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Eya1 regulates the growth of otic epithelium and interacts with Pax2 during the development of all sensory areas in the inner ear

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Abstract

Members of the Eyes absent (Eya) gene family are important for auditory system development. While mutations in human *EYA4* cause late-onset deafness at the DFNA10 locus, mutations in human *EYA1* cause branchio–oto–renal (BOR) syndrome. Inactivation of Eya1 in mice causes an early arrest of the inner ear development at the otocyst stage. To better understand the role of Eya1 in inner ear development, we analyzed the cellular and molecular basis of the early defect observed in the *Eya1* mutant embryos. We report here that $Eya1^{-/-}$ otic epithelium shows reduced cell proliferation from E8.5 and increased cell apoptosis from E9.0, thus providing insights into the cellular basis of inner ear defect which occurred in the absence of Eya1. Previous studies have suggested that Pax, Eya and Six genes function in a parallel or independent pathway during inner ear development. However, it remains unknown whether Pax genes interact with *Eya1* or *Six1* during inner ear morphogenesis. To further evaluate whether *Pax* genes function in the *Eya1–Six1* pathway or whether they interact with *Eya1* or *Six1* during inner ear morphogenesis, we have analyzed the expression pattern of *Eya1*, *Pax2* and *Pax8* on adjacent sections of otic epithelium from E8.5 to 9.5 by in situ hybridization and the inner ear gross structures of *Pax2*, *Eya1* and *Six1* compound mutants at E17.5 by latex paintfilling. Our data strongly suggest that *Pax2* interacts with *Eya1* during inner ear morphogenesis, and this interaction is critical for the development of all sensory areas in the inner ear. Furthermore, otic marker analysis in both $Eya1^{-/-}$ and $Pax2^{-/-}$ embryos indicates that *Eya1* but not *Pax2* regulates the establishment of regional specification of the otic vesicle. Together, these results show that, while Eya1 exerts an early function essential for normal growth and patterning of the otic epithelium, it also functionally synergizes with Pax2 during the morphogenesis of all sensory areas of mammalian inner ear.

Keywords

Eya1; Inner ear; Endolymphatic duct; *Six1*; *Pax2*; *Pax8*; Otic patterning; Sensory areas of the inner ear

Introduction

The Eyes absent (Eya) gene family was first identified in *Drosophila* as a key regulator for eye development and subsequently in a number of species ranging from *Arabidopsis*, race,

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C. elegans, zebrafish to higher vertebrates (Bonini et al., 1993; Abdelhak et al., 1997a; Xu et al., 1997; Zimmerman et al., 1997; Borsani et al., 1999; Sahly et al., 1999; Takeda et al., 1999; David et al., 2001). While it appears to be only a single Eya gene in *Drosophila* (Bonini et al., 1993), at least four Eya genes (*Eya1–4*) are present in the mammalian genome (Abdelhak et al., 1997a; Xu et al., 1997; Borsani et al., 1999). Expression studies have shown that all four mouse Eya genes are expressed during auditory system development (Xu et al., 1997; Wayne et al., 2001). However, only *Eya1* expression was detected in the otic epithelium from early stages, and it appears to be conserved from *Xenopus*, zebrafish to higher vertebrates (Xu et al., 1997; Sahly et al., 1999; David et al., 2001). *Eya1* is expressed in the otic vesicle, vestibuloacoustic ganglion and periotic mesenchyme (Xu et al., 1997). Subsequently, *Eya1* has been shown to be expressed in the differentiating hair and supporting cells of the sensory epithelia, as well as in the associated ganglia, and the expression persists after the differentiation has taken place (Kalatzis et al., 1998). This suggests that, in addition to a role in morphogenesis, *Eya1* could also have a role in the differentiation or survival of these inner ear cell populations. While *Eya1* mRNA has been studied previously, the onset of its expression in early otic development has not been established.

Mutations in the human *EYA1* gene cause branchio–oto–renal (BOR) syndrome, a congenital birth defect that accounts for as many as 2% of profoundly deaf children (Fraser et al., 1980; Abdelhak et al., 1997a,b; Vincent et al., 1997; Kumar et al., 1998). The otic defects in BOR syndrome include malformations of the external, middle and inner ears, and hearing loss is either sensorineural, conductive or combinations of both (Chen et al., 1995). Recently, mutations in the human *EYA4* were found to cause late-onset hearing impairment at the DFNA10 locus (Wayne et al., 2001; De Leenheer et al., 2002; Pfister et al., 2002). However, despite the identification of these Eya genes as important regulators for normal auditory system development, the developmental and cellular basis for auditory system defects occurring in the human syndromes is unclear.

We generated *Eya1* knockout mice and have previously reported that *Eya1* heterozygotes show a conductive hearing loss similar to BOR syndrome, whereas *Eya1* homozygotes lack ears due to apoptotic regression of the organ primordia (Xu et al., 1999). Inner ear development in *Eya1* homozygotes arrests at the otic vesicle stage, and all components of the inner ear fail to form (Xu et al., 1999). Therefore, it became the first described mouse mutant lacking all sensory areas of the inner ear. *Six1*, a member of the Six gene family homologous to *Drosophila so*, encodes a homeodomain protein, and its gene product physically interacts with Eya1 (Buller et al., 2001). During inner ear morphogenesis, *Six1* functions downstream of and genetically interacts with *Eya1* (Zheng et al., 2003). Consistent with this interaction, *Six1*-deficient mice show defects in all three parts of the ear similar to that observed in the *Eya1* mutants (Zheng et al., 2003) and mutations in the human *SIX1* gene also cause BOR syndrome (Ruf et al., 2004). However, how the expression of the *Eya1* and *Six1* genes is regulated and their precise mode of action in inner ear morphogenesis has not been elucidated.

In *Drosophila* eye imaginal discs, both *eya* and *so* act in the same genetic pathway downstream of *eyeless (ey*) gene, the fly *Pax6* gene (Halder et al., 1998; Kozmik et al., 2003). Recently, it was proposed that *Pax6* and *Pax2/5/8* evolved from a single ancestral diploblast pax gene that was involved in both statocyst and eye development (Kozmik et al., 2003). While we have clearly demonstrated that the Eya genes are expressed in both sensory organs and that the *Drosophila* Eya–Six cassette is evolutionarily conserved during mammalian inner ear morphogenesis (Xu et al., 1997, 1999; Zheng et al., 2003), it is unclear whether Pax genes function upstream of *Eya1* and *Six1*. In the mammalian ear, *Pax2* and *Pax8* are expressed in the otic epithelium from early stages and both gene expressions were

unaffected in *Eya1*$^{-/-}$ or *Six1*$^{-/-}$ otic epithelium (Xu et al., 1999; Zheng etal.,2003). However, the inner ear phenotype in *Pax2*$^{-/-}$mice is less severe than that seen in *Eya1*$^{-/-}$ or *Six1*$^{-/-}$ mice (Torres et al., 1995; Burton et al., 2004), and *Pax8*$^{-/-}$ mice do not exhibit an otic phenotype (Pfeffer et al., 1998). In addition, the expression of *Eya1* and *Six1* was unaffected in *Pax2*$^{-/-}$ otic epithelium (Zheng et al., 2003). These observations suggest that Pax, Eya and Six genes function in a parallel or independent pathway during inner ear development. However, it remains undetermined whether the Pax genes interact with *Eya1* or *Six1* during inner ear morphogenesis. In addition, no careful studies exist to determine the order of appearance of these mRNAs and proteins and their expression domain in the otic epithelium.

