Published in final edited form as:

Hear Res. 2013 March ; 297: 13–19. doi:10.1016/j.heares.2012.09.009.

EYA1—SIX1 complex in neurosensory cell fate induction in the mammalian inner ear

Elaine Y.M. Wong^{a,*}, Mohi Ahmed^a, and Pin-Xian Xu^{a,b,**}

^aDepartment of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York, NY 10029, USA

^bDevelopmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY 10029, USA

Abstract

The phosphatase-transactivator EYA1 interacts with the homeodomain protein SIX1 to form transcriptional activation complexes, which play essential roles in regulating cell proliferation, survival and induction of sensory and neuronal differentiation programs during inner ear development. Mutations of the *Eya1* and *Six1* genes cause profound developmental auditory defects in mice and humans. The molecular mechanisms and developmental processes controlled by the EYA1 and SIX1 complex in inner ear development and neurosensory fate induction are the focus of this review.

1. Introduction

Members of the Eyes absent (Eya) and Sine oculis (So/Six) gene families of transcription factors are important regulators for inner ear development and have been implicated in human genetic syndromes with congenital auditory system malformations. The vertebrate genome has at least 4 different Eya genes and 6 So/Six genes with diverse regulatory functions in development and disease (Borsani et al., 1999; Kawakami et al., 2000; Oliver et al., 1995a,b; Xu et al., 1997b). The original Eya (Bonini et al., 1993) and So (Cheyette et al., 1994) genes were reported in *Drosophila* as key regulators for eye development. Since then, they were identified in vertebrate and invertebrate organisms from worms, frogs, fish, and birds to higher vertebrates (Abdelhak et al., 1997b; Bessarab et al., 2004; Dozier et al., 2001; Hoshiyama et al., 2007; Kawakami et al., 2000; Pineda et al., 2000; Sahly et al., 1999; Seo et al., 1999; Serikaku and O'Tousa, 1994; Xu et al., 1997b; Zimmerman et al., 1997). While So/Six genes encode homeodomain transcription factors with direct DNA-binding capacity, Eya genes encode proteins that affect transcription indirectly by interacting with DNA-binding proteins such as SO/SIX as well as other DNA-binding proteins. The key role of EYA-SO/SIX during development was first revealed by loss- and gain-of-function studies in fruitflies. In *Drosophila*, *eya* and *so* genes function in a molecular network with the fly *Pax6* gene *eyeless* (*ey*) and *dachshund* (*dach*) to regulate eye morphogenesis (Fig. 1) (Treisman,

*Corresponding author. Elaine.wong@mssm.edu (E.Y.M. Wong). **Corresponding author. Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York, NY 10029, USA. pinxian.xu@mssm.edu (P.-X. Xu).

1999). The components of this molecular network have been highly conserved during evolution with related genes in mammals regulating the development of multiple organ systems, including the inner ear (Xu et al., 2003; Zheng et al., 2003).

The first indication of the importance of Eya and Six genes in auditory system development was the identification of human EYA1 as the gene responsible for Branchio-Oto-Renal (BOR) and Branchio-Oto (BO) syndrome, which are characterized by combinations of craniofacial defects and hearing loss with or without kidney anomalies. Approximately 93% of patients with BOR/BO syndrome show hearing loss, accounting for as many as 2% of profoundly deaf children (Abdelhak et al., 1997a). More recently, mutations in SIX1 and SIX5 have also been identified in BOR/BO patients (Hoskins et al., 2007; Ruf et al., 2004), while mutations in EYA4 cause late-onset autosomal dominant hearing loss at the DFNA10 locus (Pfister et al., 2002; Schonberger et al., 2005; Wayne et al., 2001). Recent studies have provided exciting data suggesting that EYA1 and SIX1 proteins have roles at several stages during neurosensory cell development, including cell fate specification, proliferation, differentiation and maintenance. However, many questions regarding the function and regulation of Eya and So/Six genes in the developing inner ear remain unanswered.

2. EYA and SO/SIX family proteins

The defining characteristic of the Eya proteins is the presence of a highly conserved 271–273—amino acid C-terminal domain, the Eya domain. The EYA proteins function as both transcription cofactors and phosphatases. The N-terminal domain (NTD) is highly divergent but possesses a transcriptional activation function (Xu et al., 1997a). The conserved Eya domain not only interacts with other proteins but also possesses a catalytic motif belonging to the phosphatase subgroup of the haloacid dehalogenase (HAD) superfamily of enzymes (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). This suggests that EYA is likely to regulate the phosphorylation state of its transcriptional cofactors or itself to directly modify transcriptional output within a cell. However, it is currently not understood whether the two functions depend on each other and how they operate during development in mammals. To date, the DNA damage-related histone H2AX was the only physiological relevant substrate that has been reported for EYA's tyrosine phosphatase activity (Cook et al., 2009; Krishnan et al., 2009). While the transactivation activity of the EYA–SIX complex represents a nuclear function and EYA's tyrosine phosphatase activity in DNA damage repair is also a nuclear function, there has been some evidence that the phosphatase activity of the EYAs may participate in a cytoplasmic cellular function. The cytoplasmic EYA4 plays a role in innate immunity via its threonine phosphatase activity (Okabe et al., 2009) and cytoplasmic EYA3 promotes cell motility via its tyrosine phosphatase activity (Pandey et al., 2010). Additional cytoplasmic function has been suggested for EYA proteins, as G protein subunits were isolated as binding partners for EYA2 (Embry et al., 2004; Fan et al., 2000; Ozaki et al., 2002). Future studies involving identification of more binding partners and in vivo substrates of EYA will likely reveal how its functions as both transcriptional coactivator and phosphatase are used throughout development.

