurog1* or *Neurod1* by electroporating the *Eya1.GFP* and *Six1.GFP* plasmid alone or combined into the otocyst of E9.25-9.5 embryos. Cultured rudiments were hybridized with *Neurog1* or *Neurod1* probes 24-36 hours after electroporation, followed by IHC for detecting GFP. Overexpression of both *Eya1* and *Six1* but not each plasmid alone or a control plasmid in the otocyst caused marked increase in neurogenesis as labeled by *Neurod1* expression (Fig. 3A,B). Overexpression of *Eya1* and *Six1* also resulted in ectopic neurogenesis in the head ectoderm near the Vth-VIIth ganglionic regions (Fig. 3A,B). We isolated the otocyst including the VIII/VIIth ganglion after 24-36 hours in culture and analyzed *Neurod1* expression by qRT-PCR. Transcriptional levels of endogenous *Neurod1* significantly increased with respect to the controls (Fig. 3C). Similarly, ectopic neurogenesis was induced by co-expression of *Eya1* and *Six1* in the head ectoderm and the roof plate/dorsal spinal cord or ectoderm lateral to the spinal cord as labeled by *Neurog1* and *Neurod1* and the neuronal marker TUJ1 (β-TUBULIN III; TUBB3 – Mouse Genome Informatics) (Hallworth et al., 2000; Lee et al., 1990) (supplementary material Fig. S1), further indicating that *Eya1* and *Six1* can efficiently induce transcription of *Neurog1* and *Neurod1* to promote neuronal development.

As *Eya1* and *Six1* are co-expressed in the spiral ganglion, we investigated whether overexpression of *Eya1* and *Six1* either alone or combined is sufficient to induce neuronal fate in the cochlea. The *Eya1.GFP* and/or *Six1.GFP* plasmids were electroporated into E13.5-14.0 cochleae to induce their ectopic expression in nonsensory epithelial cells located within the greater epithelial ridge (GER) or the lesser epithelial ridge (LER). After 3-4 DIV, we analyzed neuronal development by examining the expression of TUJ1. Multiple transfected cells in both the GER and the LER were observed (Fig. 3D). However, very few or no epithelial cells transfected with either *Eya1.GFP* or *Six1.GFP* alone were positive for TUJ1 (Table 1). By contrast, a subset of epithelial cells transfected with both *Eya1* and *Six1* were TUJ1^{+} (~11-12%) (Fig. 3D,F; Table 1), most of which extended long processes and many ended in growth cones (Fig. 3E,G), a morphology that is consistent with developing neurites. Transfected cells with neuronal morphologies were also positive for the microtubule-associated-protein 2 (MAP2; MTAP2 – Mouse Genome Informatics) (data not

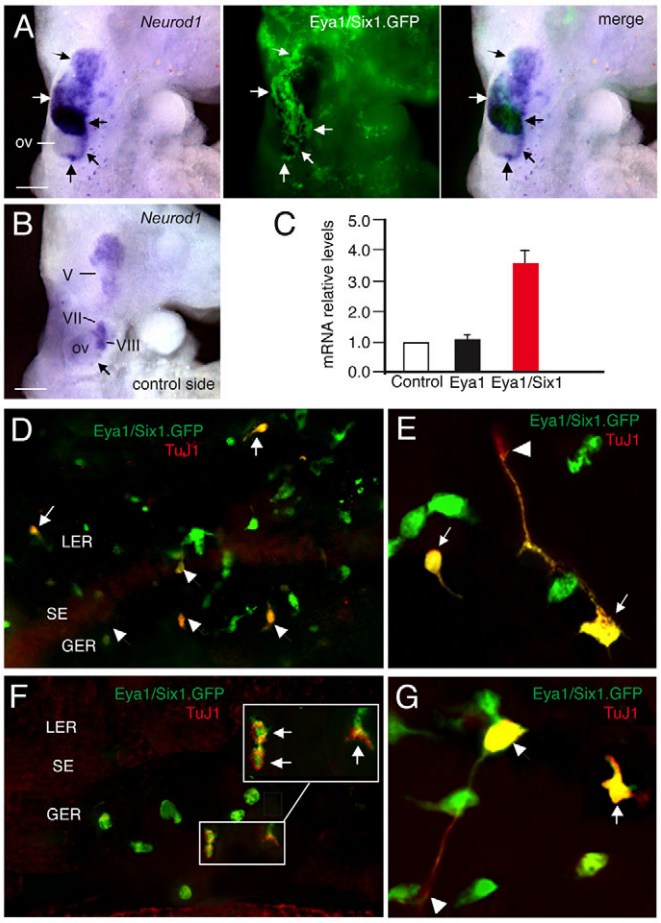

Fig. 3. Co-expression of *Eya1* and *Six1* induces neuronal phenotypes in cochlear nonsensory epithelial cells. (**A**,**B**) *Neurod1* whole-mount ISH of explants of E9.25-9.5 embryo transfected with *Eya1* and Six1 (green) in electroporated (A) and unelectroporated/control (B) sides. Arrows indicate ectopic neurogenesis in A and normal low level expression in B. (**C**) Relative mRNA levels of *Neurod1* in otocyst including the VIIIth-VIIth ganglion transfected with *Eya1* or both *Eya1* and *Six1* for 1 day. Untransfected side was used as control and *Neurod1* mRNA level was designated as 1.0. Data indicate mean ± s.e.m. (**D**,**F**) Cochlear explant transfected with *Eya1* and *Six1* and labeled with anti-TUJ1 (red). The sensory epithelium (SE) and nonsensory GER and LER are indicated. Arrows and arrowheads point to TUJ1^{+} neurons. In F, higher magnification of the boxed area is shown. (**E**,**G**) High-magnification images showing cells transfected with *Eya1* and *Six1*. Arrows point to TUJ1^{+} cells and arrowheads point to axonal growth cone. Scale bars: 100 μm.

shown), another neuron-specific marker (Hafidi et al., 1992). Cells transfected with a *control.GFP* vector were negative for TUJ1 or MAP2 (Table 1). This observation indicates that *Eya1* and *Six1* act cooperatively to induce a neuronal fate in cochlear nonsensory epithelial cells.

We examined next whether the ectopic neurons reflect an induction of endogenous *Neurog1* and *Neurod1* activation. ISH and IHC of the explants transfected with both *Eya1* and *Six1* revealed that 100% of GFP^{+}TUJ1^{+} cells were *Neurog1*$^{+}$*Neurod1*$^{+}$ (Fig. 4A,B). Furthermore, all ectopic neurons expressed the mature neuronal marker NF after 4 DIV (Fig. 4C,D; Table 1). Thus, *Eya1* and *Six1* are sufficient to drive not only neuronal fate but also neuronal differentiation/maturation.

Table 1. Neuronal induction by *Eya1*, *Six1*, *Neurog1* and *Neurod1*

Construct	No. transfected cells/no. explants 3 DIV	No. TUJ1$^+$*Neurog1*$^+$/ no. TUJ1$^+$*Neurog1*$^-$ cells	No. TUJ1$^+$*Neurod1*$^+$/ no. TUJ1$^+$*Neurod1*$^-$ cells	Percentage TUJ1$^+$ cells (no. TUJ1$^+$:no. GFP$^+$) 3 DIV	Percentage NF$^+$/*Neurod1*$^+$ cells (no. NF$^+$:no. GFP$^+$/ no. *Neurod1*$^+$:no. GFP$^+$) (no. explants) 4 DIV
Eya1.GFP	312/6	1/2	0/3	1.0 (3:312)	0/0 (0:253/0:253) (3)
Six1.GFP	282/5	0/0	0/0	0 (0:282)	0/0 (0:218/0:218) (3)
Eya1/Six1	414/7	49/0	–	11.8 (49:414)	10.8/10.8 (20:185/20:185) (3)
Eya1/Six1	338/6	–	41/0	12.1 (41:338)	–
Eya1/Neurog1	118/2	–	0/32	27.1 (32:118)	0/0 (0:263/0:263) (3)
Six1/Neurog1	133/2	–	0/36	27.1 (36:133)	0/0 (0:237/0:237) (3)
Eya1/Neurod1	199/3	0/142	–	71.3 (142:199)	4.0/4.0 (18:448/–) (6)
Six1/Neurod1	128/2	0/91	–	71.1 (91:128)	3.8/3.8 (21:548/–) (8)
Neurog1/Neurod1	197/3	–	–	98.5 (194:197)	1.0/– (2:208/–) (3)
Neurog1.GFP	131/2	–	–/36	27.5 (36:131)	0/0 (0:135/0:135 (2)
Neurod1.GFP	210/3	0/144	–	68.6 (144:210)	0/– (0:215/–) (3)
Control.GFP	164/3	0	0	0	0/0 (0:183/0:183) (3)

Nonsensory cells located in either the GER or the LER that were transfected with the indicated constructs were identified based on expression of GFP. Neuronal identity was established based on the expression of TUJ1 or NF.