In this study, we have established the onset of cellular defects occurred in *Eya1*$^{-/-}$ otic epithelium and further evaluated whether Pax genes function in the *Eya1–Six1* pathway during inner ear morphogenesis. Our results provide strong evidence that *Pax2* interacts with *Eya1* during inner ear development, and this interaction is critical for normal morphogenesis of all sensory areas of the inner ear. Finally, our results show that *Eya1* but not *Pax2* regulates the establishment of regional specification of the otic vesicle. Together, these analyses establish the possible cellular and molecular mechanism by which *Eya1* acts in early otic patterning and in the morphogenesis of all six sensory regions of mammalian inner ear.

Materials and methods

Animals and genotyping

Eya1;Pax2, *Six1;Pax2*, *Eya1;Six1;Pax2* or *Pax2;Pax8* mice were generated by crossing mice carrying mutant alleles of *Eya1*, *Six1*, *Pax2* and *Pax8*.

Genotyping of mice and embryos was performed as described (Torres et al., 1995; Mansouri et al., 1998; Xu et al., 1999, 2002).

TUNEL assay and BrdU labeling

TUNEL assay was performed as described (Xu et al., 1999). BrdU labeling was performed as described (Zheng et al., 2003). Briefly, paraffin sections of 6 μm were prepared and denatured with 4 N HCl for 1 h at 37°C. Mouse anti-BrdU monoclonal antibody and goat anti-mouse IgG coupled with HRP or Cy3 were used for detection. The number of apoptotic or proliferating cells was counted in serial sections from each otic placode or vesicle, and at least 5 embryos (10 ears) of each genotype were counted.

Phenotype analyses and in situ hybridization

Embryos for histology and in situ hybridization were dissected out in PBS and fixed with 4% PFA at 4°C overnight. Embryonic membranes were saved in DNA isolation buffer for genotyping. Histology was performed as described (Xu et al., 1999).

For whole-mount and section in situ hybridization, we used 6 wild-type or mutant embryos at each stage for each probe as described (Wilkson and Green, 1990; Rosen and Beddington, 1993).

The latex paintfilling of the ears at E17.5 was performed as described (Morsli et al., 1998). For *Pax2*$^{-/-}$ ears, the ears were paintfilled laterally, and the brains were removed after paintfilling because of their abnormal brain development. The paintfilled inner ears were dissected out and photographed.

Result

Eya1$^{-/-}$ otic epithelial cells undergo abnormal cell death from E9.0

In our earlier work, we described that *Eya1*$^{-/-}$ otic epithelial cells undergo abnormal apoptosis from E10.5; the earliest stage examined (Xu et al., 1999). To determine the exact time point at when the otic epithelial cells begin to undergo programmed cell death in *Eya1*$^{-/-}$ embryos, we analyzed the mutant embryos at younger stages, from E8.5 to 9.5, using TUNEL detection method of apoptotic nuclei. Increased cell death in *Eya1*$^{-/-}$ otic epithelium was first observed at around E9.0 (Fig. 1). Apoptotic cells were increased in the rims of *Eya1*$^{-/-}$ otic cup (arrows, Figs. 1B, E), whereas very few apoptotic cells were seen in the ventrolateral rim of the otic cup in E9.0 control embryos (Fig. 1A). By E9.5, increased cell death became apparent in the lateral wall of *Eya1*$^{-/-}$ otic vesicle (Figs. 1D, E), while a few apoptotic cells were also seen in the medial wall of *Eya1*$^{-/-}$ otic vesicle at this stage, and a day later, apoptotic cells were found throughout the otic vesicle (Xu et al., 1999). These data indicate that, in the absence of Eya1, the otic epithelial cells undertake apoptotic pathway starting as early as E9.0, thus establishing the onset of abnormal cell death in early *Eya1*$^{-/-}$ otic development.

Eya1 regulates proliferation of otic epithelial cells

We have previously shown that Six1 regulates cell proliferation in the otic epithelium (Zheng et al., 2003). Although *Eya1* functions upstream of *Six1* during early otic development, it is unknown whether Eya1 is also required for normal proliferation of otic epithelial cells. We therefore tested whether *Eya1*$^{-/-}$ otic epithelial cells proliferate appropriately by assaying BrdU incorporation in the mutant otic placode and vesicle. S-phase cells in E8.5 to 9.5 otic epithelium were pulse-labeled with BrdU for 4 h, and BrdU-positive cells were scored under a microscope. In E8.5 wild-type embryos, BrdU labeled cells were seen throughout the otic placode (Fig. 2A). However, in *Eya1*$^{-/-}$embryos, the number of BrdU-labeled cells was reduced in the otic placode (Figs. 2B, G). At E9.0 and 9.5, BrdU-positive cells were markedly reduced in *Eya1*$^{-/-}$ otic cup (Figs. 2C, D) and vesicle (Figs. 2E, F). Using an image analysis system, we next counted the number of BrdU-positive cells from 10 wild-type and 10 *Eya1*$^{-/-}$ ears at each stage on serial sections and performed statistic analysis (Fig. 2G). At E8.5, the number of BrdU-positive cells in *Eya1*$^{-/-}$ otic placode was approximately 80% of wild-type embryos, and by E9.0 and 9.5, it was reduced to approximately 60% and 40% of that in wild-type embryos respectively (Fig. 2G). Thus, similar to *Six1*, *Eya1* is also required for normal growth of the otic epithelium by regulating cell proliferation during early inner ear development.

Malformation of endolymphatic duct in Eya1$^{-/-}$ embryos

At E10.5 to 11.5, the endolymphatic duct pinches off from the dorsomedial aspect of the otic vesicle (Kaufman, 1990; Morsli et al., 1998). The endolymphatic duct/sac belongs to the non-sensory part of the membranous labyrinth, and this component of the inner ear is thought to be involved in endolymph circulation (Guild, 1927; Hendriks and Toerien, 1973). A normal endolymphatic duct was clearly present in all of E10.5 to 11.5 control embryos that we examined by histological analysis (Fig. 3A and data not shown). In all *Eya1*$^{-/-}$ embryos, the normal endolymphatic duct was absent (in all 20 ears of 10 embryos) but a vesicular structure formed posteroventrally was observed in 10 ears of 7 embryos (arrow in Fig. 3D). To determine whether this vesicular structure is fated to become the endolymphatic duct/sac, we performed marker gene analysis. The first marker we used is *Sall1*, a mammalian homolog of *Drosophila spalt*, which is a regulator in sensory organ development in flies (de Celis et al., 1999; Buck et al., 2000; Dong et al., 2003). *Sall1* encodes a zinc finger protein and mutations in the human *SALL1* cause Townes–Brocks Syndrome (TBS) (reviewed by Kohlhase, 2000; Kiefer et al., 2003), which has strong

phenotypic overlap with BOR. Interestingly, *Sall1* is strongly expressed in the region fated to form the endolymphatic duct at E10.5 (arrow, Fig. 3B), and its expression is preserved in the dislocated vesicular structure in *Eya1*$^{-/-}$ embryos (arrow, Fig. 3E), suggesting that this vesicular structure is fated to form the endolymphatic duct. *Foxi1*, which encodes a winged helix/forkhead transcription factor, is expressed in the endolymphatic duct/sac epithelium from early stages, and lack of *Foxi1* causes an expansion of the endolymphatic duct (Hulander et al., 2003). The developing endolymphatic duct labeled by *Foxi1* was evident in wild-type embryos at E10.5 and 11.5 (arrow, Fig. 3C and data not shown). In *Eya1*$^{-/-}$ embryos, although this structure was not observed (Fig. 3F), *Foxi1* expression was observed in the dorsal region of the otic vesicle in all 6 embryos analyzed (arrow, Fig. 3F). This suggests that the development of endolymphatic duct is initiated, but it fails to form its normal structure in the mutant.