The SO/SIX family represents a divergent group of the homeodomain (HD) superfamily of transcription factors and it contains two evolutionarily conserved domains, a 60—amino acid

HD and a 110–115—amino acid SO/SIX-specific domain (SD), which interacts with EYA and other proteins (Pignoni et al., 1997). SO/SIX family members have been divided into three subfamilies (SIX1/2, SIX4/5 and SIX3/6) on the basis of *Drosophila* SO, which belongs to the same subfamily as vertebrate SIX1/2. The SO/SIX HD binds to a TGATAC consensus sequence, differing from the classic TAAT core sequence because it lacks two highly conserved amino acid residues typical of most HDs (Kawakami et al., 1996a,b; Serikaku and O'Tousa, 1994; Spitz et al., 1998). Depending on the context, SO/SIX proteins function as either transcriptional activators (Ohto et al., 1999; Silver et al., 2003) or repressors (Li et al., 2002). When coexpressed with EYA, EYA-SO/SIX interactions lead to formation of a bipartite transcription factor, with the HD of SO/SIX providing the DNA-binding activity and the NTD of EYA providing the transactivation potential, which leads to potent transcriptional activation—even under conditions in which SO/SIX alone acts as a repressor (Ahmed et al., 2012b.; Li et al., 2002; Ohto et al., 1999; Silver et al., 2003).

To date, several binding partners of EYA and SO/SIX proteins have been identified by yeast two-hybrid screens and immunoprecipitation. Recent studies have demonstrated that SOX2 is an essential factor directly interacting with EYA1 or SIX1 both in vitro and in vivo to synergize transcriptional output of EYA1–SIX1 during sensory hair cell induction and neurogenesis in the inner ear (Ahmed et al., 2012a,b; Zou et al., 2008). In addition to SOX2, components of the SWI/SNF chromatin-remodeling complex BRG1 and BAF170 and the helix-loop-helix (bHLH) transcription factors, NEUROG1 and NEUROD1 are found to be binding partners of EYA1 and SIX1 in the inner ear (Ahmed et al., 2012a). Two novel EYA1-binding partners Sipl1 (Shank-interacting protein-like 1) and Rbck1 (RBCC protein interacting with PKC1) have been recently identified through yeast two-hybrid screens and they may interact with EYA1 during craniofacial development (Landgraf et al., 2010). However, how these protein–protein interactions modify transcriptional activities remains to be elucidated. Since *Eya1* and *Six1* are coexpressed and interact in neurosensory cells of the inner ear, and mutants of *Eya1* and *Six1* exhibit a very similar spectrum of deficiencies in mice and humans as well as in lower vertebrates (Abdelhak et al., 1997b; Bricaud and Collazo, 2006; Brugmann et al., 2004; Kozlowski et al., 2005; Laclef et al., 2003; Li et al., 2003,2010; Nica et al., 2006; Ozaki et al., 2004; Ruf et al., 2004; Schlosser, 2005, 2006; Xu et al., 1999; Zheng et al., 2003; Zou et al., 2004), below we will focus on specific functions of EYA1–SIX1 complex during neurosensory cell development.

3. *Eya1* and *Six1* family genes in early neurosensory lineage development

The inner ear is a beautiful yet complex sensory organ that originates from the otic vesicle (also called otocyst). Its development begins ~E8.0 in mice as a placodal thickening (the otic placode) in the surface ectoderm next to the hindbrain neuronal tube that is induced by the adjacent dorsal hindbrain and the underlying mesoderm. The otic placode invaginates to form the otic cup and vesicle, which subsequently undergoes elaborate processes that include patterning, cellular growth, apoptosis, migration and differentiation to eventually give rise to different regions of the inner ear. In the mouse, the basic architecture of the inner ear is fully established by E14.5 (Morsli et al., 1998).

The otocyst is specified and diversified by combinatorial gene expression and gives rise to three main cell types: neuronal, sensory (future hair cells and supporting cells) and nonsensory. The neuronal lineage is the earliest cell fate specified within the otic epithelium (~E9.0–E9.5) (Fekete and Wu, 2002). Soon after the otocyst forms, the neuroblasts delaminate from the anteroventral region and migrate into the adjacent mesoderm where they coalesce to form the auditory and vestibular ganglion (gVIII), which provides the sensory innervation for the inner ear (Barald and Kelley, 2004). During the delamination of otic neuroblasts, gene expression studies suggest that the otocyst simultaneously becomes regionally patterned along several different axes, also referred to as compartmentalization (Fekete and Wu, 2002). From ~E11.0, the ventral region of the otocyst elongates and coils to form the cochlear duct.

The essential roles of *Eya1* and *Six1* in neurosensory cell lineage development have been demonstrated by loss-of-function studies in mice. *Eya1* and *Six1* are coexpressed in otic placode from as early as E8.5–8.75 and in neuroblast precursors before their delamination. In the otocyst, *Eya1* and *Six1* are coexpressed in the ventral region where vestibular and auditory sensory epithelia form and in delaminating neuroblasts as well as the vestibuloacoustic ganglion (Fig. 2) (Xu et al., 1997a,b; Zheng et al., 2003). At later stages, these two genes are expressed in all the six sensory epithelia of the inner ear. Inactivation of either gene leads to malformation of the outer, middle and inner ears (Xu et al., 1999; Zheng et al., 2003). The inner ear development in *Eya1*^{-/-} or *Six1*^{-/-} embryos arrested at the otocyst stage and all components of the inner ear failed to form due to increased cell death and reduced cell proliferation in the otic epithelium (Xu et al., 1999; Zheng et al., 2003; Zou et al., 2006). Both genes are not only required for normal growth and maintenance but also for regional specification of the otocyst, as all sensory or neuronal markers failed to be expressed in their mutant otocysts (Xu et al., 1999; Zheng et al., 2003; Zou et al., 2006), thus causing ventral shift of expression of dorsal markers.

In addition to the inner ear, EYA1 or SIX1 is required for normal cell proliferation and survival for several other organs that were affected in their mutants. Recent studies have suggested that SIX1 may promote proliferation by interaction with cell cycle control genes such as *CyclinA1*, *CyclinD1*, and *c-Myc* (Coletta et al., 2004; Ford et al., 1998; Li et al., 2003; Yu et al., 2006) and that EYA1 may modulate apoptosis and survival decisions by dephosphorylating histone H2AX (Cook et al., 2009). Future studies are necessary to characterize the precise roles of *Eya1* and *Six1* in regulating proliferation and cell survival.