EYA1 and SIX1 act synergistically with NEUROG1 and NEUROD1 to regulate neuronal differentiation

We tested next whether EYA1 and SIX1 interact with NEUROG1 or NEUROD1 to induce NF$^+$ neurons. Co-expression of *Neurog1* and *Eya1*, or *Neurog1* and *Six1*, in cochlear explants was incompetent to activate *Neurod1* expression and induce NF$^+$ cells (Fig. 4E,F; Table 1), further indicating that both *Eya1* and *Six1* are required for activating *Neurod1* expression. Interestingly, however, the combination of *Neurod1* and *Eya1*, or *Neurod1* and *Six1*, was capable of inducing NF$^+$ neurons at lower efficiency (~4% of transfected GFP$^+$ cells) (Fig. 4G,H; Table 1), demonstrating that *Eya1* and *Six1* act synergistically with *Neurod1* to mediate neuronal differentiation/maturation. By contrast, consistent with previous observations (Puligilla et al., 2010), combination of *Neurog1* and *Neurod1* induced ~98.5% of transfected cells into TUJ1$^+$ neurons, but <1% differentiated into NF$^+$ neurons (Table 1), indicating that *Neurog1* and *Neurod1* expression is insufficient to mediate differentiation/maturation to produce NF$^+$ neurons. Thus, these observations demonstrate that both EYA1 and SIX1 are not only necessary for activating *Neurog1* and *Neurod1* expression but also act synergistically with their gene products to mediate neuronal differentiation.

EYA1 and SIX1 cooperatively interact with the SWI/SNF subunits BAF170 and BRG1 to promote neuronal development

The relatively low efficiency of neuronal induction by EYA1 and SIX1 in the GER suggests that other factors might be required to induce neuronal fate. The SWI/SNF ATP-dependent chromatin-remodeling complex is important for vertebrate neurogenesis and the ATPase BRG1 is a key subunit of this complex (Lessard et al., 2007; Seo et al., 2005). It interacts physically with and mediates transcriptional activities of *Neurog1* and *Neurod* in *Xenopus* (Seo et al., 2005). Through our yeast two-hybrid screen

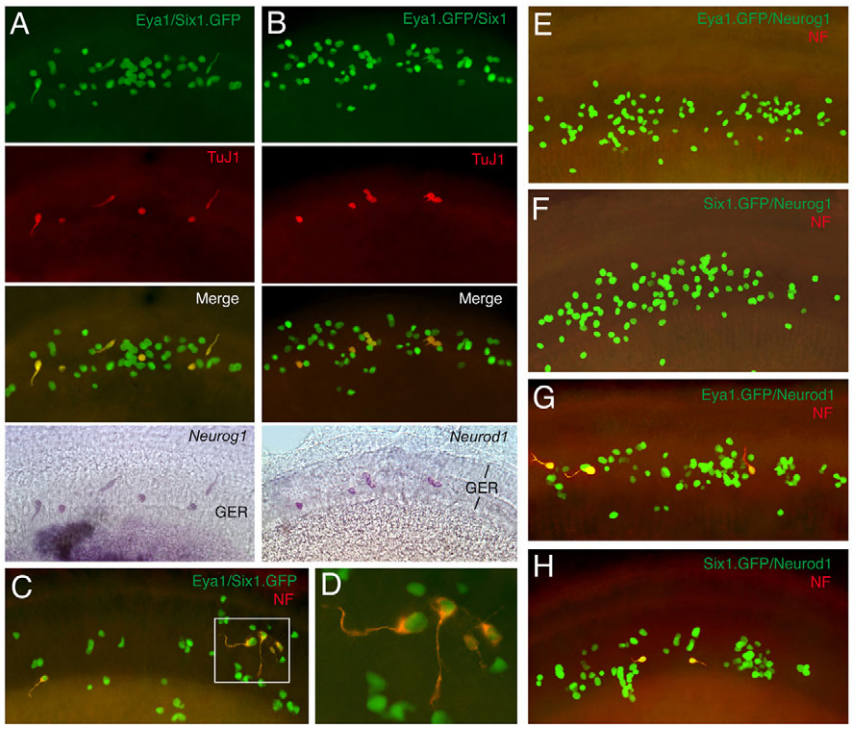

Fig. 4. *Eya1* and *Six1* induce *Neurog1* and *Neurod1* expression and are required for neuronal differentiation. (**A-C**) Cochlear explants transfected with *Eya1* and *Six1* (green) stained with anti-TUJ (red) and *Neurog1* (A) or *Neurod1* (B) ISH or anti-NF (red, C). (**D**) Higher magnification of boxed area in C. (**E-H**) Cochlear explants transfected with *Eya1* and *Neurog1* (E), *Six1* and *Neurog1* (F), *Eya1* and *Neurod1* (G) or *Six1* and *Neurod1* (H) and stained for NF.

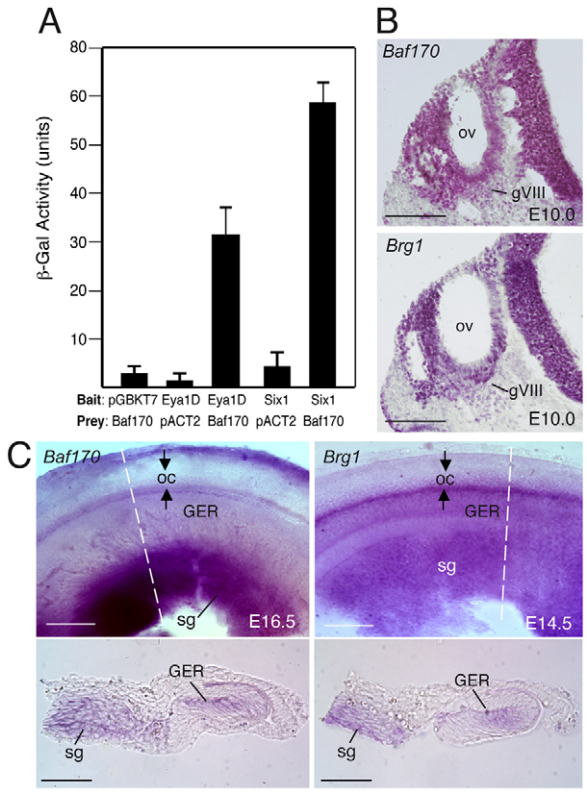

Fig. 5. Yeast two-hybrid and in situ hybridization analyses. (**A**) SIX1 or EYA1 domain was used as 'bait'. pGBKT7 or pACT2 vector alone was used as negative control. Co-transformation was analyzed for ability to activate *lacZ* expression by liquid β-gal assay. Strength of interactions was judged by the units of β-gal activity. A result representing an example data set of three independent experiments (performed in triplicate) is shown with standard deviation. (**B**,**C**) Section ISH for *Baf170* and *Brg1* in otocyst (ov) and VIIIth ganglion at E10.0 (B) and in developing cochlea (C). Lower panels in C are sections of upper panels and the plane of sections are indicated. oc, organ of Corti; sg, spiral ganglion. Scale bars: 100 μm.

of mouse E11 cDNA library using SIX1 or EYA1 domain as 'bait', subunits of the BAF complexes, including BRG1 and BRG/BRM-associated factor 170 (BAF170; SMARCC2 – Mouse Genome Informatics), were isolated as co-factors for SIX1 or EYA1 (Fig. 5A; data not shown). *Brg1* has been shown to be expressed in a widespread manner during mouse embryogenesis (Randazzo et al., 1994) and is required for early embryogenesis (Bultman et al., 2000). We analyzed the expression of *Baf170* and *Brg1* by ISH and found that both are highly expressed in the developing otocyst and VIIIth ganglion at E10.0 and in the spiral ganglion at E14.5-16.5 (Fig. 5B,C). In addition, their expression was observed in the GER cells but not in the organ of Corti (Fig. 5C). These observations led us to test whether the ectopic neurons induced by EYA1 and SIX1 require SWI/SNF ATPase activity in both mammalian cells and cochlear explants. Surprisingly, ~30.4% of 3T3 fibroblast cells 4 days post-transfection with *Eya1* and *Six1* were TUJ1$^+$ or NF$^+$ (Fig. 6A,B; Table 2), whereas neither factor alone induced any NF$^+$ neurons (data not shown). Thus, the combined action of EYA1 and SIX1 is also capable of activating neuronal differentiation from fibroblast cells. Remarkably, however, combination of *Eya1* and *Six1* with either *Baf170* or *Brg1* resulted in a large increase in the number of TUJ1$^+$ or NF$^+$ neurons (~82.6% or ~89.3% of transfected cells, respectively) (Fig. 6C-F; Table 2). When all four factors were combined, ~96.6% of transfected 3T3 cells differentiated into TUJ1$^+$ or NF$^+$ neurons (Fig. 6G,H; Table 2). By contrast, co-expression of *Eya1* and *Brg1* induced very few TUJ1$^+$ or NF$^+$ neurons (Table 2), whereas co-expression of *Six1* and *Brg1*, *Six1* and *Baf170*, *Eya1* and *Baf170*, or *Brg1* and *Baf170* was incapable of promoting neuronal development (Table 2).

Co-expression of *Eya1* and *Six1* resulted in upregulation of BRG1 expression levels compared with untransfected 3T3 cells, whereas addition of *Baf170* together with *Eya1* and *Six1* led to a large increase in the levels of BRG1 expression (supplementary material Fig. S2A). These results indicate that EYA1 and SIX1, or BAF170, EYA1 and SIX1, can upregulate BRG1 expression. Thus, our results demonstrate that EYA1 and SIX1 act cooperatively with BRG1 and BAF170 to drive neuronal differentiation from 3T3 fibroblast cells.