To further investigate the abnormal development of the endolymphatic duct/sac in the mutant, we performed paintfilling to reveal its gross structure at E10.5 to 11.5. The tube-like endolymphatic duct projecting dorsally from the medial aspect of the otocyst was evident in all control embryos (Figs. 3G–J). In *Eya1*$^{-/-}$ embryos, outgrowth of the endolymphatic duct was not observed in all 8 ears analyzed (Figs. 3K–N). In addition, all *Eya1*$^{-/-}$ embryos lacked visible development of the vestibule and the cochlea (Figs. 3K–N). These data indicate that normal morphogenesis of the endolymphatic duct/sac is blocked in the absence of Eya1.

Examination of E12.5 revealed that the inner ear formation and the cartilage primordium of the temporal bone in *Eya1*$^{-/-}$mutants were more severely affected than that in *Six1*$^{-/-}$ mutants (Figs. 3O–T). E12.5 *Eya1*$^{-/-}$ ears showed two vesicle-like structures, and the one located medially showed strong *Foxi1* expression in all 6 embryos examined (Figs. 3P, S), indicating that this structure is the endolymphatic duct/sac. Taken together, our marker gene analyses at different stages show that the primordia fated to form the endolymphatic duct are present in the mutant but fail to outgrow normally, thus leading to its abnormal morphogenesis.

We further confirmed that *Eya1* is not expressed in the region fated to form the endolymphatic duct/sac on both coronal and transverse sections of wild-type embryos at E10.5 to 12.5 (data not shown), indicating that Eya1 is unlikely to directly regulate the formation of endolymphatic duct/sac.

Eya1, Pax2 and Pax8 expression in relation to otic placode and otocyst development

A central prediction of the hypothesis that Pax genes function in the *Eya1–Six1* regulatory pathway during early otic morphogenesis involves the expression of *Pax2*, *Pax8*, *Eya1* and *Six1* in early otic development in wild-type and respective mutant embryos. However, no careful studies exist to determine the order of appearance of these mRNAs and proteins and their expression domain in the otic epithelium. Detailed *Six1* expression during otic development was recently described, and its expression in the otic vesicle is *Eya1*-dependent (Zheng et al., 2003). To further evaluate this pathway, we first performed in situ hybridization experiments using *Pax2*, *Pax8* and *Eya1* probes on adjacent sections of otic epithelium between E8.0 and 9.5. At E8.5, all three genes are expressed in the thickened otic placode (Figs. 4A–C). Among these three genes, only *Eya1* expression was observed in the periotic mesenchyme from as early as E8.5 and persists until late stages (Figs. 4A, D, G, J). At around E8.75 when the otic placode begins to invaginate to form the otic cup, strong *Eya1* expression was detected in the otic epithelium (Fig. 4D). However, its expression became weaker in the dorsal tip of the otic epithelium (arrow, Fig. 4D). In contrast, *Pax2* expression was undetectable in the ventrolateral region (arrow, Fig. 4E), while *Pax8* expression in the ventral half is also slightly weaker than in the dorsal half of the otic

epithelium (Fig. 4F). At E9.0 before the vesicle is completely closed up, *Eya1* is strongly expressed in the medial and ventral region but is absent from the dorsal region (arrow, Fig. 4G). At this stage, *Pax2* is expressed strongly in the medial region and weakly in the dorsomedial tip of the otic cup (arrow, Fig. 4H). By contrast, *Pax8* expression is restricted to the dorsal region, complementary to that of *Eya1* (arrow, Fig. 4I). At E9.5 after vesicle formation, *Eya1* expression remains strongly in the medial and ventral otic vesicle within which the vestibular and auditory sensory epithelia form but is excluded from the dorsal region where the semicircular canals form (arrow, Fig. 4J). By contrast, *Pax2* expression remains strongly in the medial otic vesicle and weakly in both the dorsal- and ventral-most walls (arrows, Fig. 4K). However, its expression is excluded from the lateral otic vesicle. The strongest *Pax8* expression domain is confined to the dorsal aspect at this stage (arrow, Fig. 4L). Taken together, these data show that all three genes are expressed in the otic placode at E8.5. When the otic placode begins to invaginate, *Eya1* and *Pax2* expressions only partially overlap in the ventromedial region, while *Eya1* and *Pax8* are not coexpressed in the otic epithelium from E9.0. This suggests that *Eya1* is unlikely to synergistically interact with *Pax8* in early otic development from E9.0 because of non-overlapping expression pattern.

Pax2 interacts with Eya1 during mammalian inner ear morphogenesis

We further tested whether *Pax2* interacts with *Eya1* or *Six1* in a molecular pathway during mammalian inner ear morphogenesis by examining the inner ear gross structures of $Pax2^{+/-}$; $Eya1^{+/-}$, $Pax2^{+/-}$;$Six1^{+/-}$ and $Pax2^{+/-}$;$Eya1^{+/-}$;$Six1^{+/-}$ compound heterozygotes using latex paintfilling (Table 1 and Fig. 5). At E17.5, the membranous labyrinth developed to its mature shape and the cochlea reached 1.75 turns (Fig. 5A; Morsli et al., 1998). It was previously reported that inactivation of *Pax2* results in cochlear agenesis by histological analysis (Torres et al., 1995). To further confirm this, we analyzed the gross structure of E17.5 $Pax2^{-/-}$ ears by paintfilling. Because of brain defects that occurred in $Pax2^{-/-}$ mice (Torres et al., 1995), the brains were removed after their ears were paintfilled. A single latex paint solution injected into the lateral or anterior ampulla region of $Pax2^{-/-}$ ears showed a protrusion of the cochlea into the brain because of the lack of the temporal bone (arrows, Fig. 5B). Close examination of the inner ears revealed three semicircular canals with ampullae (Fig. 5C). However, the saccule and utricle were in a large single chamber without subdivision in $Pax2^{-/-}$ ears (arrowhead, Fig. 5C). Although a cochlea-like structure is present, it is severely malformed (arrow, Figs. 5B, C). Consistent with recent observation (Burton et al., 2004), this result further indicates that *Pax2* is required for normal inner ear morphogenesis.