Recently, studies have identified *Sox2* as a key gene for sensory and neuronal cell specification in the inner ear (Kiernan et al., 2005; Puligilla et al., 2010). *Eya1* and *Six1* are coexpressed with *Sox2* in the otic placode and the ventral otocyst (Fig. 2) (Zheng et al., 2003; Zou et al., 2008). All three genes have early roles in the inner ear and are required for inner ear morphogenesis (Puligilla et al., 2010; Zou et al., 2004). New insights into the important roles of EYA1–SIX1 complex in combination with SOX2 in triggering neuronal and sensory developmental programs have recently been obtained (Ahmed et al., 2012a,b). Below, EYA1–SIX1 complex and its interaction with SOX2 in inducing sensory and neuronal cell fate as well as their differentiation will be discussed.

4. EYA1–SIX1 complex in inducing hair cell development

Besides the early roles of EYA1 and SIX1 in inner ear development, the EYA1–SIX1 transcriptional activation complex has recently emerged as a key regulator of hair cell differentiation. The ventral portion of the otocyst elongates and begins to coil ~E12 to reach a full 1.5 turns of cochlear duct by E17.5. Previous studies have revealed that the prosensory progenitors that give rise to the entire organ of Corti are specified between E12–E14 in a region termed the prosensory domain that is distinctively marked by the expression of cyclin-dependent kinase inhibitor p27^{Kip1} (Chen and Segil, 1999; Fekete, 2000; Ruben, 1967) (Fig. 2). The progenitor cells exit the cell cycle from apex toward base of the cochlear duct between E12 to E14 (Chen et al., 2002; Lee et al., 2006). Functional studies have shown that *Sox2* is required for specifying the precursors, as inner ear structures derived from the prosensory progenitors are not formed in *Sox2*-deficient mice (Kiernan et al., 2005). Nonsensory cells develop adjacent to the sensory epithelium and reside within the greater and lesser epithelial ridges (GER/LER) flanking the organ of Corti. *Eya1* and *Six1* are coexpressed with *Sox2* in the entire cochlear duct by E12.5 and are required for cochlear duct formation, as inner ear development arrests at otocyst stage in their null mutants. All three genes also show differential expression in the nonsensory epithelium with *Eya1* and *Sox2* in the GER and *Six1* in both the GER and LER, suggesting that they may play essential roles in regulating differentiation of the organ of Corti.

In the nascent organ of Corti, hair cell differentiation initiates from base toward apex, as expression of the hair cell differentiation factor gene *Atoh1* begins in the base of the cochlea between E13.5 and E14.5, and reaches the apex at ~E17.5 (Chen et al., 2002). Recent findings have provided evidence that such developmental patterns of *Atoh1* expression are likely to be achieved by combinatorial regulation of *Eya1*, *Six1* and *Sox2* (Ahmed et al., 2012b), as discussed below.

Between E12.5 and 13.5 when the progenitors within the prosensory domain begin to exit the cell cycle, a high level of *Eya1* and *Sox2* expression becomes restricted to the postmitotic progenitors, whereas *Six1* expression disappears in the postmitotic progenitors within the prosensory domain (Fig. 2). From E13.5, *Six1* shows a gradient of expression that is similar to the pattern of *Atoh1* but appears to occur before the onset of *Atoh1* expression (Ahmed et al., 2012b). This pattern of *Six1* expression in the organ of Corti suggests that it may serve as a critical positive regulator for stimulating *Atoh1* activation near the basal cochlear duct. Supporting evidence for this hypothesis comes from both gain- and loss-of-function studies in mice.

In a gain-of-function experiment in cochlear explants, Ahmed et al. (2012b) demonstrated that nonsensory epithelial cells within the GER electroporated with *Eya1/Six1* expression plasmids can be induced to become sensory hair cells. Approximately 89% of the electroporated cells differentiated into Myo7a⁺ hair cells. One would expect all the ectopic hair cells to express *Atoh1*, however this was not the case. The vast majority (~61%) were in fact *Atoh1*[−], suggesting that there exists an *Atoh1*-independent pathway for hair cell development. Unlike *Atoh1*, all the ectopic hair cells were POU4F3⁺. Before this study, ATOH1 was the only known and accepted factor responsible for hair cell fate determination

(Bermingham et al., 1999). This study reveals that there is an *Atoh1* - dependent and - independent pathway for hair cell development, and POU4F3 appears to be the common downstream factor that is activated in both pathways. The authors further demonstrated that the ability of EYA1/SIX1 to induce endogenous *Atoh1* expression to induce a hair cell fate depends on endogenous SOX2 activity.

To directly address the requirement of *Eya1/Six1* in hair cell development, the authors used an inducible system to delete these genes in vivo by crossing transgenic mice expressing tamoxifen regulated Cre recombinase under control of the 1.4 kb *Atoh1* enhancer (*Atoh1CreERT2*) with either *Eya1^{flox}* or *Six1^{flox}* mice. No hair cells were induced when either gene function was abolished and hair cells that had already formed were eventually lost when cultured in vitro.

The molecular link between *Eya1*, *Six1*, *Sox2* and *Atoh1* has been revealed (Fig. 3). SOX2 and SIX1 are DNA-binding proteins and binding sites for SOX2 and SIX1 were found in the 1.4 kb *Atoh1* enhancer, which is located ~3.4 kb 3' of the *Atoh1* coding sequence and contains two conserved elements (element A and B) and can direct expression of reporter transgenes to the inner ear hair cells (Chow et al., 2006; Helms et al., 2000). Two specific regions being the essential elements for mediating SOX2- or SIX1- dependent transcriptional activation were found to be located in the conserved element A and B respectively. The authors found that EYA1, SIX1 and SOX2 cooperatively interact via direct protein-protein interaction and that the ability of each transcription factor to induce the endogenous *Atoh1* expression is partially dependent upon the other. Endogenous SOX2 activity in the GER cells is not only necessary for endogenous *Atoh1* activation but also for activating the 1.4 kb *Atoh1*-GFP reporter. The authors proposed that SIX1 may stimulate the initial expression of *Atoh1* in cooperation with SOX2 and EYA1 to initiate hair cell differentiation within the organ of Corti. In the absence of SIX1, EYA1/SOX2 alone may not lead to the formation of an active complex capable of transcriptional activation, which explains why in the primordial organ of Corti, *Atoh1* activation is not initiated in all prosensory precursors that express both EYA1 and SOX2. This study also revealed that SOX2 represses POU4F3⁺ cells to differentiation into MYO7A⁺ hair cell (Fig. 3).