In cochlear explants, co-expression of either *Brg1* and *Eya1*, or *Brg1* and *Six1*, is incapable of inducing neuronal development (Table 2). However, combination of *Eya1* and *Six1* with either *Baf170* or *Brg1* in the GER increased the number of NF$^+$*Neurod1*$^+$ neurons induced to ~45% (Fig. 7A,B; Table 2), whereas combination of four factors resulted in ~85.9% of NF$^+$*Neurod1*$^+$ neurons (Table 2). Thus, similar to 3T3 cells, EYA1 and SIX1 cooperate with BRG1 and BAF170 to coordinate neuronal development in the cochlea.

Previous studies have shown that BAF170 only exists in committed neuronal lineage and forms a heterodimer with BAF155 (SMARCC1 – Mouse Genome Informatics) (Chen and Archer, 2005; Ho and Crabtree, 2010), another subunit of the complex that is highly homologous to BAF170 (Wang et al., 1996). In contrast to *Baf170*, co-expression of *Baf155* did not show obvious synergy with the *Brg1*, *Eya1*, *Six1* combination (Table 2), indicating that BAF155 cannot substitute BAF170 to interact with EYA1 and SIX1. However, when *Baf155*, *Baf170*, *Brg1*, *Eya1* and *Six1* are combined, almost all transfected 3T3 cells were positive for NF and a robust neuronal induction was also observed in cochlear explants (Fig. 7C; Table 2). Co-staining of NF with MYO7A, a specific marker for differentiating hair cells, revealed that a subset of the ectopic neurons induced in the GER innervated hair cells (Fig. 7D), indicating that they are spiral ganglion neurons. Thus, BAF155 is likely to interact with BAF170 to cooperatively regulate neuronal development activated by EYA1, SIX1, BAF170 and BRG1.

The specification of neuronal developmental program initiated by EYA1 and SIX1 requires BRG1 ATPase activity

Next, we tested the hypothesis that activation of *Neurog1* and *Neurod1* by EYA1 and SIX1 might require the ATPase activity of BRG1 to remodel chromatin at their promoters/enhancers. Western blot analysis revealed that co-expression of *Eya1* and *Six1* increased the levels of BRG1 expression compared with untransfected 3T3 cells, and addition of *Baf170* together with *Eya1* and *Six1* resulted in a large upregulation of BRG1 levels (supplementary material Fig. S2A). This result indicates that EYA1 and SIX1 with or without BAF170 require BRG1 activity to induce neurogenesis. Co-expression of *Brg1K798R* mutant construct, which contains a mutation in the ATP-binding site of BRG1, together with *Eya1* and *Six1*, or *Baf170*, *Eya1* and *Six1* failed to induce neuronal development (Fig. 8A,B; Table 2), indicating that the BRG1K798R mutant has a dominant-negative effect, which completely blocks neurogenesis.

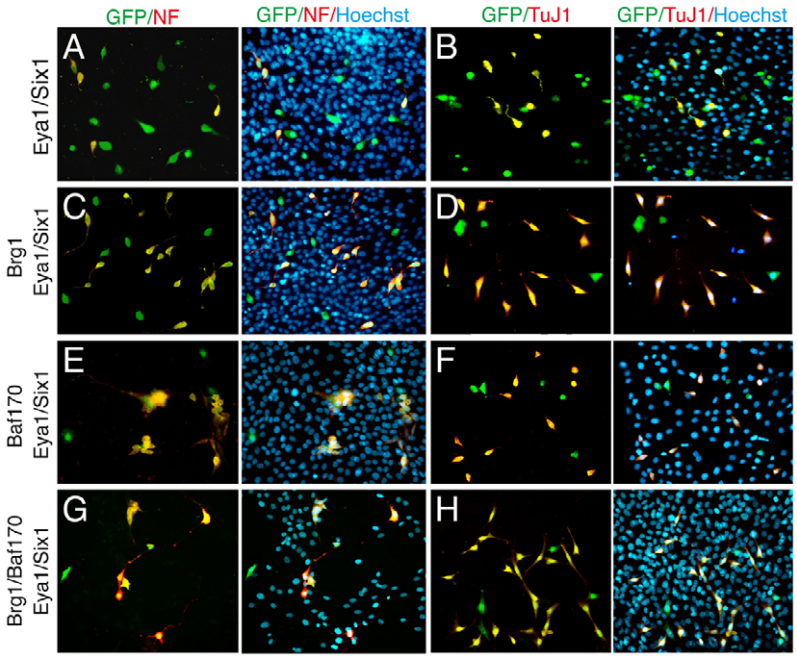

Fig. 6. EYA1 and SIX1 cooperatively interact with BRG1 and BAF170 to drive neuronal differentiation in 3T3 fibroblast cells. (**A-H**) 3T3 cells were transfected with the indicated constructs (green) and stained with anti-NF (red) (A,C,E,G) or anti-TUJ1 (B,D,F,H). Hoechst stains the nuclei.

We then co-transfected *Brg1* shRNA (named *shBrg1*) (Ramirez-Carrozzi et al., 2006) to knock down endogenous BRG1 activity. Surprisingly, co-expression of *shBrg1* with *Eya1* and *Six1* completely blocked neuronal differentiation in either 3T3 cells or cochlear explants (Table 2). By contrast, when *Baf170* was co-expressed with *Eya1* and *Six1* with or without *Sox2*, a higher dose of *shBrg1* was required to deplete BRG1 activity completely and block neurogenesis in 3T3 cells or cochlear GER cells (supplementary material Fig. S2A; Fig. 8C,E; Table 2), whereas co-expression of control shRNA (named *shControl*) (Sarbassov et al., 2005) did not interfere with ectopic neurogenesis (Fig. 8D,F; Table 2). Together, our results show that activation of *Neurog1* and *Neurod1* induced by EYA1 and SIX1 depends on the SWI/SNF ATPase subunit BRG1.

Normal otic neurogenesis also requires BRG1 ATPase activity

We then addressed whether normal otic neurogenesis also requires BRG1 activity by transfecting *shBrg1* into the otic region at ~E9.25-9.5, specifically targeting the neurogenic domain. Blocking endogenous BRG1 activity led to marked reduction of neurogenesis not only in the otocyst and VIIIth ganglion but also in the VIIth/Vth ganglionic regions as labeled by *Neurod1* (Fig. 8G,K), whereas *shControl* had no effect (Fig. 8H,K). In addition, co-expression of *shBrg1* with *Eya1* and *Six1* not only blocked ectopic neurogenesis but also severely impaired normal neurogenesis in the otocyst, VIIIth, VIIth and Vth ganglia (Fig. 8I, compare with Fig. 3A and Fig. 8J), similar to that observed with *shBrg1* alone (Fig. 8G). By contrast, *shControl* did not interfere with ectopic neurogenesis induced by *Eya1* and *Six1* (data not shown). Together, these results demonstrate that the BRG1 ATPase activity is necessary for normal neurogenesis of the ectoderm-derived cranial ganglia, which also require EYA1 and SIX1 function.

EYA1 and SIX1 interact directly with BAF170, BRG1, NEUROG1 and NEUROD1

The synergism between BAF170-EYA1-SIX1, BRG1-EYA1-SIX1, NEUROG1-EYA1-SIX1 and NEUROD1-EYA1-SIX1 suggests that these factors interact physically. We performed an in vitro pulldown assay to test for a direct interaction between these proteins. GST-EYA1 (EYA domain only) or -SIX1 was able to pulldown in vitro translated FLAG-BAF170, FLAG-BRG1, NEUROG1 or NEUROD1 (Fig. 9), demonstrating that EYA1 or SIX1 directly interacts with BAF170, BRG1, NEUROG1 or NEUROD1. Co-IP of extracts from 3T3 cells transfected with *HA-Flag-Eya1/His-Six1/Flag-Brg1/Flag-Baf170* or *HA-Flag-Eya1/His-Six1/Flag-Brg1* confirmed that these proteins were physically associated (Fig. 9). Co-IP of extracts prepared from cochlea at E13.5 confirmed that these proteins interact in vivo independent of DNA (supplementary material Fig. S2). Collectively, these results demonstrate a direct interaction between EYA1 and/or SIX1 and BAF170, BRG1, NEUROG1 or NEUROD1.