The inner ear gross structures in all $Pax2^{+/-}$ mice were normal (Table 1 and Fig. 5D), although some ears showed a slight reduction in their overall volume with thinner ducts and 6 of 24 ears showed slightly shortened cochlea (Fig. 5D and Table 1). Among the 20 *Pax2;Six1* double heterozygous ears (10 embryos) analyzed, all revealed normal gross structures (Fig. 5E and Table 1). However, approximately half of them exhibited slightly shortened cochlea but reached 1.5 turns and only one ear completed between 1 turn and 1.25 turn (Table 1). By contrast, *Pax2* and *Eya1* compound heterozygous ears were severely affected (Table 1). 18 of 24 $Pax2^{+/-}$;$Eya1^{+/-}$ ears (10 of 12 embryos) showed smaller or mal-shaped saccule (Fig. 5F and data not shown). Approximately, 75% of $Pax2^{+/-}$;$Eya1^{+/-}$ ears revealed small or morphologically unidentifiable ampullae (arrowhead, Fig. 5F). The cochlea was also severely affected in *Pax2;Eya1* double heterozygotes. 25% of the ears completed between 1 turn and 1.25 turns (Fig. 5F). Among these affected cochlea, some showed a malformed distal tip but all coiled correctly (arrow, Fig. 5F). Interestingly, the inner ear structures were more severely affected in *Pax2;Eya1;Six1* triple heterozygotes (Table 1). 100% of the triple heterozygous animals showed small or malformed saccule,

small or missing ampullae and a truncation of the semicircular canals (Figs. 5G–I). Within the semicircular canals, the lumen in some areas became extremely narrow and it took much longer (up to 24 h) for the paint solution to passage through (asterisks, Figs. 5G–I). Among the 8 ears analyzed, only one cochlea reached 1 turn (Fig. 5I), and all 8 ears showed severely malformed distal tips (arrows, Figs. 5G–I). This defect was not seen in each single or *Pax2; Six1*, *Pax2;Eya1* or *Eya1;Six1* double heterozygotes (Table 1; Zheng et al., 2003). In summary, although it is unclear whether *Pax2* interacts with *Six1* during inner ear morphogenesis because of only slight enhancement of the cochlear phenotype observed in *Pax2;Six1* double heterozygotes, our data strongly suggest that *Pax2* interacts with *Eya1* during inner ear morphogenesis and this interaction is critical for normal morphogenesis of both auditory and vestibular systems.

Eya1 but not Pax2 is required for normal patterning of the otic vesicle

We have previously shown that *Six1* is required for normal patterning of the otic vesicle, and the expression of *Fgf3* and *Six1* in the otic epithelium is *Eya1*-dependent (Xu et al., 1999; Zheng et al., 2003). However, at present, the molecular mechanism by which *Pax2* acts during inner ear development is unknown. To further understand the relation between *Eya1*, *Six1* and *Pax2* during inner ear morphogenesis and explore the effects of the activities of these genes on sensory organ patterning, we analyzed several otic markers that are known to be important for inner ear patterning and sensory organ formation at early stages. At E10.5, *Hmx3* (previously called *Nkx5.1*) is expressed in the dorsolateral otic vesicle that will give rise to the vestibular apparatus of the inner ear, and its expression shifted ventrally in $Six1^{-/-}$ otic vesicle (Fig. 6A; Hadrys et al., 1998; Wang et al., 1998; Zheng et al., 2003). In $Eya1^{-/-}$ embryos, *Hmx3* expression was excluded from the dorsolateral region at E10.5 (arrow, Fig. 6B) and its expression also shifted ventrally (Figs. 6A, B). By contrast, *Hmx3* expression was unaltered in $Pax2^{-/-}$ otic vesicle (Fig. 6C). *Gata3* is expressed strongly in the dorsolateral region and weakly in the ventromedial region at E10.5, and its expression in the dorsolateral region also shifted ventrally in $Six1^{-/-}$ otic vesicle (Fig. 6C; Karis et al., 2001; Lawoko-Kerali et al., 2002; Zheng et al., 2003). In $Eya1^{-/-}$embryos, no significant difference of *Gata3* expression was detected by E9.5 (data not shown). However, similar to *Hmx3*, *Gata3* expression in the dorsolateral region also shifted or expanded ventrally in $Eya1^{-/-}$ otic vesicle at E10.5 (Figs. 6D, E). In addition, its medial expression was also slightly reduced in $Eya1^{-/-}$ otic vesicle (Figs. 6D, E). By contrast, *Gata3* expression was also unaffected in $Pax2^{-/-}$ otic vesicle at these stages (Fig. 6F). These data strongly suggest that *Eya1* but not *Pax2* acts together with *Six1* to regulate the establishment of regional specification of the otic vesicle. We next examined the expression of growth factors that are known to be important for early otic morphogenesis, such as Fgfs and Bmps at E9.5 and 10.5, after the formation of otic vesicle. *Fgf10*, a member of the Fgf superfamily, is expressed in the otic placode and vesicle and facioacoustic ganglionic complex (Fig. 6G; Pirvola et al., 2000; Pauley et al., 2003), and its expression was markedly reduced in E10.5 $Six1^{-/-}$ otic vesicle (Zheng et al., 2003). Similarly, only residual *Fgf10* expression was detected in E10.5 $Eya1^{-/-}$ otic vesicle (arrow, Fig. 6H). However, its expression was normal in $Pax2^{-/-}$ otic vesicle (Fig. 6I). *Fgf3*, another member of the Fgf superfamily, is also expressed in the otic vesicle and VIIIth ganglion in an overlapping pattern with *Fgf10* expression, and both *Fgf3* and *Fgf10* are required for normal otic development (Mansour et al., 1993; Pauley et al., 2003; Wright and Mansour, 2003). Previous studies have shown that *Fgf3* expression was undetectable in $Eya1^{-/-}$ or $Six1^{-/-}$ otic vesicle (Xu et al., 1999; Zheng et al., 2003). However, its expression was also unaffected in $Pax2^{-/-}$ otic vesicle at E9.5 and 10.5 (data not shown). Bmp4, a member of the Tgfβ superfamily, has been shown to play a role in otic development (Chang et al., 1999; Gerlach et al., 2000). At E10.5, *Bmp4* expression is normally restricted to two domains that mark the sensory anlagen of the cristae (Fig. 6J; Wu and Oh, 1996), and its dorsal expression domain disappeared in $Six1^{-/-}$otic

vesicle (Zheng et al., 2003). Interestingly, *Bmp4* expression was undetectable in *Eya1*$^{-/-}$ otic vesicle at E10.5 (Fig. 6K). However, its expression was unaffected in *Pax2*$^{-/-}$ otic vesicle (Fig. 6L). Thus, these results strongly suggest that Eya1 but not Pax2 regulates the Fgf and Bmp signaling pathways during early otic development. In addition, our results strongly suggest that Eya1 and Six1 function together to regulate normal growth and patterning of the otic epithelium because of similar molecular and cellular defects detected in both mutants.