5. Molecular mechanism by which EYA1–SIX1 complex drives neuronal differentiation

New findings have revealed that EYA1–SIX1 appears to operate through different mechanisms to induce neuronal versus sensory fates (Ahmed et al., 2012a), with the former induced several days earlier during development than the latter. In the auditory system, neuronal precursors become distributed in a spiral pattern (referred to as the spiral ganglion) that mirrors the distribution of their peripheral targets, the mechanosensory hair cells located along the cochlea. Spiral ganglion neurons extend dendritic processes that innervate both inner and outer hair cells with 95% of all afferent synapses occurring on inner hair cells. Loss of spiral ganglion neurons is believed to contribute to decreased hearing acuity and presence of intact spiral ganglion neurons is required for cochlear implant function. Despite

their crucial role in auditory function, the molecular pathways that mediate spiral ganglion neuron formation are not fully understood.

Eya1 and *Six1* stay coexpressed in differentiating cochlear neurons in the spiral ganglia. As discussed above, both genes are required for the neuroblast specification as *Neurog1* and *Neurod1* are not expressed in *Eya1;Six1* double mutant otocyst, which still express dorsal marker such as *Dlx5* (Ahmed et al., 2012a).

Recent studies have shown that the cochlear nonsensory GER cells are not only competent to develop as sensory hair cell fate but also as neurons since ectopic expression of *Neurog1* or *Neurod1* within these cells is sufficient to induce a neuronal phenotype (Puligilla et al., 2010). Overexpression of *Sox2* in the GER cells can also induce a neuronal phenotype (Puligilla et al., 2010). However, neither factor alone nor combination of all three factors together is capable of inducing Neurofilament⁺ cells, indicating that additional factors are necessary for promoting neuronal differentiation. In a recent gain-of-function study, Ahmed et al. (2012a) demonstrated that overexpression of both *Eya1* and *Six1* is sufficient to convert cochlear GER cells into neurons that not only express *Neurog1*, *Neurod1* and TuJ1 but also mature neuronal markers such as Neurofilament. The authors further demonstrated that overexpression of *Eya1/Six1* can also convert non-neuronal epithelial cells within the otocyst, head ectoderm and 3T3 fibroblast cells into neurons (Ahmed et al., 2012a).

When *Eya1* and *Six1* are ectopically expressed in the GER cells, only ~11% of transfected cells were induced to neurons (Ahmed et al., 2012a), while the other ~89% of transfected cells were induced to differentiate into hair cells (Ahmed et al., 2012a). Interestingly, EYA1 and SIX1 were found to interact directly with the SWI/SNF chromatin-remodeling subunits BRG1 and BAF170 to drive neurogenesis cooperatively in 3T3 cells and cochlear non-sensory epithelial cells. SOX2 also cooperates with these factors to mediate neuronal differentiation, as when all five factors are overexpressed together, ~100% of transfected GER cells or 3T3 cells became neurons. Furthermore, the ATPase activity of BRG1 is required for not only EYA1- and SIX1-induced ectopic neurogenesis but also normal neurogenesis in the otocyst (Ahmed et al., 2012a). Thus, EYA1 and SIX1 appear to be key transcription factors in initiating the neuronal developmental program, mostly likely by recruiting and interacting with the SWI/SNF chromatin-remodeling complex to specifically mediate *Neurog1* and *Neurod1* transcription (Fig. 4). EYA1 and SIX1 activities are also required for neuronal differentiation based on their synergistic interactions with NEUROG1 or NEUROD1 during neuronal maturation (Fig. 4) (Ahmed et al., 2012a). How the SWI/SNF chromatin-remodeling complex is recruited to chromatin, and the direct targets of EYA1-SIX1 in neuronal differentiation are interesting areas for future studies.

6. Conclusion and perspectives

The studies summarized here have begun to elucidate the important functions that *Eya1* and *Six1* play during multiple stages of inner ear development. These multiple functions such as maintaining a proliferative state of cells, triggering neuronal and sensory hair cell differentiation programs, and mediating cell survival, suggest a diversity of activity at the cellular level. This diversity is reflected at the molecular level by complex modes of

interaction with chromatin, multiple binding partners and sophisticated transcriptional activation and repression activities as well as dephosphorylation and phosphorylation activities. However, their precise mode of action and direct downstream targets in mediating proliferation and survival of neurosensory progenitors and inducing neuronal and sensory differentiation programs in the inner ear have left much to be revealed. Future studies of cell type- and stage-specific knockouts, genetic lineage tracing, proteomics and ChIP-seq analysis are essential for fully understanding how the EYA–SIX complexes work in different biological processes and disease states. New findings of a robust neuronal or hair cell induction via interaction with different factors may not only have new therapeutic implications but also reveal a way to reprogram new hair cells or auditory neurons for restoring sensory or neuronal function in the inner ear.

Acknowledgments

The authors wish to thank all of the past and present members of our lab for their many contributions and valuable discussions. Some of the work presented was supported by the NIH grant RO1 DC005824 (P-X. X.).