SOX2 acts synergistically with EYA1 and SIX1 and with BAF170 and BRG1 to regulate neuronal development cooperatively

SOX2 has been shown to be sufficient for inducing TUJ1$^+$ but not NEUROG1$^+$NEUROD1$^+$NF$^+$ cells in cochlear nonsensory epithelial cells, and combination of *Sox2*, *Neurog1* and *Neurod1* is also incapable of inducing NF$^+$ neurons (Puligilla et al., 2010). As we found that EYA1 can interact physically with SOX2 in P19 cells (Zou et al., 2008) and that EYA1, SIX1 and SOX2 form a complex in vivo and directly interact in vitro (Ahmed et al., 2012), we sought to test whether SOX2 acts synergistically with EYA1 and SIX1 to coordinate neurogenesis. Co-expression of *Sox2* with *Eya1* and *Six1* in 3T3 cells increased the number of NF$^+$ neurons from 30.4% of cells transfected with *Eya1* and *Six1* to ~48% (Table 2). Similarly, co-expression of *Sox2* with *Eya1* and *Six1* resulted in ~24.5% of *Neurod1*$^+$NF$^+$ neurons (Fig. 10A; Table 2). A further increase in the number of NF$^+$ neurons was observed when *Sox2* was co-expressed with *Baf170*, *Eya1* and *Six1*, or *Brg1*, *Eya1* and *Six1* in 3T3 cells (Table 2) and with *Neurod1*$^+$NF$^+$ neurons in cochlear explants (Fig. 10B; Table 2). When all five factors were combined, robust neurogenesis was observed in 3T3 cells (98.1%) and in cochlear GER cells (95.9%) (Fig. 10D; Table 2). In the absence of EYA1 and SIX1, SOX2 also appears to cooperate with BAF170 and BRG1 as co-

Table 2. *Eya1* and *Six1* cooperate with SWI/SNF complex and *Sox2* to drive neurogenesis

Construct	Percentage TUJ1$^+$ cells (no. TuJ1$^+$:no. GFP$^+$) 3T3 cells	Percentage NF$^+$ cells (no. NF$^+$:no. GFP$^+$) 3T3 cells	Percentage TUJ1$^+$ cells (no. TuJ1$^+$:no. GFP$^+$) (no. explants)	Percentage NF$^+$ cells (no. NF$^+$:no. GFP$^+$) (no. explants)
Eya1/Six1	30.5 (32:105)	30.4 (31:102)	10.8 (25:231) (3)	10.8 (20:185) (3)
Baf170/Eya1/Six1	82.5 (94:114)	82.6 (90:109)	44.4 (76:171) (3)	44.5 (185:416) (6)
Brg1/Eya1/Six1	89.0 (97:109)	89.3 (109:122)	44.7 (92:206) (3)	44.9 (106:236) (3)
Brg1K798R/Eya1/Six1*	0 (0:142)	0 (0:129)	0 (0:210) (3)	0 (0:219) (3)
Brg1/Baf170/Eya1/Six1	95.9 (117:122)	96.6 (113:117)	85.4 (193:226) (3)	85.9 (293:341) (5)
Brg1K798R/Baf170/Eya1/Six1*	0 (0:103)	0 (0:161)	0 (0:215) (3)	0 (0:222) (3)
Baf170/Eya1	–	0 (0:104)	–	0 (0:202) (3)
Baf170/Six1	–	0 (0:120)	–	0 (0:129) (3)
Brg1/Baf170	–	–	–	6.7 (33:491) (7)
Baf170	–	0 (0:140)	–	0 (0:249) (3)
Brg1	–	0 (0:126)	–	0 (0:234) (3)
Brg1/Eya1	–	1.5 (2:130)	–	0 (0:257) (3)
Brg1/Six1	–	0 (0:125)	–	0 (0:206) (3)
Baf155/Eya1/Six1	–	30.1 (34:113)	–	11.6 (36:311) (5)
Baf155/Brg1/Eya1/Six1	–	78.2 (86:110)	–	46.2 (174:377) (6)
Baf155/Baf170/Brg1/Eya1/Six1	–	99.1 (108:109)	–	98.4 (435:442) (6)
Brg1/Baf155	–	–	–	0 (0:224) (3)
shBrg1 (1.0x)/Eya1/Six1	–	0 (0:125)	–	0.3 (1:286) (3)
shControl (1.0x)/Eya1/Six1	–	28.8 (33:114)	–	10.7 (23:214) (3)
shBrg1 (1.0x)/Eya1/Six1/Baf170	–	40.5 (49:121)	–	7.6 (32:421) (6)
shBrg1 (1.5x)/Eya1/Six1/Baf170	–	–	–	0.5 (2:359) (5)
shBrg1 (2.0x)/Eya1/Six1/Baf170	–	0 (0:171)	–	0.2 (1:369) (5)
shControl (1.0x)/Eya1/Six1/Baf170	–	82.0 (91:111)	–	44.8 (198:442) (6)
shControl (2.0x)/Eya1/Six1/Baf170	–	81.8 (189:231)	–	44.1 (56:127) (2)
Sox2	–	0 (0:121)	–	0 (0:297) (4)
Sox2/Baf170	–	–	–	0 (0:295 (3)
Sox2/Brg1	–	–	–	0 (0:215) (3)
Sox2/Eya1/Six1	–	48.1 (50:104)	–	24.5 (48:196) (3)
Sox2/Baf170/Brg1	–	22.7 (25:110)	–	19.7 (61:310) (5)
Sox2/Baf170/Eya1/Six1	–	93.8 (90:96)	–	73.9 (263:356) (6)
Sox2/Brg1/Eya1/Six1	–	96.1 (99:103)	–	75.8 (182:240) (3)
Sox2/Brg1/Baf170/Eya1/Six1	–	98.1 (103:105)	–	95.9 (327:341) (5)
Sox2/Brg1K798R/Baf170/Eya1/Six1*	–	0 (0:166)	0.4 (1:206) (3)	0.9 (2:219) (3)
shBrg1 (1.0x)/Sox2/Eya1/Six1	–	0.5 (1:119)	–	0.5 (1:186) (3)
shControl (1.0x)/Sox2/Eya1/Six1	–	48.0 (59:123)	–	24.3 (49:202) (3)
shBrg1 (1.0x)/Sox2/Baf170/Eya1/Six1	–	52.7 (68:129)	–	6.8 (39:578) (7)
shBrg1 (1.5x)/Sox2/Baf170/Eya1/Six1	–	–	–	0.3 (1:347) (5)
shBrg1 (2.0x)/Sox2/Baf170/Eya1/Six1	–	0 (0:168)	–	0 (0:349) (5)
shControl (1.0x)/Sox2/Baf170/Eya1/Six1	–	–	–	73.2 (161:220) (2)
shControl (2.0x)/Sox2/Baf170/Eya1/Six1	–	93.5 (100:107)	–	73.4 (94:128) (2)
shSox2 (1.0x)/Baf170/Brg1/Eya1/Six1	–	87.6 (113:129)	–	77.7 (283:364) (6)
shSox2 (2.0x)/Baf170/Brg1/Eya1/Six1	–	27.8 (44:158)	–	19.7 (27:137) (2)
shSox2 (1x)/Eya1/Six1	–	26.4 (24:91)	–	3.2 (9:284) (4)
shSox2 (2x)/Eya1/Six1	–	0 (0:102)	–	0 (0:127) (2)

GER cells transfected with the indicated constructs were identified based on the expression of GFP. Neuronal identity was established based on the expression of NF and *Neurod1*. The initial dose of each plasmid at 2 μg/μl for electroporation was designated as 1× (all unspecified samples were 1×).
*The Brg1K798R mutant has a dominant-negative effect, which completely blocks neurogenesis induced not only by *Eya1* and *Six1*, but also by *Baf170*, *Eya1* and *Six1*, or by *Sox2*, *Baf170*, *Eya1* and *Six1*.

expression of *Sox2*, *Baf170* and *Brg1* enhanced neurogenesis (Fig. 10C; Table 2). Notably, co-IP with 3T3 cells transfected with all five factors or extracts prepared from E13.5 cochleae revealed that these factors form a complex (Fig. 10H; supplementary material Fig. S2). Thus, SOX2 appears to work cooperatively with EYA1 and SIX1 and with BRG1 and BAF170 to mediate neuronal development.

In contrast to loss of BRG1 function, which completely abolished the ability of EYA1 and SIX1 (with or without SOX2) to drive neurogenesis (Fig. 10E; Table 2), co-expression of *Sox2* shRNA (Sarbassov and Sabatini, 2005) only reduced the number of *Neurod1*$^+$NF$^+$ neurons induced by EYA1 and SIX1 or by BAF170, BRG1, EYA1 and SIX1 (Fig. 10F; Table 2). However, a higher dose of *shSox2* was also able to completely block neurogenesis induced by EYA1 and SIX1 (Table 2), but not by the combination of BAF170, BRG1, EYA1 and SIX1 (Table 2). EYA1- and SIX1-dependent neurogenesis was unaffected by *shControl* (Fig. 10G; Table 2). Together, these observations indicate that SOX2 cooperates with EYA1 and SIX1 and the SWI/SNF chromatin-remodeling complex to mediate neuronal development.