Discussion

Role of Eya1 in otic patterning

Although many genes are implicated in inner ear development (reviewed by Fekete and Wu, 2002), the mechanisms governing the morphogenetic processes and cellular events including differentiation, proliferation and apoptosis that are required to transform the otic placode into the highly organized structures of the adult inner ear are currently unclear. *Eya1* expression is turned on in the otic placode before invagination, and our results clearly show that Eya1 regulates proliferation from placodal stage (Fig. 2). After invagination of the otic placode to form the otocyst, Eya1 is required for cell survival in the otic cup and vesicle. The lack of visible development of the vestibular and auditory systems in *Eya1*$^{-/-}$ embryos can be explained by the failure of expansion of a population of epithelial cells that is destined to form the vestibular apparatus and the cochlea due to abnormal proliferation and apoptosis. We did detect alterations of certain gene expression in *Eya1*$^{-/-}$ otic vesicle. Among the markers analyzed, *Bmp4*, *Fgf10* and *Gata3* were expressed normally at E9.5, and their expression was either undetectable or altered in E10.5 *Eya1*$^{-/-}$ otic vesicle. Although *Eya1* is clearly required for maintenance of their expression, it may not have a genetic relation with these genes, and without Eya1, the cells normally expressing these genes may be missing at E10.5. Consistent with this view, we have shown that the initial cell fate determination for the vestibuloacoustic neurons and their delamination is unaffected in the absence of Eya1 or Six1 as judged by the expression of the basic helix–loop–helix genes *Neurog1* and *Neurod* (Zou et al., 2004), but the neurogenesis fails to maintain likely due to abnormal apoptosis and proliferation (Zou et al., 2004; Friedman et al., 2005). Nonetheless, it should be noted that, among the markers analyzed so far, only *Fgf3* expression was undetectable in *Eya1*$^{-/-}$ or *Six1*$^{-/-}$ otic vesicle at E9.5 (Xu et al., 1999; Zheng et al., 2003). Since *Fgf3* and *Fgf10*, both required for normal inner ear development (Mansour et al., 1993; Pauley et al., 2003; Wright and Mansour, 2003), share overlapping expression domain in the otic vesicle and *Fgf10* expression was unaffected in *Eya1*$^{-/-}$ or *Six1*$^{-/-}$ otic vesicle at E9.5, we would like to speculate that *Fgf3* expression in the otic vesicle is regulated by both Eya1 and Six1. Thus, *Fgf3* may be the common downstream target for both Eya1 and Six1. Further expression studies of *Eya1* and *Six1* in *Fgf3*$^{-/-}$ embryos should be performed to clarify the epistatic relation between these genes.

Shh loss-of-function also results in severe malformation or absence of the vestibular and auditory systems (Riccomagno et al., 2002). In both *Eya1*$^{-/-}$ and *Shh*$^{-/-}$ mutants, *Hmx3* expression is expanded ventrally (Fig. 6; Riccomagno et al., 2002). However, the failure of auditory system development of *Eya1*$^{-/-}$ and *Shh*$^{-/-}$ embryos may result from independent mechanisms because *Pax2* expression was downregulated in *Shh*$^{-/-}$ but not in *Eya1*$^{-/-}$ embryos (Xu et al., 1999; Riccomagno et al., 2002). Alternatively, these two molecules may crosstalk to regulate the cochlear development. In support of this, we found that *Eya1* and *Pax2* genetically interact during the morphogenesis of the cochlea duct as well as the sensory organs in the inner ear. In addition to the otic epithelium, the periotic mesenchyme has also been shown to respond to Shh signaling (Riccomagno et al., 2002). Since *Eya1* is also expressed in the periotic mesenchyme that was also severely affected in the *Eya1* mutant (Figs. 3H, K), it could potentially function cell autonomously and/or cell non-

autonomously during inner ear development. What the relative contribution of epithelial versus mesenchymal expression of *Eya1* is to inner ear development will require tissue-specific deletion of Eya1.

Interaction between Pax, Eya and Six genes

The *Drosophila* Pax–Eya–Six regulatory pathway has been suggested to operate during mammalian inner ear development based on the evidence that all these genes are expressed during inner ear development. Although our previous studies have clearly demonstrated that the Eya–Six regulatory cassette is evolutionarily conserved during mammalian inner ear development (Zheng et al., 2003), it remains unclear whether Pax genes function in the Eya–Six regulatory pathway. Existing data show that the expression of both *Pax2* and *Pax8* does not require Eya1 or Six1 function. Since *Eya1* or *Six1* expression is normal in $Pax2^{-/-}$ otic vesicle (Zheng et al., 2003) and $Pax2^{-/-}$ mice show less severe inner ear phenotype than that seen in $Eya1^{-/-}$ or $Six1^{-/-}$ mice (Torres et al., 1995; Burton et al., 2004), it has been suggested that *Pax2* and *Pax8* may function redundantly during early otic morphogenesis. We have previously shown that *Six1* begins to be expressed in the invaginating otic pit from E8.75 (Zheng et al., 2003). Here, we show that all three genes, *Eya1*, *Pax2* and *Pax8*, are coexpressed in the thickened otic placode before invagination. This raises the possibility that *Eya1* and Pax genes may act together to regulate Six gene expression. It is also possible that Eya and Six genes act downstream of Pax in a genetic cascade leading to the initiation of the otic differentiation program by activating other otic genes. Detailed expression studies of Eya and Six genes in the *Pax2;Pax8* mutant at early stages is underway in my laboratory to clarify the regulatory relation between these genes.

From E9.0, *Pax2* expression partially overlaps with *Eya1* and *Six1* in the ventromedial region, suggesting that these genes may interact in the ventromedial region during inner ear morphogenesis. In support of this, we have found that the all *Pax2;Eya1;Six1* triple heterozygous mutants showed more severe phenotype in the cochlea duct and all sensory regions than in each single or double heterozygous mice. Although the molecular and cellular mechanisms by which these genes act together to regulate the development of the cochlea duct and sensory regions remain unknown, it is possible that these genes function together to control the expression of certain downstream target genes that are involved in the morphogenesis of the cochlea and sensory regions. As all three genes have been shown to regulate cell proliferation and survival, they may directly regulate the expression of genes that are involved in the cell proliferation and survival. More analysis will be required to elucidate their precise mode of action in multiple cell lineages in the inner ear.

In summary, we have demonstrated that *Eya1* is expressed in the otic epithelium earlier than that of *Six1*, which is turned on in the invaginating otic placode at E8.75 (Zheng et al., 2003). However, our results show that both genes function closely together to regulate the morphogenetic and cellular events involved in the inner ear development. Since Eya1 requires DNA-binding proteins to activate a downstream target, its cofactor(s) involved in the activation of Six1 expression in the otic epithelium remains to be identified. Furthermore, because our data show that *Eya1* and *Six1* are not required for the initiation of inner ear organogenesis, *Eya1* expression in the otic placode is likely to be regulated by signals that establish positional identity of the otic placode. Our results strongly suggest that Eya1 is able to link the positional identity to otic morphogenesis.