References

- Abdelhak S, Kalatzis V, Heilig R, Compain S, Samson D, Vincent C, Levi-Acobas F, Cruaud C, Le Merrer M, Mathieu M, Konig R, Vigneron J, Weissenbach J, Petit C, Weil D, 1997a Clustering of mutations responsible for branchio-oto-renal (BOR) syndrome in the eyes absent homologous region (eyaHR) of EYA1. *Hum. Mol. Genet* 6, 2247–2255. [PubMed: 9361030]
- Abdelhak S, Kalatzis V, Heilig R, Compain S, Samson D, Vincent C, Weil D, Cruaud C, Sahly I, Leibovici M, Bitner-Glindzicz M, Francis M, Lacombe D, Vigneron J, Charachon R, Boven K, Bedbeder P, Van Regemorter N, Weissenbach J, Petit C, 1997b A human homologue of the *Drosophila* eyes absent gene underlies branchio-oto-renal (BOR) syndrome and identifies a novel gene family. *Nat. Genet* 15, 157–164. [PubMed: 9020840]
- Ahmed M, Xu J, Xu PX, 2012a EYA1 and SIX1 drive the neuronal developmental program in cooperation with the SWI/SNF chromatin-remodeling complex and SOX2 in the mammalian inner ear. *Development* 139, 1965–1977. [PubMed: 22513373]
- Ahmed M, Wong EY, Sun J, Xu J, Wang F, Xu PX, 2012b Eya1-six1 interaction is sufficient to induce hair cell fate in the cochlea by activating Atoh1 expression in cooperation with Sox2. *Dev. Cell* 22, 377–390. [PubMed: 22340499]
- Barald KF, Kelley MW, 2004 From placode to polarization: new tunes in inner ear development. *Development* 131, 4119–4130. [PubMed: 15319325]
- Bermingham NA, Hassan BA, Price SD, Vollrath MA, Ben-Arie N, Eatock RA, Bellen HJ, Lysakowski A, Zoghbi HY, 1999 Math1: an essential gene for the generation of inner ear hair cells. *Science* 284, 1837–1841. [PubMed: 10364557]
- Bessarab DA, Chong SW, Korzh V, 2004 Expression of zebrafish six1 during sensory organ development and myogenesis. *Dev. Dyn* 230, 781–786. [PubMed: 15254912]
- Bonini NM, Leiserson WM, Benzer S, 1993 The eyes absent gene: genetic control of cell survival and differentiation in the developing *Drosophila* eye. *Cell* 72,379–395. [PubMed: 8431945]
- Borsani G, DeGrandi A, Ballabio A, Bulfone A, Bernard L, Banfi S, Gattuso C, Mariani M, Dixon M, Donnai D, Metcalfe K, Winter R, Robertson M, Axton R, Brown A, van Heyningen V, Hanson I, 1999 EYA4, a novel vertebrate gene related to *Drosophila* eyes absent. *Hum. Mol. Genet* 8, 11–23. [PubMed: 9887327]
- Bricaud O, Collazo A, 2006 The transcription factor six1 inhibits neuronal and promotes hair cell fate in the developing zebrafish (*Danio rerio*) inner ear. *J. Neurosci* 26, 10438–10451. [PubMed: 17035528]

- Brugmann SA, Pandur PD, Kenyon KL, Pignoni F, Moody SA, 2004 Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a transcriptional activator and repressor. *Development* 131, 5871–5881. [PubMed: 15525662]
- Chen P, Segil N, 1999 p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. *Development* 126, 1581–1590. [PubMed: 10079221]
- Chen P, Johnson JE, Zoghbi HY, Segil N, 2002 The role of Math1 in inner ear development: uncoupling the establishment of the sensory primordium from hair cell fate determination. *Development* 129, 2495–2505. [PubMed: 11973280]
- Cheyette BN, Green PJ, Martin K, Garren H, Hartenstein V, Zipursky SL, 1994 The *Drosophila* sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. *Neuron* 12, 977–996. [PubMed: 7910468]
- Chow LM, Tian Y, Weber T, Corbett M, Zuo J, Baker SJ, 2006 Inducible Cre recombinase activity in mouse cerebellar granule cell precursors and inner ear hair cells. *Dev. Dyn* 235, 2991–2998. [PubMed: 16958097]
- Coletta RD, Christensen K, Reichenberger KJ, Lamb J, Micomono D, Huang L, Wolf DM, Muller-Tidow C, Golub TR, Kawakami K, Ford HL, 2004 The Six1 homeoprotein stimulates tumorigenesis by reactivation of cyclin A1. *Proc. Natl. Acad. Sci. U. S. A* 101, 6478–6483. [PubMed: 15123840]
- Cook PJ, Ju BG, Telese F, Wang X, Glass CK, Rosenfeld MG, 2009 Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. *Nature* 458, 591–596. [PubMed: 19234442]
- Dozier C, Kagoshima H, Niklaus G, Cassata G, Burglin TR, 2001 The *Caenorhabditis elegans* Six/sine oculis class homeobox gene *ceh-32* is required for head morphogenesis. *Dev. Biol* 236, 289–303. [PubMed: 11476572]
- Embry AC, Glick JL, Linder ME, Casey PJ, 2004 Reciprocal signaling between the transcriptional cofactor Eya2 and specific members of the Galphai family. *Mol. Pharmacol* 66, 1325–1331. [PubMed: 15308761]
- Fan X, Brass LF, Poncz M, Spitz F, Maire P, Manning DR, 2000 The alpha subunits of Gz and Gi interact with the eyes absent transcription cofactor Eya2, preventing its interaction with the six class of homeodomain-containing proteins. *J. Biol. Chem* 275, 32129–32134. [PubMed: 10906137]
- Fekete DM, 2000 Making sense of making hair cells. *Trends Neurosci.* 23, 386.
- Fekete DM, Wu DK, 2002 Revisiting cell fate specification in the inner ear. *Curr. Opin. Neurobiol* 12, 35–42. [PubMed: 11861162]
- Ford HL, Kabingu EN, Bump EA, Mutter GL, Pardee AB, 1998 Abrogation of the G2 cell cycle checkpoint associated with overexpression of HSIX1: a possible mechanism of breast carcinogenesis. *Proc. Natl. Acad. Sci. U. S. A* 95, 12608–12613. [PubMed: 9770533]
- Helms AW, Abney AL, Ben-Arie N, Zoghbi HY, Johnson JE, 2000 Autoregulation and multiple enhancers control Math1 expression in the developing nervous system. *Development* 127, 1185–1196. [PubMed: 10683172]
- Hoshiyama D, Iwabe N, Miyata T, 2007 Evolution of the gene families forming the Pax/Six regulatory network: isolation of genes from primitive animals and molecular phylogenetic analyses. *FEBS Lett.* 581, 1639–1643. [PubMed: 17383640]
- Hoskins BE, Cramer CH, Silvius D, Zou D, Raymond RM, Orten DJ, Kimberling WJ, Smith RJ, Weil D, Petit C, Otto EA, Xu PX, Hildebrandt F, 2007 Transcription factor SIX5 is mutated in patients with branchio-oto-renal syndrome. *Am. J. Hum. Genet* 80, 800–804. [PubMed: 17357085]
- Kawakami K, Ohto H, Takizawa T, Saito T, 1996a Identification and expression of six family genes in mouse retina. *FEBS Lett.* 393, 259–263. [PubMed: 8814301]
- Kawakami K, Ohto H, Ikeda K, Roeder RG, 1996b Structure, function and expression of a murine homeobox protein AREC3, a homologue of *Drosophila* sine oculis gene product, and implication in development. *Nucleic Acids Res.* 24, 303–310. [PubMed: 8628654]
- Kawakami K, Sato S, Ozaki H, Ikeda K, 2000 Six family genes—structure and function as transcription factors and their roles in development. *Bioessays* 22, 616–626. [PubMed: 10878574]

