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 Eya and Six 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).
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 Eya1lox2 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

Eya1EGFP or Six1EGFP 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), Brg1K798 (Addgene plasmid 1864), scramble shRNA in pLKO.1 lentiviral vector (Addgene plasmid 1864) (Sarbassov et al., 2005), mU6-Brg/Brm shRNA (pRVG-P-BB) (Addgene plasmid 15380) (Ramirez-Carrozzi et al., 2006), Sox2 shRNA (Addgene plasmid 26353) (Bass et al., 2009) and Neurod1-lo-GFP (Addgene plasmid 19414). The full-length cDNA of Six1 or the Eya domain of Eya1 was cloned into the pGBK7 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 msec 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’-CCCTAGATCACTAACAA-3’ (forward) and 5’-CTAATTGAAGT TCGATGCT-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 (colp), GST pull-down assay and western blot

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

For colP 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 (Bullman 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 colP.

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-BRGI, NEUROG1 or NEUROD1 protein made using TNT-coupled rabbit reticulocyte lysate (Promega).

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’-CCCTAGATCACTAACAA-3’ (forward) and 5’-CTAATTGAAGT TCGATGCT-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.

RESULTS

Otocyst-derived neuroblasts are not specified in mice lacking both Eya1 and Six7

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, Neurod1 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 Eya1lacZ/+ or Six1lacZ/+ 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 Eya1lacZ/+ or Six1lacZ/+ 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. 2. Eya1 and Six1 are expressed in otic neuroblast precursors and spiral ganglion. (A–F) Sections of X-gal-stained E8.75 Eya1lacZ/embryo (A), E14.5 Eya1lacZ+ cochlear (B), E16.5 Eya1lacZ+ cochlear stained with anti-NEUROD1 (red, C), E9.5 Six1lacZ+ embryo (arrow points to differentiating neurons within the VIIIth ganglionic anlagen) (D), E15.5 Six1lacZ+ cochlear (E), E15.5 Six1lacZ+ cochlear stained with anti-NEUROD1 (red, F). GER and LER are indicated. oc, otic cup; sg, spiral ganglion. Scale bars: 50 μm.

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 VIIth-VIIIth ganglionic regions (Fig. 3A,B). We isolated the otocyst including the VIIth-VIIIth 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 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.
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 Neurod1 in Xenopus (Seo et al., 2005). Through our yeast two-hybrid screen

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

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

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.
Representing an example data set of three independent experiments for ability to activate alone was used as negative control. Co-transformation was analyzed neither factor alone induced any NF+ neurons (data not shown).

~30.4% of 3T3 fibroblast cells 4 days post-transfection with \( \text{Brg1} \) and \( \text{Sixl} \) induced by EYA1 and SIX1 require SWI/SNF ATPase activity in developing otocyst and VIIIth ganglion at E10.0 and in the spiral ganglion. Scale bars: 100 μm.

Remarkably, however, combination of \( \text{Eya1} \) and \( \text{Sixl} \) 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 \( \text{Eya1} \) and \( \text{Brg1} \) induced very few TUJ1+ or NF+ neurons (Table 2), whereas co-expression of \( \text{Sixl} \) and \( \text{Baf170} \), \( \text{Eya1} \) and \( \text{Baf170} \), or \( \text{Brg1} \) and \( \text{Baf170} \) was incapable of promoting neuronal development (Table 2).

Co-expression of \( \text{Eya1} \) and \( \text{Sixl} \) resulted in upregulation of BRG1 expression levels compared with untransfected 3T3 cells, whereas addition of \( \text{Baf170} \) together with \( \text{Eya1} \) and \( \text{Sixl} \) led to a large increase in the levels of BRG1 expression (supplementary material Fig. S2A). These results indicate that \( \text{Eya1} \) and \( \text{Sixl} \), or \( \text{Baf170} \), \( \text{Eya1} \) and \( \text{Sixl} \), 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 \( \text{Brg1} \) and \( \text{Eya1} \), or \( \text{Brg1} \) and \( \text{Sixl} \), is incapable of inducing neuronal development (Table 2). However, combination of \( \text{Eya1} \) and \( \text{Sixl} \) with either \( \text{Baf170} \) or \( \text{Brg1} \) 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 \( \text{Brg1} \), \( \text{Eya1} \), \( \text{Sixl} \) combination (Table 2), indicating that BAF155 cannot substitute BAF170 to interact with EYA1 and SIX1. However, when \( \text{Baf155} \), \( \text{Baf170} \), \( \text{Brg1} \), \( \text{Eya1} \) and \( \text{Sixl} \) 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 \( \text{Eya1} \) and \( \text{Sixl} \) increased the levels of BRG1 expression compared with untransfected 3T3 cells, and addition of \( \text{Baf170} \) together with \( \text{Eya1} \) and \( \text{Sixl} \) 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 \( \text{Brg1K798R} \) mutant construct, which contains a mutation in the ATP-binding site of BRG1, together with \( \text{Eya1} \) and \( \text{Sixl} \), or \( \text{Baf170} \), \( \text{Eya1} \) and \( \text{Sixl} \) failed to induce neuronal development (Fig. 8A,B; Table 2), indicating that the BRG1K798R mutant has a dominant-negative effect, which completely blocks neurogenesis.
EYA1 and SIX1 in neuronal induction

Figure 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 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 (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.

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-
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 cochlea 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+ 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 Neurod1+ neurons 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 normal phenotype.
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.
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.
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, Eya1, Eya2 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 BAF170, 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 cofactors 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 placoid 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 eya/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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References