Eya1 and *Six1* appear to operate through different mechanisms to induce neuronal versus sensory fate

Because the GER cells in the cochlea can also adopt a sensory hair cell fate (Izumikawa et al., 2005; Zheng and Gao, 2000), we tested whether NF$^-$ GER cells transfected with *Eya1* and *Six1* showed a

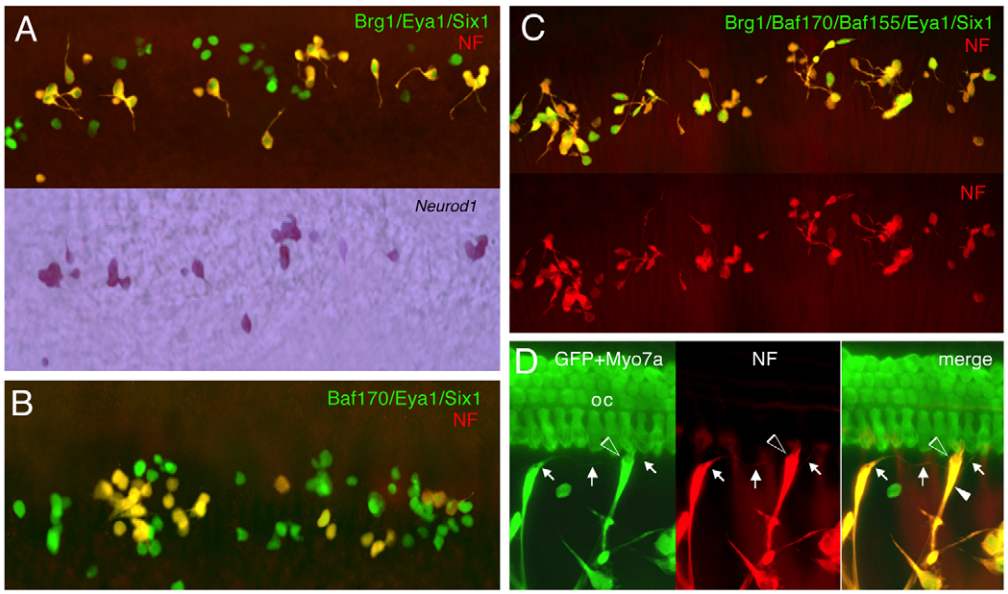

Fig. 7. EYA1 and SIX1 cooperatively interact with BAF170 and BRG1 and with BAF155 to drive neurogenesis in cochlear GER cells. (**A-D**) Explants transfected with *Brg1*, *Eya1* and *Six1* (A), *Baf170*, *Eya1* and *Six1* (B), or *Baf155*, *Baf170*, *Brg1*, *Eya1* and *Six1* (C,D) and hybridized with *Neurod1* probe and stained with anti-NF (red), anti-GFP (green) and/or anti-MYO7A (green in organ of Corti) (oc). Arrows and open arrowheads point to two ectopic neurons induced in the GER that innervate hair cells.

hair cell-like phenotype. Indeed, we found that *Eya1* and *Six1* can induce ~89% of transfected GER cells into hair cells, which express MYO7A (Table 3) (Ahmed et al., 2012), of which ~34% were *Atoh1*$^{+}$ (Table 3) (Ahmed et al., 2012), a gene encoding a bHLH transcription factor required for hair cell differentiation. When co-expressed in 3T3 cells, *Eya1* and *Six1* were not able to induce MYO7A or *Atoh1*. In contrast to the synergistic effect observed between *Sox2* and *Eya1-Six1* in inducing the GER cells to differentiate into neurons, *Sox2* antagonizes differentiation of *Atoh1*$^{+}$ cells into MYO7A^{+} hair cells (Table 3) (Ahmed et al., 2012). To validate our overexpression explant system further, we tested whether the NF^{+} GER cells transfected with *Eya1* and *Six1*, or with *Baf170*, *Baf155*, *Brg1* and *Sox2* might be MYO7A^{+} or *Atoh1*$^{+}$. We found that none of the NF^{+} GER cells were *Atoh1*$^{+}$ (Table 3) and combination of *Eya1*, *Six1*, *Baf170*, *Baf155* and *Brg1* was able to induce almost ~99% of transfected GER cells into neurons (Table 2). Together, these results clearly show that EYA1 and SIX1 can work together with the SWI/SNF complex and SOX2 to efficiently reprogram the cochlear neurosensory stem cells to differentiate into neurons instead of hair cells (Table 2). Thus, *Eya1* and *Six1* are likely to operate through different mechanisms to induce neuronal versus sensory fate, with the former induced several days earlier during development than the latter.

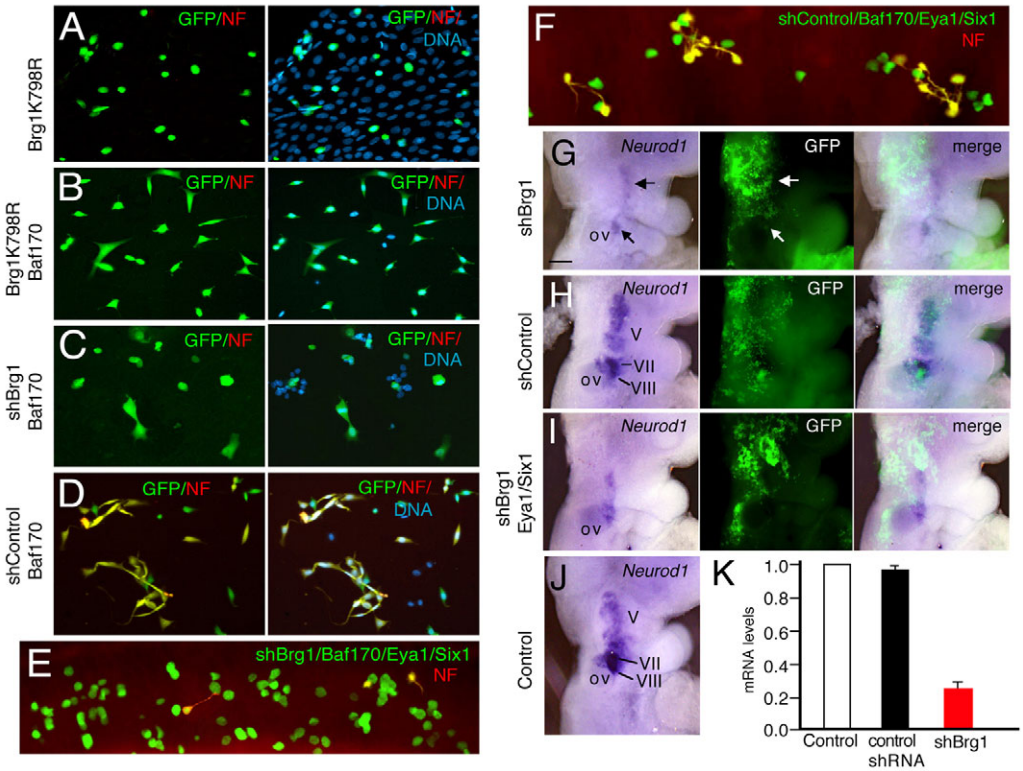

Fig. 8. BRG1 ATPase activity is necessary for EYA1 and SIX1 to initiate neuronal development and normal neurogenesis in the otocyst. (**A-D**) 3T3 cells transfected with indicated constructs and stained with anti-GFP (green) for detecting transfected cells and anti-NF (red) for neurons. Hoechst was used for nuclear staining. (**E**,**F**) Explants transfected with indicated constructs and stained with anti-NF and anti-GFP. (**G-J**) Embryos at ~E9.5 transfected with *shBrg1* (G), control shRNA (H), *shBrg1*, *Eya1* and *Six1* (I) or unelectroporated (J) and hybridized with *Neurod1* probe and stained with anti-GFP. ov, otic vesicle; V-VIII, Vth-VIIIth ganglion. (**K**) The relative mRNA levels of *Neurod1* in the otocyst and VIII/VIIth ganglion transfected with *shBrg1*, control shRNA and unelectroporated control were quantified by qRT-PCR. Data indicate mean ± s.e.m. $P<0.05$. Scale bar: 100 μm.

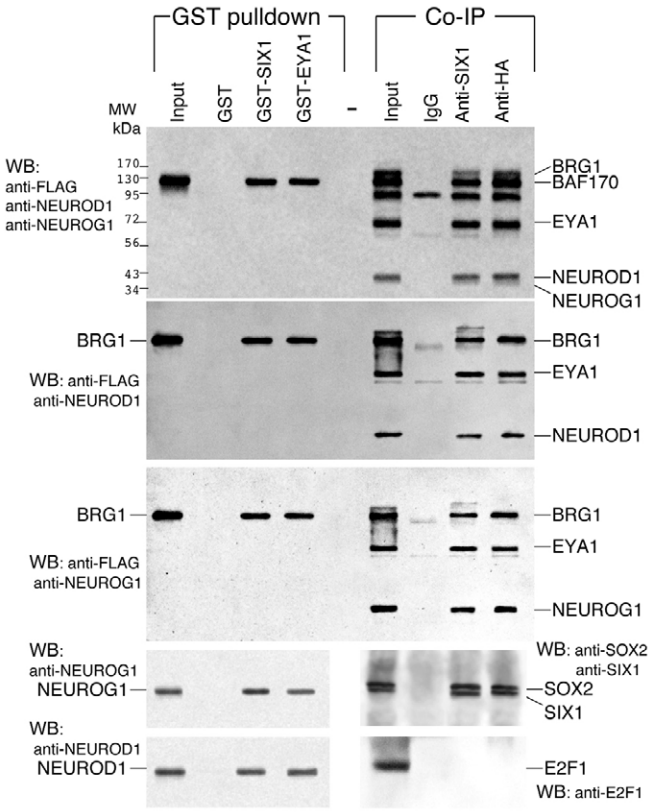

Fig. 9. EYA1 and SIX1 interact directly with BRG1 and BAF170. GST, GST-EYA1 or GST-SIX1 fusion protein was incubated with FLAG-BAF170, FLAG-BRG1, NEUROG1 or NEUROD1 protein and analyzed by western blot using anti-FLAG, anti-NEUROG1 or anti-NEUROD1 antibodies. CoIP of BAF170, BRG1, EYA1, NEUROG1, NEUROD1, SOX2 or SIX1 detected by western blot in SIX1 or HA immunoprecipitates from 3T3 cells transfected with *Flag-Baf170*, *Flag-Brg1*, *HA-Flag-Eya1*, *His-Six1* and *Sox2*. Anti-E2F1 was used as a negative control for detection of the transcription factor E2F1.