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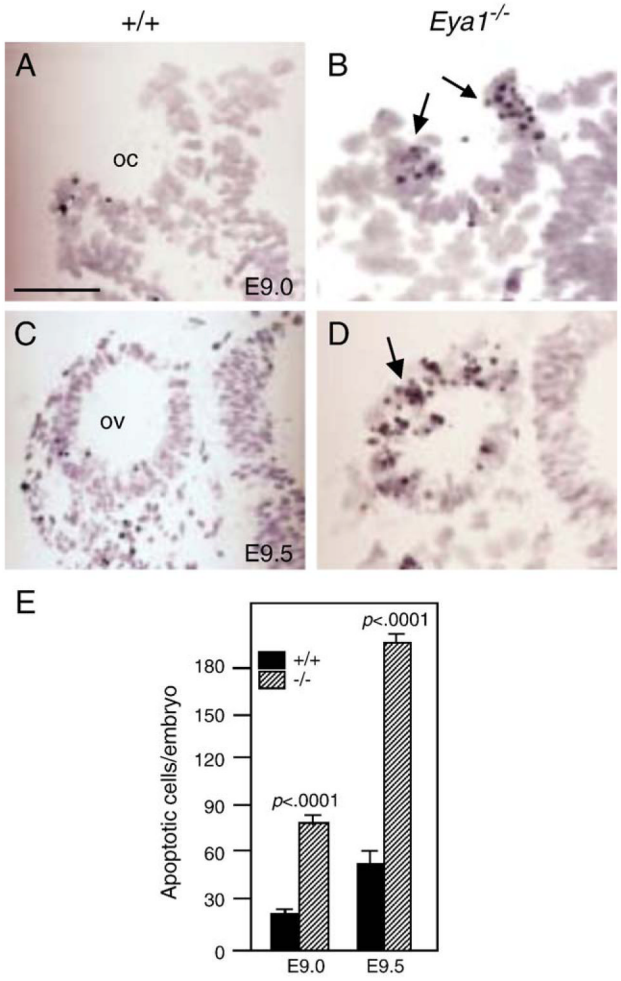

Fig. 1.

Eya1$^{-/-}$ otic epithelial cells undergo abnormal apoptosis from E9.0. (AD) TUNEL analysis of transverse sections through the ear region of wild-type and *Eya1*$^{-/-}$ at E9.0 and 9.5 for labeling apoptotic bodies (brown staining). Arrows point to numerous apoptotic bodies detected in the mutant. (E) Statistic analysis of apoptotic cells. Data refer to the average of 5 embryos per genotype; *P* values were calculated using StatView *t* test. Error bars indicate standard deviation. Scale bars: 100 μm.

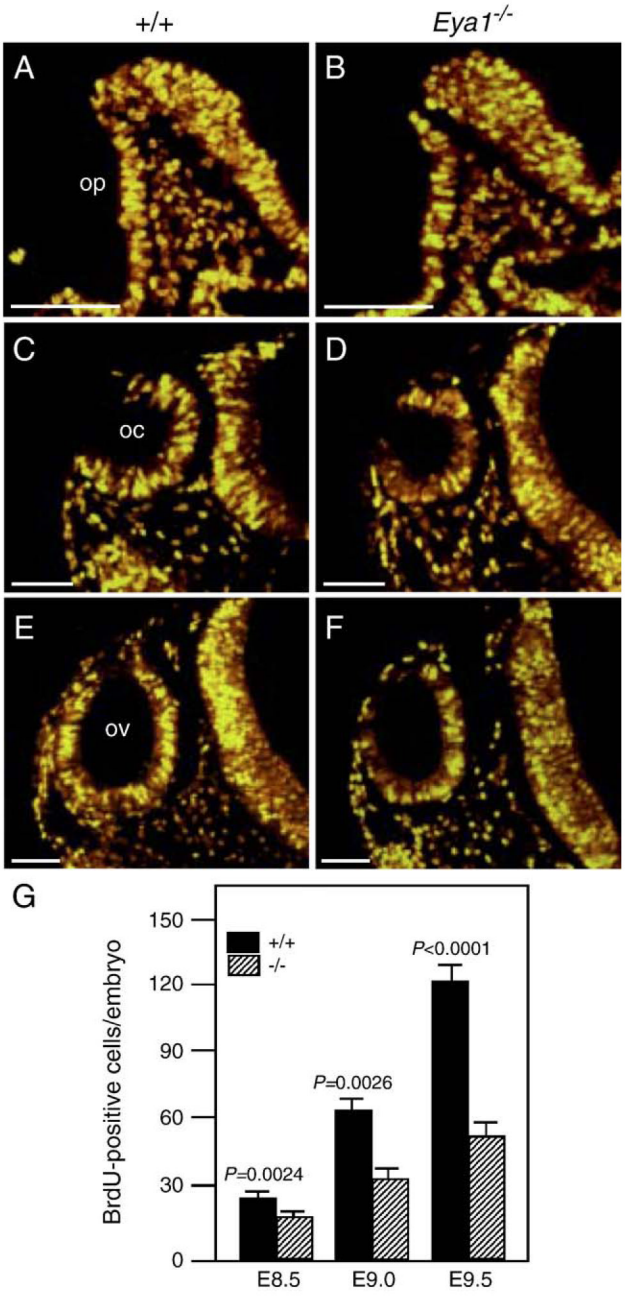

Fig. 2.

Eya1 controls proliferation of otic epithelial cells during early inner ear development. Transverse sections of otic regions from E8.5 to 9.5 wild-type (A, C, E) and *Eya1*$^{-/-}$ (B, D, F) embryos showing BrdU-labeled cells (orange). (G) Statistic analysis of BrdU-positive cells from each otic placode, cup or vesicle. Data refer to the average of 5 embryos per genotype; *P* values were calculated using StatView *t* test. Error bars indicate standard deviation. Scale bars: 100 μm.