- Kiernan AE, Pelling AL, Leung KK, Tang AS, Bell DM, Tease C, Lovell-Badge R, Steel KP, Cheah KS, 2005 Sox2 is required for sensory organ development in the mammalian inner ear. *Nature* 434, 1031–1035. [PubMed: 15846349]
- Kozłowski DJ, Whitfield TT, Hukriede NA, Lam WK, Weinberg ES, 2005 The zebrafish dog-eared mutation disrupts *eya1*, a gene required for cell survival and differentiation in the inner ear and lateral line. *Dev. Biol* 277, 27–41. [PubMed: 15572137]
- Krishnan N, Jeong DG, Jung SK, Ryu SE, Xiao A, Allis CD, Kim SJ, Tonks NK, 2009 Dephosphorylation of the C-terminal tyrosyl residue of the DNA damage-related histone H2A.X is mediated by the protein phosphatase eyes absent. *J. Biol. Chem* 284, 16066–16070. [PubMed: 19351884]
- Laclef C, Souil E, Demignon J, Maire P, 2003 Thymus, kidney and craniofacial abnormalities in Six 1 deficient mice. *Mech. Dev* 120, 669–679. [PubMed: 12834866]
- Landgraf K, Bollig F, Trowe MO, Besenbeck B, Ebert C, Kruspe D, Kispert A, Hanel F, Englert C, 2010 *Sip1* and *Rbck1* are novel *Eya1*-binding proteins with a role in craniofacial development. *Mol. Cell. Biol* 30, 5764–5775. [PubMed: 20956555]
- Lee YS, Liu F, Segil N, 2006 A morphogenetic wave of p27Kip1 transcription directs cell cycle exit during organ of Corti development. *Development* 133, 2817–2826. [PubMed: 16790479]
- Li X, Perissi V, Liu F, Rose DW, Rosenfeld MG, 2002 Tissue-specific regulation of retinal and pituitary precursor cell proliferation. *Science* 297, 1180–1183. [PubMed: 12130660]
- Li X, Oghi KA, Zhang J, Krones A, Bush KT, Glass CK, Nigam SK, Aggarwal AK, Maas R, Rose DW, Rosenfeld MG, 2003 *Eya* protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. *Nature* 426, 247–254. [PubMed: 14628042]
- Li Y, Manaligod JM, Weeks DL, 2010 EYA1 mutations associated with the branchio-oto-renal syndrome result in defective otic development in *Xenopus laevis*. *Biol. Cell* 102, 277–292. [PubMed: 19951260]
- Morsli H, Choo D, Ryan A, Johnson R, Wu DK, 1998 Development of the mouse inner ear and origin of its sensory organs. *J. Neurosci* 18, 3327–3335. [PubMed: 9547240]
- Nica G, Herzog W, Sonntag C, Nowak M, Schwarz H, Zapata AG, Hammerschmidt M, 2006 *Eya1* is required for lineage-specific differentiation, but not for cell survival in the zebrafish adenyohypophysis. *Dev. Biol* 292, 189–204. [PubMed: 16458879]
- Ohto H, Kamada S, Tago K, Tominaga SI, Ozaki H, Sato S, Kawakami K, 1999 Cooperation of six and *eya* in activation of their target genes through nuclear translocation of *Eya*. *Mol. Cell. Biol* 19, 6815–6824. [PubMed: 10490620]
- Okabe Y, Sano T, Nagata S, 2009 Regulation of the innate immune response by threonine-phosphatase of Eyes absent. *Nature* 460, 520–524. [PubMed: 19561593]
- Oliver G, Mailhos A, Wehr R, Copeland NG, Jenkins NA, Gruss P, 1995a Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. *Development* 121, 4045–4055. [PubMed: 8575305]
- Oliver G, Wehr R, Jenkins NA, Copeland NG, Cheyette BN, Hartenstein V, Zipursky SL, Gruss P, 1995b Homeobox genes and connective tissue patterning. *Development* 121, 693–705. [PubMed: 7720577]
- Ozaki H, Watanabe Y, Ikeda K, Kawakami K, 2002 Impaired interactions between mouse *Eya1* harboring mutations found in patients with branchio-oto-renal syndrome and Six, Dach, and G proteins. *J. Hum. Genet* 47, 107–116. [PubMed: 11950062]
- Ozaki H, Nakamura K, Funahashi J, Ikeda K, Yamada G, Tokano H, Okamura HO, Kitamura K, Muto S, Kotaki H, Sudo K, Horai R, Iwakura Y, Kawakami K, 2004 Six1 controls patterning of the mouse otic vesicle. *Development* 131, 551–562. [PubMed: 14695375]
- Pandey RN, Rani R, Yeo EJ, Spencer M, Hu S, Lang RA, Hegde RS, 2010 The Eyes Absent phosphatase-transactivator proteins promote proliferation, transformation, migration, and invasion of tumor cells. *Oncogene* 29, 3715–3722. [PubMed: 20418914]
- Pfister M, Toth T, Thiele H, Haack B, Blin N, Zenner HP, Sziklai I, Nurnberg P, Kupka S, 2002 A 4-bp insertion in the *eya*-homologous region (*eyaHR*) of EYA4 causes hearing impairment in a Hungarian family linked to DFNA10. *Mol. Med* 8, 607–611. [PubMed: 12477971]