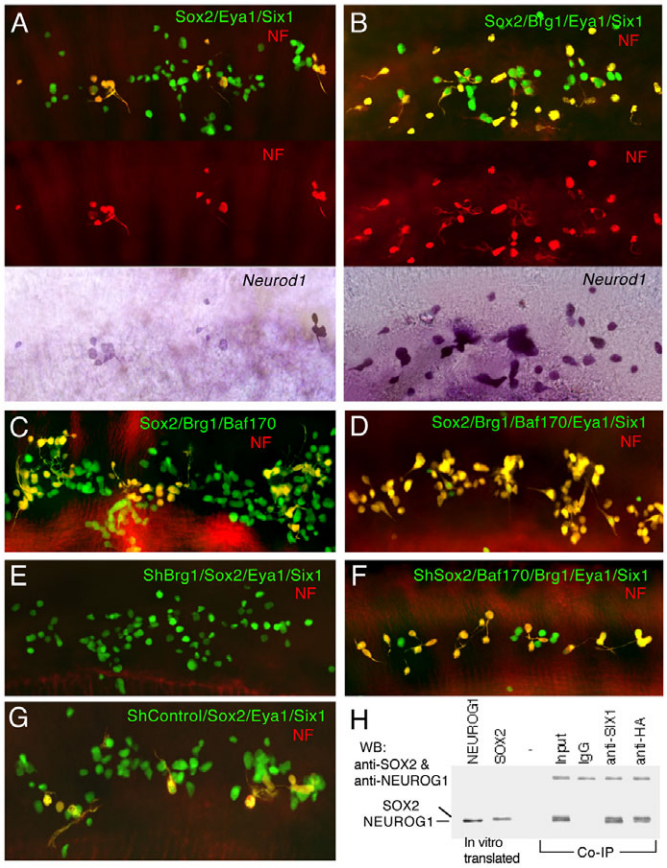

Fig. 10. SOX2 cooperates with EYA1 and SIX1 and the SWI/SNF chromatin-remodeling complex to coordinate neuronal differentiation. (**A-G**) Cochlear explants transfected with the indicated constructs and stained with anti-NF (red) and *Neurod1* probe. (**H**) CoIP of SOX2 and NEUROG1 detected by western blot in SIX1 or HA immunoprecipitates from 3T3 cells transfected with *Sox2*, *Flag-BAF170*, *Flag-BRG1*, *HA-Flag-Eya1* and *His-Six1*. In vitro translated NEUROG1 and SOX2 were loaded as size control.

DISCUSSION

The regulatory relationship among different factors and the transcriptional networks that control otocyst-derived neuroblast specification, differentiation and maturation are ill-defined. This study demonstrates that EYA1 and SIX1 specifically interact with the SWI/SNF ATPase chromatin-remodeling complex to activate the *Neurog1-Neurod1*-regulatory network to induce neuronal fate and that SOX2 works cooperatively with these proteins in this process.

Eya1 and *Six1* are necessary and sufficient for otocyst-derived neuroblast specification

In the gain-of-function experiments, overexpression of *Eya1* and *Six1* converts 3T3 fibroblast cells and cochlear epithelial cells in non-neurogenic regions into neurons. Remarkably, overexpression of *Eya1* and *Six1* is sufficient to activate transcription of *Neurog1* and *Neurod1*, indicating that *Eya1* and *Six1* function upstream of and in the same pathway as *Neurog1-Neurod1* to induce a neuronal fate.

A subpopulation of otic ectodermal cells are programmed for neurogenesis as early as ~E9.0 when the placode begins to invaginate. The neuroblast precursors are among the first cell lineages specified within the ectoderm and they undergo committed neuronal differentiation to delaminate and form the vestibulocochlear ganglion. *Eya1* is highly expressed in a subset of cells near the center of the otic cup and ventral otocyst, which coincides with the neurogenic domain. Similar expression pattern was observed for *Six1* (Zheng et al., 2003). Thus, *Eya1* and *Six1* might play a crucial role in a very early event in selecting a subset of ectodermal cells to adopt a neuronal fate by inducing the expression of *Neurog1*. In the absence of both genes, the neuroblast precursors might not be specified, leading to a complete absence of neuronal development in the double mutant. This would explain why *Neurog1* expression was undetectable at any stages examined in the double mutant.

How do we explain the initial presence of *Neurog1* expression in either $Eya1^{-/-}$ or $Six1^{-/-}$ single mutant otocyst? As either *Eya1* or *Six1* alone could not induce ectopic neurons in cochlear explant, the most likely explanation for the observed phenotype in the single mutants is that *Eya1* or *Six1* might interact with other Eya or Six genes to activate *Neurog1* expression. Indeed, *Six4* and *Eya4* are also expressed in the otic placode (Borsani et al., 1999; Ozaki et al., 2001). We are currently testing these combinations in cochlear explants. Nonetheless, our loss- and gain-of-function studies have demonstrated that EYA1 and SIX1 are key transcription factors in initiating the neuronal developmental program in the mammalian inner ear.

Table 3. *Eya1* and *Six1* reprogram cochlear nonsensory epithelial cells to differentiate into neurons instead of sensory hair cells via interaction with the SWI/SNF complex

Construct	Percentage *Atoh1*$^+$ cells (no. *Atoh1*$^+$:no. GFP$^+$) 3T3 cells 5 DIV	Percentage *Myo7a*$^+$ cells (no. *Myo7a*$^+$:no. GFP$^+$) 3T3 cells 5 DIV	Percentage *Atoh1*$^+$NF$^+$ cells (no. *Atoh1*$^+$NF$^+$:no. GFP$^+$) (no. explants) 5 DIV	Percentage NF$^+$ cells (no. NF$^+$:no. GFP$^+$) (no. explants) 5 DIV	Percentage *Atoh1*$^+$ cells (no. *Atoh1*$^+$:no. GFP$^+$) (no. explants) 5 DIV	Percentage *Myo7a*$^+$ cells (no. *Myo7a*$^+$:no. GFP$^+$) (no. explants) 5 DIV
Eya1/Six1	0 (0:157)	0 (0:161)	0 (0:237) (3)	10.2±0.8 (24:237) (3)	34.2±0.3 (81:237) (3)	89.1±0.4 (206:231) (3)
Brg1/Baf170/Eya1/Six1	0 (0:150)	0 (0:166)	0 (0:209) (3)	85.2±1.4 (178:209) (3)	11.6±1.6 (22:209) (3)	12.0±1.2 (26:216) (3)
Brg1/Baf170/Baf155/Eya1/Six1	0 (0:143)	0 (0:151)	0 (0:241) (3)	98.0±1.3 (236:241) (3)	2.1±0.8 (5:241) (3)	3.1±0.2 (6:191) (3)
Sox2/Eya1/Six1	0 (0:153)	0 (0:163)	0 (0:209) (3)	24.3±0.3 (51:209) (3)	5.3±0.8 (11:209) (3)	9.6±1.0 (20:209) (3)
Sox2/Brg1/Baf170/Eya1/Six1	0 (0:164)	0 (0:158)	0 (0:222) (3)	95.1±0.7 (211:222) (3)	2.8±0.5 (6:222) (3)	3.6±0.5 (8:221) (3)
Sox2/Brg1/Baf170/Baf155/ Eya1/Six1	0 (0:155)	0 (0:165)	0 (0:224) (3)	99.1±0.5 (221:224) (3)	0.7±0.7 (2:224) (3)	1.4±0.7 (7:204) (3)

3T3 cells or nonsensory cells in the GER of cochlear explants transfected with the indicated constructs were identified based on expression of GFP. Sensory hair cell identity was established based on expression of *Atoh1*, which is required for hair cell differentiation in the inner ear, and Myosin 7a (*Myo7a*), which is a specific marker for differentiating hair cells after 5 DIV. Neuronal identity in the cochlear explants was established based on expression of neurofilament (NF). In cochlear explants, double staining of *Atoh1* and NF revealed no transfected GFP$^+$ cells were positive for both *Atoh1* and NF. The ratio of NF$^+$; *Atoh1*$^+$ or *Myo7a*$^+$ to total GFP$^+$ cells from each explant was quantified using StatView's *t*-test; and values are expressed as mean ± s.d. Significance was determined by comparing each of the sample groups with Eya1 and Six1. *P*-values were between $P<0.0001$ and $P=0.005$.