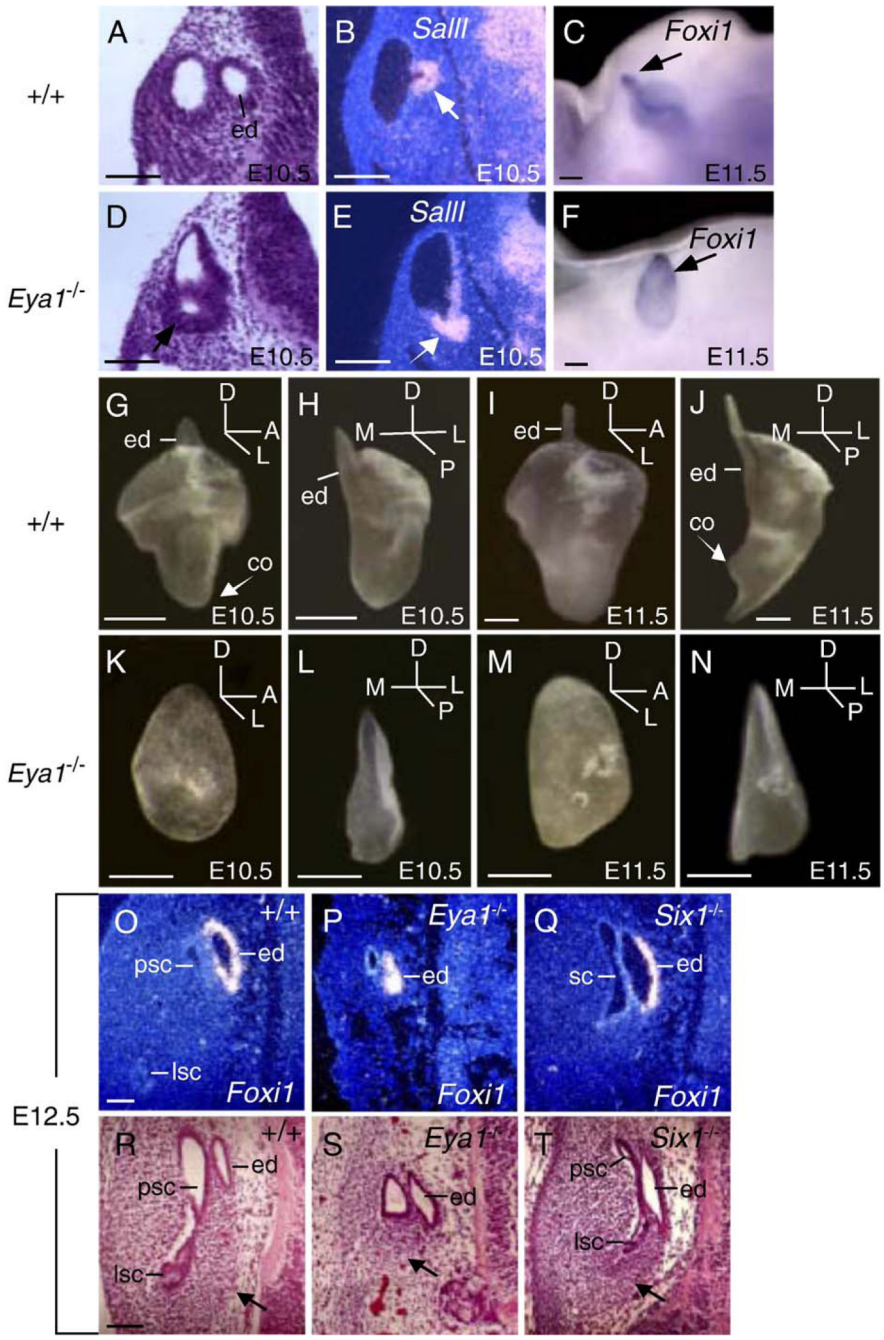

Fig. 3.

Eya1$^{-/-}$ embryos exhibit malformation or absence of the endolymphatic duct. (A) H&E-stained transverse section showing the formation of endolymphatic duct (ed) in wild-type embryos at E10.5 (A), and (D) *Eya1*$^{-/-}$ embryos show a malformed vesicle (arrow). (B) In situ hybridization showing *Sall1* expression in the endolymphatic duct at E10.5 (arrow), and (E) its expression is preserved in the malformed vesicular structure of *Eya1*$^{-/-}$ embryos (arrow). (C, F) Whole-mount in situ hybridization showing *Foxi1* expression in wild-type and *Eya1*$^{-/-}$ otocysts at E11.5. (G, I, K, M) Lateral and (H, J, L, N) posterior view of paintfilled otocysts at E10.5 and E11.5. Orientation is indicated for all panels. (G–J) Wild-

type otocysts showing the developing endolymphatic duct projecting from medial aspect. (K–N) *Eya1*$^{-/-}$otocysts, which lack normal outgrowth of the endolymphatic duct, show narrower dorsal tips (L, N). Note the significant size difference of the otocysts between wild-type and *Eya1*$^{-/-}$ embryos at E11.5. co, cochlea. (O–T) Transverse sections of E12.5 ears stained with *Foix1* probe (O–Q) or H&E (R–T) in wild-type (O, R), *Eya1*$^{-/-}$(P, S) and *Six1*$^{-/-}$ (Q, T) embryos. psc, posterior semicircular canal; lsc, lateral semicircular canal. Note that the formation of endolymphatic duct/sac and semicircular canals was less affected in *Six1*$^{-/-}$ embryos than in *Eya1*$^{-/-}$ embryos. Arrows point to the cartilage primordium. Scale bars: 50 μm for panels G–N and 100 μm for all other panels.

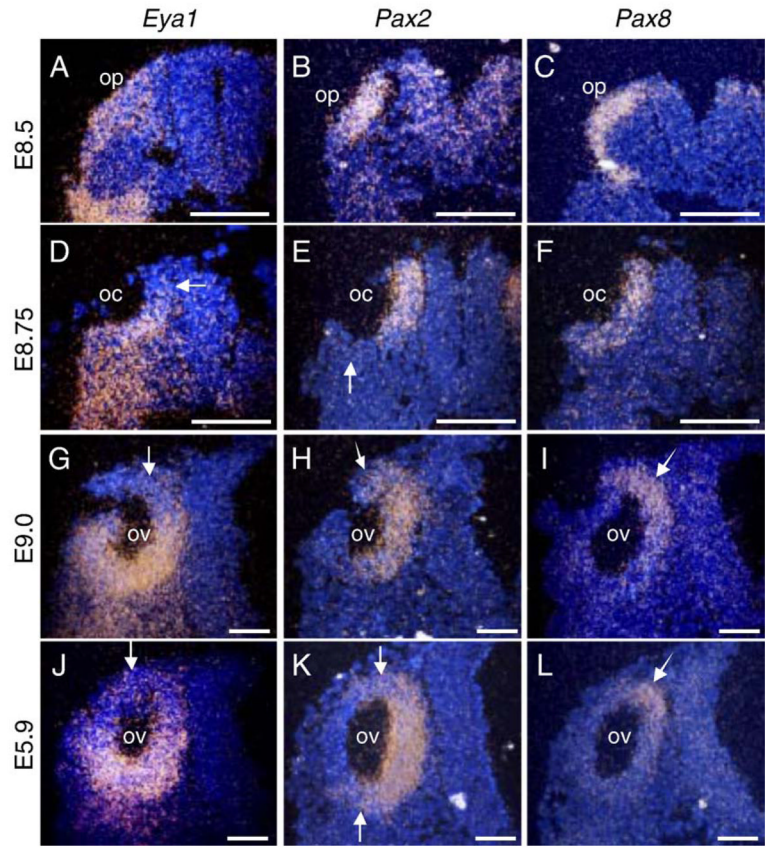

Fig. 4.

Eya1, *Pax2* and *Pax8* expression in relation to otic placode and otocyst development. All panels are transverse sections. (A) *Eya1* is expressed in the otic placode and in the periotic mesenchyme. (B, C) *Pax2* and *Pax8* are expressed in the otic placode at E8.5. (D) *Eya1* expression in the dorsal region of the otic cup (oc) is disappearing (arrow) at E8.75. (E) *Pax2* expression is excluded from the ventral and lateral otic cup (arrow) at E8.75, (F) while *Pax8* expression is stronger in the dorsal region and weaker in the ventral region of the otic cup. (G–L) At E9.0 and 9.5, *Eya1* expression is excluded from the dorsal region (arrows in panels G and J), while *Pax2* expression is excluded from the lateral region and weaker in both dorsal- and ventral-most walls (arrows in K). In contrast, *Pax8* is expressed strongly in the dorsomedial region (arrows in panels I and L). ov, otic vesicle. Scale bars: 100 μm.