- Pignoni F, Hu B, Zavitz KH, Xiao J, Garrity PA, Zipursky SL, 1997 The eye-specification proteins *So* and *Eya* form a complex and regulate multiple steps in *Drosophila* eye development. *Cell* 91, 881–891. [PubMed: 9428512]
- Pineda D, Gonzalez J, Callaerts P, Ikeo K, Gehring WJ, Salo E, 2000 Searching for the prototypic eye genetic network: *sine oculis* is essential for eye regeneration in planarians. *Proc. Natl. Acad. Sci. U. S. A* 97, 4525–4529. [PubMed: 10781056]
- Puligilla C, Dabdoub A, Brenowitz SD, Kelley MW, 2010 *Sox2* induces neuronal formation in the developing mammalian cochlea. *J. Neurosci* 30, 714–722. [PubMed: 20071536]
- Rayapureddi JP, Kattamuri C, Steinmetz BD, Frankfort BJ, Ostrin EJ, Mardon G, Hegde RS, 2003 *Eyes absent* represents a class of protein tyrosine phosphatases. *Nature* 426, 295–298. [PubMed: 14628052]
- Ruben RJ, 1967 Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. *Acta Otolaryngol. (Suppl. 220)*, 1–44. [PubMed: 6030182]
- Ruf RG, Xu PX, Silvius D, Otto EA, Beekmann F, Muerb UT, Kumar S, Neuhaus TJ, Kemper MJ, Raymond RM Jr., Brophy PD, Berkman J, Gattas M, Hyland V, Ruf EM, Schwartz C, Chang EH, Smith RJ, Stratakis CA, Weil D, Petit C, Hildebrandt F, 2004 *SIX1* mutations cause branchio-otorenal syndrome by disruption of *EYA1-SIX1*-DNA complexes. *Proc. Natl. Acad. Sci. U. S. A* 101, 8090–8095. [PubMed: 15141091]
- Sahly I, Andermann P, Petit C, 1999 The zebrafish *eya1* gene and its expression pattern during embryogenesis. *Dev. Genes Evol.* 209, 399–410. [PubMed: 10370123]
- Schlosser G, 2005 Evolutionary origins of vertebrate placodes: insights from developmental studies and from comparisons with other deuterostomes. *J. Exp. Zool B Mol. Dev. Evol* 304, 347–399.
- Schlosser G, 2006 Induction and specification of cranial placodes. *Dev. Biol* 294, 303–351. [PubMed: 16677629]
- Schonberger J, Wang L, Shin JT, Kim SD, Depreux FF, Zhu H, Zon L, Pizard A, Kim JB, Macrae CA, Mungall AJ, Seidman JG, Seidman CE, 2005 Mutation in the transcriptional coactivator *EYA4* causes dilated cardiomyopathy and sensorineural hearing loss. *Nat. Genet* 37, 418–422. [PubMed: 15735644]
- Seo HC, Curtiss J, Mlodzik M, Fjose A, 1999 Six class homeobox genes in *Drosophila* belong to three distinct families and are involved in head development. *Mech. Dev* 83, 127–139. [PubMed: 10381573]
- Serikaku MA, O'Tousa JE, 1994 *Sine oculis* is a homeobox gene required for *Drosophila* visual system development. *Genetics* 138, 1137–1150. [PubMed: 7896096]
- Silver SJ, Davies EL, Doyon L, Rebay I, 2003 Functional dissection of *eyes absent* reveals new modes of regulation within the retinal determination gene network. *Mol. Cell. Biol* 23, 5989–5999. [PubMed: 12917324]
- Spitz F, Demignon J, Porteu A, Kahn A, Concordet JP, Daegelen D, Maire P, 1998 Expression of myogenin during embryogenesis is controlled by *Six/sine oculis* homeoproteins through a conserved MEF3 binding site. *Proc. Natl. Acad. Sci. U. S. A* 95, 14220–14225. [PubMed: 9826681]
- Tootle TL, Silver SJ, Davies EL, Newman V, Latek RR, Mills IA, Selengut JD, Parlikar BE, Rebay I, 2003 The transcription factor *eyes absent* is a protein tyrosine phosphatase. *Nature* 426, 299–302. [PubMed: 14628053]
- Treisman JE, 1999 A conserved blueprint for the eye? *Bioessays* 21, 843–850. [PubMed: 10497334]
- Wayne S, Robertson NG, DeClau F, Chen N, Verhoeven K, Prasad S, Tranebjarg L, Morton CC, Ryan AF, Van Camp G, Smith RJ, 2001 Mutations in the transcriptional activator *EYA4* cause late-onset deafness at the *DFNA10* locus. *Hum. Mol. Genet* 10, 195–200. [PubMed: 11159937]
- Xu PX, Cheng J, Epstein JA, Maas RL, 1997a Mouse *Eya* genes are expressed during limb tendon development and encode a transcriptional activation function. *Proc. Natl. Acad. Sci. U. S. A* 94, 11974–11979. [PubMed: 9342347]
- Xu PX, Woo I, Her H, Beier DR, Maas RL, 1997b Mouse *Eya* homologues of the *Drosophila* *eyes absent* gene require *Pax6* for expression in lens and nasal placode. *Development* 124, 219–231. [PubMed: 9006082]