The role of *Eya1* and *Six1* in neuronal differentiation

During inner ear neurogenesis, both *Eya1* and *Six1* are expressed in differentiating neurons even at late embryonic or postnatal stages. *Neurod1* has been suggested to act as a differentiation factor downstream of *Neurog1* based on the onset of their expression and loss of *Neurod1* expression in *Neurog1*$^{-/-}$ embryos (Ma et al., 1998). Consistent with such regulatory relationship, previous gain-of-function studies in *Xenopus* have demonstrated that misexpression of *Neurog1* can induce ectopic expression of *Neurod1* (Lee et al., 1995; Ma et al., 1996). However, in the cochlear explants, *Neurog1* is insufficient to induce *Neurod1* expression (Table 1) (Puligilla et al., 2010). Our observation that co-expression of *Eya1* and *Six1* is sufficient to induce the expression of both *Neurog1* and *Neurod1* indicates that the combined action of EYA1 and SIX1 might first transactivate the expression of *Neurog1*, which in turn might interact with EYA1 and SIX1 to induce *Neurod1* expression to drive the neuronal differentiation program (supplementary material Fig. S3). In support of this, we found that combination of *Neurog1* and *Eya1*, or *Neurog1* and *Six1* is insufficient to activate *Neurod1* expression, and that EYA1 and SIX1 interact physically with NEUROG1. This explains why *Neurod1* expression is also absent in the *Eya1*$^{-/-}$;*Six1*$^{-/-}$ double mutant and *Neurog1*$^{-/-}$ otocyst.

All *Neurog1*$^+$ or *Neurod1*$^+$ cells transfected with *Eya1* and *Six1* are not only TUJ1$^+$ but also NF$^+$, indicating that the nonsensory epithelial cells in the cochlea are competent to become mature neurons. As combination of *Neurog1* and *Neurod1* is incompetent to induce the expression of NF in cochlear explants (Table 1) (Puligilla et al., 2010), EYA1 and SIX1 are also likely to interact with NEUROD1 and other downstream bHLH factors to regulate neuronal differentiation/maturation (supplementary material Fig. S3). Indeed, we found that EYA1 and SIX1 interact physically with NEUROD1 and that these three factors act synergistically to induce NF$^+$ neurons.

Requirement of the SWI/SNF complex for mediating the transcriptional activities of *Neurog1* and *Neurod1* induced by EYA1 and SIX1

The specification of developmental programs by transcription factors requires epigenetic changes necessary for the activation of silent genes. Our results show that in order to accomplish this, EYA1 and SIX1 interact with SWI/SNF subunits BAF170 and BRG1, and require the BRG1 ATPase activity. Knocking down endogenous BRG1 activity in the otocyst not only severely impaired normal neurogenesis but also blocked ectopic neurogenesis induced by *Eya1* and *Six1*. Thus, endogenous functional SWI/SNF enzymes are necessary for EYA1 and SIX1 binding to the *Neurog1* and *Neurod1* promoters to drive otic neurogenesis.

Consistent with previous observations that BAF155 and BAF170 form a heterodimer in committed neuronal lineage (Chen and Archer, 2005; Ho and Crabtree, 2010), co-expression of *Baf155* together with *Baf170*, *Brg1*, *Eya1* and *Six1* led to a robust neuronal induction, and such synergy is probably achieved through interaction with BAF170 as no interaction was found between BAF155 and EYA1, SIX1, EYA1 and SIX1, or BRG1, EYA1 and SIX1 (supplementary material Fig. S3). Nonetheless, the physical interaction between EYA1 and SIX1, BRG1, BAF170, and NEUROG1 and NEUROD1 suggests that the SWI/SNF complex are recruited to *Neurog1* and *Neurod1* target loci and remodel the chromatin structure to activate transcription of these genes. Thus, co-expression of EYA1 and SIX1 might first lead to upregulation of chromatin-remodeling activities and then recruit and require the chromatin-remodeling activities for stable binding to the regulatory regions of *Neurog1* and *Neurod1* or other neuron-specific genes. Future studies will be required to elucidate how the SWI/SNF remodeling complexes are recruited to their site of action and whether they are recruited by transient interactions with EYA1, SIX1, SOX2, NEUROG1 and NEUROD1 and with DNA-binding proteins that recognize specific DNA sequences. Furthermore, identification of regulatory sequences of *Neurog1* and *Neurod1* and elucidation of their regulations by SIX1 and EYA1 and their co-factors at molecular levels will be absolutely necessary.

SOX2 cooperates with EYA1 and SIX1 and with the SWI/SNF complex to coordinate neuronal development

Sox2 is co-expressed with *Eya1* and *Six1* in placodal cells, neuroblasts and differentiating cochlear neurons (Kalatzis et al., 1998; Puligilla et al., 2010; Zheng et al., 2003; Zou et al., 2008). However, the functional significance of their expression is not understood. Previous studies have shown that SOX1-3 maintain neural progenitors in an undifferentiated state and suppress neuronal differentiation in CNS because of antagonistic interaction between SOX and proneural bHLH proteins (Bylund et al., 2003), and that eya1/six1 appear to interact with sox2 and sox3 for this process in *Xenopus* cranial placodes (Schlosser et al., 2008). In *Eya1* or *Six1* knockouts, proliferation of otic ectodermal progenitors is significantly reduced, leading to arrest of inner ear development at the otocyst stage (Zheng et al., 2003; Zou et al., 2006). Thus, early in otic development, EYA1 and SIX1 might act cooperatively with SOX2 to regulate cell proliferation in order to expand the ectodermal progenitors. During neuronal induction and differentiation, EYA1 and SIX1 function as transcriptional activators and interact with the SWI/SNF complex to induce the expression of *Neurog1* and *Neurod1* to determine the transition from ectoderm to neuronal fate. The activity of SOX2 appears to synergize with the effect of EYA1 and SIX1 and the SWI/SNF complex in activating *Neurog1* and *Neurod1* transcription and neuronal differentiation, and when all five proteins SOX2, EYA1, SIX1, BAF170 and BRG1 are present, a robust production of NF^+ neurons is observed. Such synergy suggests that SOX2 might interact physically with EYA1, SIX1, BAF170 and BRG1. Indeed, we found that these proteins are physically associated as demonstrated by Co-IP analysis (Fig. 9), and that SOX2 interacts directly with EYA1 or SIX1 (Ahmed et al., 2012).

The GER cells in the cochlea have been suggested to be neurosensory stem cells as they can be induced to differentiate into neurons (present study) (Puligilla et al., 2010) or sensory hair cells (Izumikawa et al., 2005; Zheng and Gao, 2000). We have recently shown that EYA1 and SIX1 can induce ~89% of transfected GER cells into hair cells (Ahmed et al., 2012). In contrast to the synergistic effect observed between SOX2 and EYA1 and SIX1 in inducing the GER cells to differentiate into neurons, SOX2 antagonizes differentiation of $ATOH1^+$ cells into $MYO7A^+$ hair cells (Dabdoub et al., 2008). Our results clearly show that EYA1 and SIX1 can cooperate with the SWI/SNF complex and SOX2 to efficiently reprogram the cochlear neurosensory stem cells to differentiate into neurons instead of hair cells (Table 3). Thus, EYA1 and SIX1 are likely to operate through different mechanisms to induce the earlier neuronal versus the later sensory fate during development.

It is currently unknown whether BRG1 or BAF170 play any role during inner ear development. As *Brg1*-null mice die before implantation (Bultman et al., 2000), inner ear-specific deletion of *Brg1* or *Baf170* is required to address their importance in the inner ear. Our loss-of-function approach by knocking down endogenous BRG1 activity identifies its requirement during early inner ear neurogenesis as well as neurogenesis in other ectoderm-derived sensory placodes. A recent report has shown that the ATP-dependent chromatin-remodeling enzyme CHD7 regulates proneural gene expression and neurogenesis in the inner ear but might function in parallel with *Eya1* (Hurd et al., 2010). Another ATP-dependent chromatin-remodeling protein CECR2 has also been reported to be involved in inner ear development (Dawe et al., 2011). It will be interesting to test whether there is functional redundancy or crosstalk between these different ATP-dependent chromatin-remodeling complexes in mediating neuronal differentiation in the inner ear. The fact that SOX2 interacts with CDH7 (Engelen et al., 2011) and our observation that SOX2 interacts with BRG1 and BAF170 suggest a strong link between different complexes, indirectly linking EYA1 and SIX1 to other chromatin-remodeling complexes.

In summary, our findings define EYA1 and SIX1 as key factors for initiating neuronal development in the inner ear by inducing the expression of *Neurog1* and *Neurod1*, which is accomplished by interaction with the SWI/SNF chromatin-remodeling complex. We demonstrate that EYA1 and SIX1 work together with the complex and SOX2 to efficiently reprogram cochlear GER cells to become neurons instead of hair cells. Our loss-of-function study using *shBrg1* demonstrates a requirement of BRG1 activity for normal neurogenesis in the otocyst. Thus, EYA1 and SIX1 are likely to interact with the SWI/SNF complex to induce a subset of otic ectodermal cells to develop into neurons. Our findings of a robust neuronal induction by a combination of EYA1, SIX1, BAF155, BAF170 and BRG1, or EYA1, SIX1, BAF170, BRG1 and SOX2 might have regenerative and therapeutic implications for restoring neuronal function in sensory systems.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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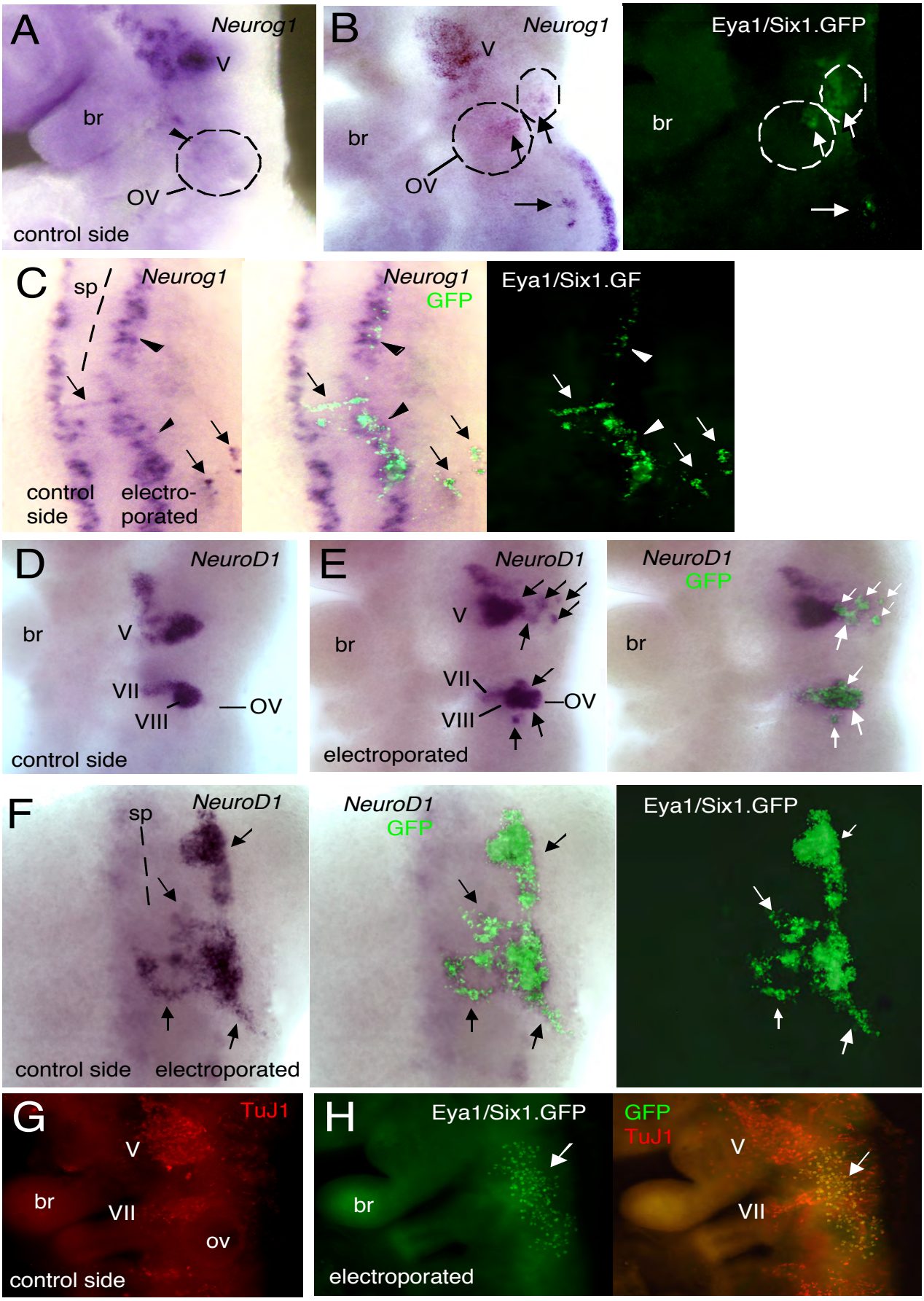
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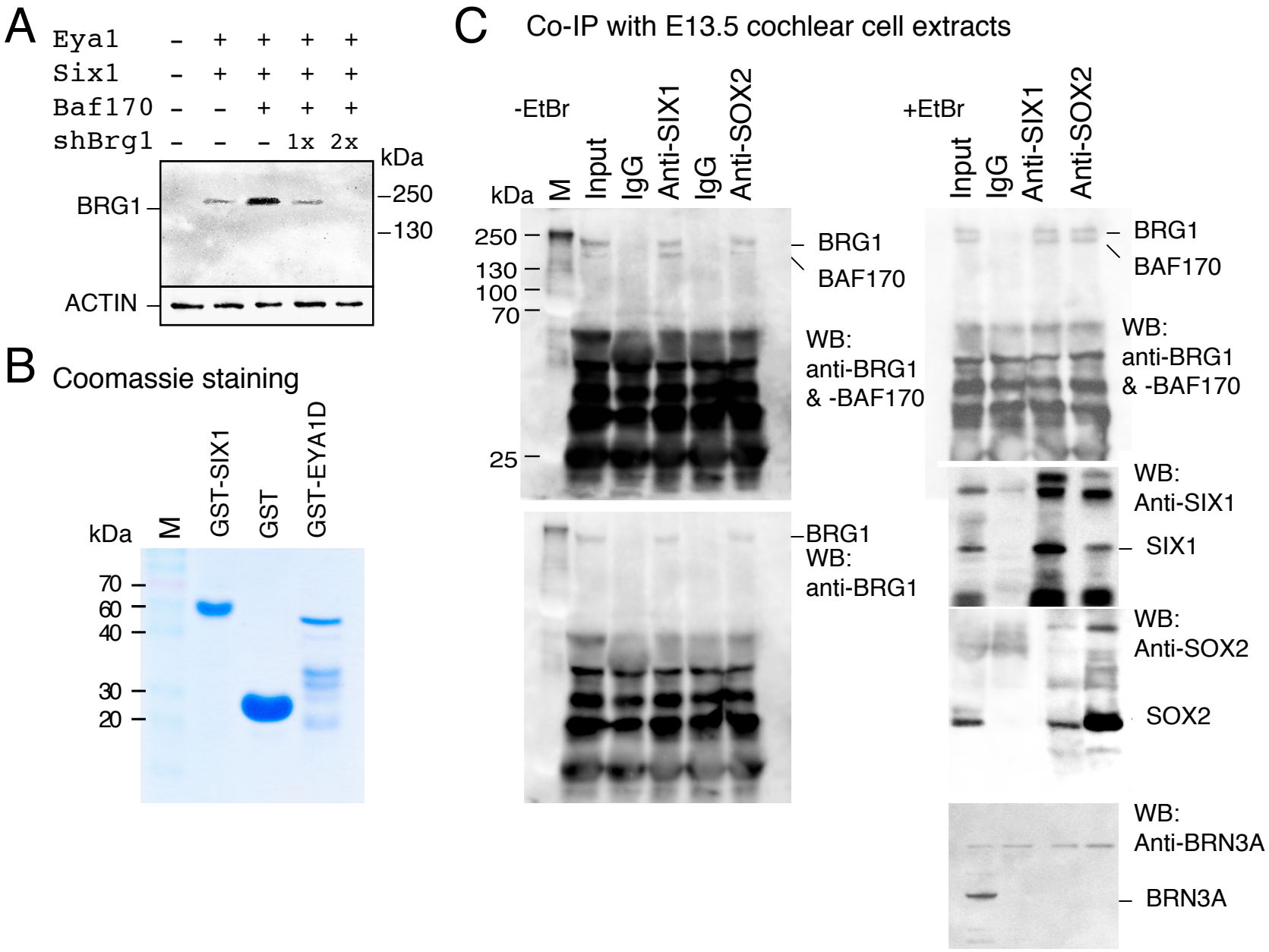
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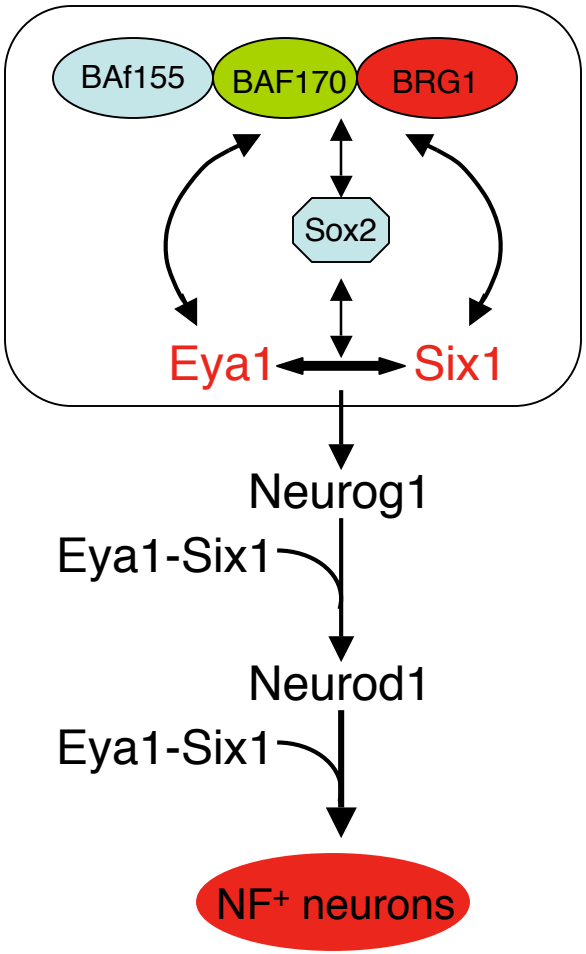
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