- Xu PX, Adams J, Peters H, Brown MC, Heaney S, Maas R, 1999 Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat. Genet* 23, 113–117. [PubMed: 10471511]
- Xu PX, Zheng W, Huang L, Maire P, Laclef C, Silvius D, 2003 Six1 is required for the early organogenesis of mammalian kidney. *Development* 130, 3085–3094. [PubMed: 12783782]
- Yu Y, Davicioni E, Triche TJ, Merlino G, 2006 The homeoprotein six1 transcriptionally activates multiple protumorigenic genes but requires ezrin to promote metastasis. *Cancer Res.* 66, 1982–1989. [PubMed: 16488997]
- Zheng W, Huang L, Wei ZB, Silvius D, Tang B, Xu PX, 2003 The role of Six1 in mammalian auditory system development. *Development* 130, 3989–4000. [PubMed: 12874121]
- Zimmerman JE, Bui QT, Steingrimsson E, Nagle DL, Fu W, Genin A, Spinner NB, Copeland NG, Jenkins NA, Bucan M, Bonini NM, 1997 Cloning and characterization of two vertebrate homologs of the *Drosophila* eyes absent gene. *Genome Res.* 7, 128–141. [PubMed: 9049631]
- Zou D, Silvius D, Fritzsche B, Xu PX, 2004 Eya1 and Six1 are essential for early steps of sensory neurogenesis in mammalian cranial placodes. *Development* 131, 5561–5572. [PubMed: 15496442]
- Zou D, Silvius D, Rodrigo-Blomqvist S, Enerback S, Xu PX, 2006 Eya1 regulates the growth of otic epithelium and interacts with Pax2 during the development of all sensory areas in the inner ear. *Dev. Biol* 298, 430–441. [PubMed: 16916509]
- Zou D, Erickson C, Kim EH, Jin D, Fritzsche B, Xu PX, 2008 Eya1 gene dosage critically affects the development of sensory epithelia in the mammalian inner ear. *Hum. Mol. Genet* 17, 3340–3356. [PubMed: 18678597]

Fig. 1.

The EYA-SO/SIX regulatory network. (A) The transcriptional hierarchy among the key regulators in the fly eyes. Feedback loops and protein–protein interactions (double-headed arrows) are indicated. (B) EYA and SIX form transcriptional activation complexes to control cell proliferation and survival as well as to trigger particular differentiation programs. EYA–SIX also interact with other proteins (×), which may vary depending on distinct developmental and cellular contexts to modify the specificity of EYA–SIX function.

Fig. 2.

Schematic drawing of *Eya1* and *Six1* expression domains in relation to *Sox2* expression in the otocyst and developing cochlea, cristae and maculae and their possible roles in sensory cell development. In the otocyst, *Eya1* (red), *Six1* (red) and *Sox2* (green) are co-expressed in the ventral otocyst where the prosensory epithelia are formed. These genes may act together to specify a common prosensory area in the otocyst at this early stage by turning on other sensory genes including *Jag1*, *Lfng*, *Bmp4* and *Fgfs*, which are not expressed in *Eya1*- or *Six1*-null embryos. In the developing cochlea, at E12.5, the floor of cochlear duct will give rise to the organ of Corti (oc), the greater epithelial ridge (GER) and lesser epithelial ridge (LER). *Eya1* and *Sox2* are coexpressed in the prosensory progenitors of the OC, and this colocalization suggests that both genes may act together to regulate the cell cycle of the

prosensory progenitors. *Six1* expression disappears in the postmitotic progenitors but reappears in differentiating hair cells in a pattern similar to that of *Atoh1*. Once the sensory progenitor cells become postmitotic, they express p27Kip1, and at around E13.5–14.5, *Atoh1* is activated in the postmitotic progenitors that become committed to hair cell fate and is coexpressed with *Eya1* and *Six1* in the differentiating hair cells. However, *Sox2* expression becomes restricted to supporting cells and border cells in the GER. Based on published data, *Eya1*, *Six1* and *Sox2* act together to regulate hair cell commitment by activating *Atoh1*; however, *Eya1* and *Six1* but not *Sox2* act to maintain or upregulate *Atoh1* expression during hair cell differentiation. Similar expression pattern of these genes is found in the sensory cell development of the cristae and maculae.

Fig. 3.

EYA1–SIX1 complex in inducing hair cell fate. (A) Molecular relationships among the key transcription factors for hair cell differentiation. A direct interaction between EYA1/SIX1/SOX2 proteins coordinately regulates *Atoh1* expression, and that POU4F3 is a common downstream factor of the *Atoh1*-dependent and -independent pathways. Dashed lines indicate that SOX2 may repress *Pou4f3* or downstream factors of *Pou4f3* to inhibit hair cell differentiation. (B) Possible mechanisms for *Atoh1* activation by *Sox2/Eya1/Six1*. EYA1/SIX1 in collaboration with SOX2 activity in prosensory progenitors can induce *Atoh1* activation via direct binding to the Sox and Six binding sites within enhancer A and B, respectively. These three factors may directly interact (model a), or EYA1 may bridge SIX1 and SOX2 (model b). These three factors may also form an active complex to regulate *Atoh1* activation via enhancer A (model c), whereas EYA1/SIX1 efficiently upregulate *Atoh1* via enhancer B. Question mark indicates that the involvement of the factor is unclear.

Fig. 4.

Possible mechanism for *Neurog1* and *Neurod1* activation by *Eya1/Six1*. EYA1/SIX1 in collaboration with the SWI/SNF complex in progenitor cells induce *Neurog1*—*Neurod1* activation. *Eya1/Six1* also interact with *Neurog1* to regulate *Neurod1* activation. In addition, *Eya1/Six1* interact with *Neurod1* to regulate neuronal differentiation. *Sox2* works cooperatively with *Eya1/Six1* or the SWI/SNF complex to promote neuronal differentiation. These factors are physically associated.