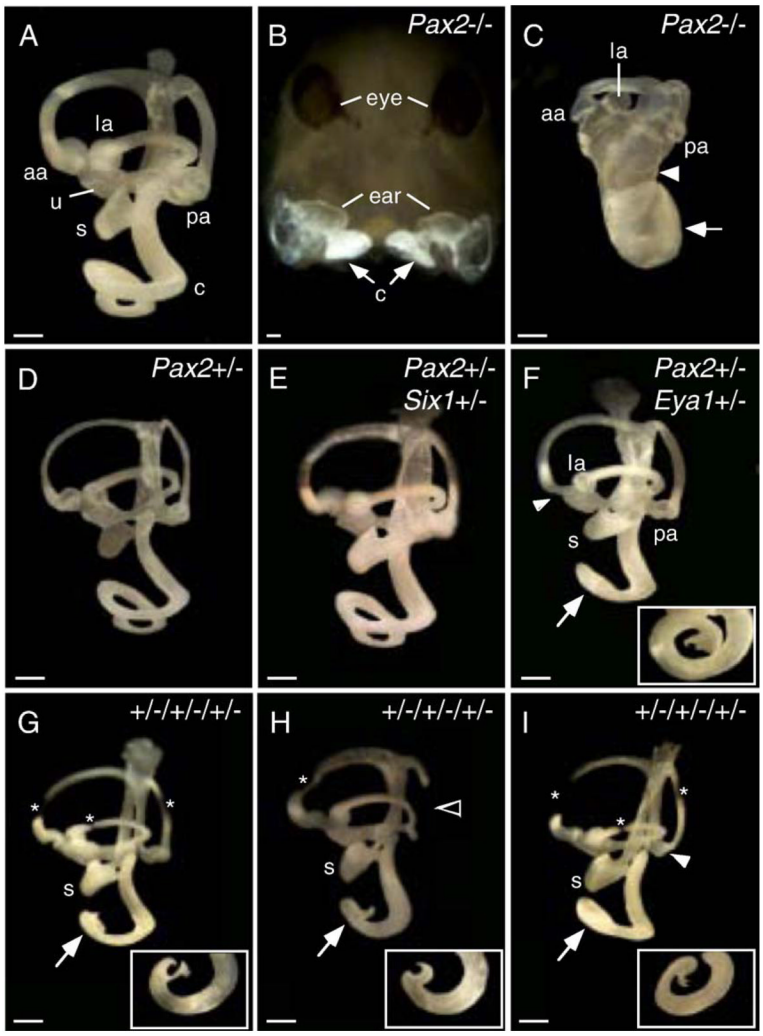

Fig. 5.

Enhancement of inner ear defects in *Pax2;Eya1* or *Pax2;Eya1;Six1* compound heterozygotes at E17.5 revealed by paintfilling. (A) Medial view of wild-type inner ear. All structures of the inner ear reached to their mature shape. The cochlea completed 1.75 turns by this stage. aa, anterior ampulla; co, cochlea; la, lateral ampulla; pa, posterior ampulla; s, saccule; u, utricle. (B) Dorsal view of $Pax2^{-/-}$ head showing the paintfilled inner ears. Note that these ears were filled by only one injection from the lateral to the posterior ampulla, and the brains were removed after paintfilling because of their brain abnormality. (C) Medial view of a $Pax2^{-/-}$ ear dissected from the head shown in panel B. No normal endolymphatic duct is visible. (D) Medial view of a $Pax2^{+/-}$ inner ear showing normal structures. The reason that the endolymphatic duct/sac is unclear in this sample is due to insufficient paint solution passaged through this structure, but it is present normally. (E) Medial view of a $Pax2^{+/-}$;$Six1^{+/-}$ inner ear showing normal structures. (F) Medial view of a $Pax2^{+/-}$;$Eya1^{+/-}$ inner ear showing morphologically unidentifiable anterior ampulla (arrowhead), small saccule and malformed cochlea (arrow), which completed between 1 and 1.25 turns, and its distal tip was enlarged and mal-shaped (inset). The endolymphatic duct/sac is relatively normal in this ear. (G–I) Inner ears from $Pax2^{+/-}$;$Eya1^{+/-}$;$Six1^{+/-}$ animals showing severely affected structures. 100% of the triple heterozygous animals showed malformed saccule, small or missing ampullae and a truncation of the semicircular canals (open arrow). Within

the semicircular canals, the lumen in some areas became narrower and it took longer time for the paint solution to passage through (asterisks). All 8 ears showed malformed distal tips of the cochlea (arrows and insets). The endolymphatic duct/sac is relatively normal in panel G and absent in panel H. The endolymphatic sac is slightly malformed in panel I. Scale bars: 200 μm.

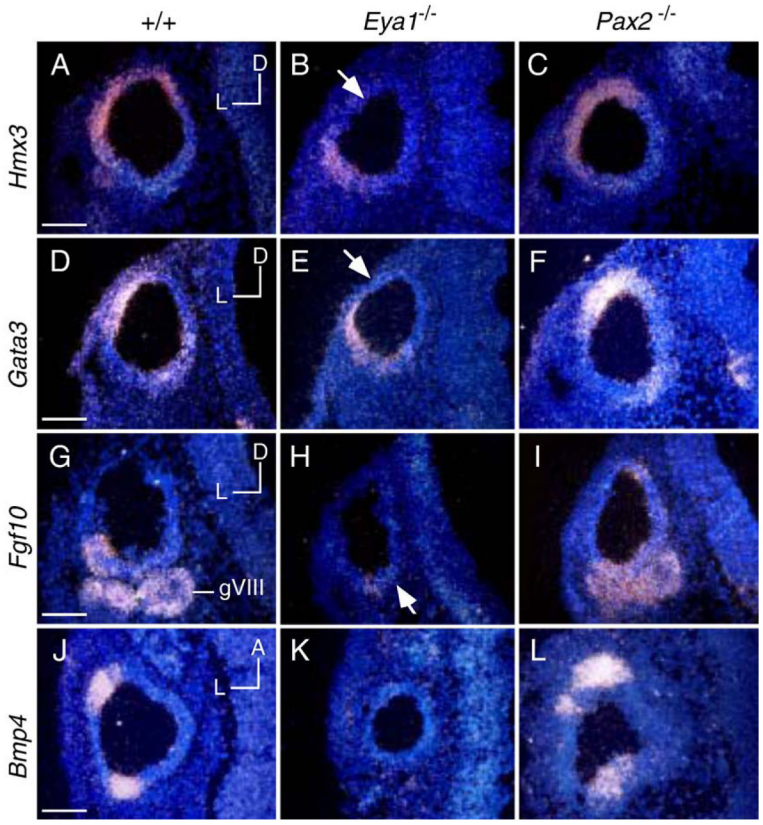

Fig. 6.

Eya1 but not *Pax2* is required for normal expression of *Hmx3*, *Gata3*, *Fgf10* and *Bmp4* in the otic vesicle at E10.5. Panels A–I are transverse sections; panels J–L are horizontal sections. Orientation is indicated for all panels. (A–C) In situ hybridization showing *Hmx3* expression in wild-type (A), *Eya1*$^{-/-}$ (B) and *Pax2*$^{-/-}$(C) embryos. *Hmx3* expression domain is shifted ventrally in *Eya1*$^{-/-}$ embryos. (D–F) In situ hybridization showing *Gata3* expression in wild-type (D), *Eya1*$^{-/-}$ (E) and *Pax2*$^{-/-}$ (F) embryos. In *Eya1*$^{-/-}$ otic vesicle, its dorsolateral expression domain is shifted ventrally and its ventromedial expression is also slightly reduced. (G–I) In situ hybridization showing *Fgf10* expression in wild-type (G), *Eya1*$^{-/-}$ (H) and *Pax2*$^{-/-}$ embryos. In *Eya1*$^{-/-}$ embryos, only residual *Fgf10* expression was detected (arrow). gVIII, VIIIth ganglion. (J–L) In situ hybridization showing *Bmp4* expression in wild-type (J), *Eya1*$^{-/-}$ (K) and *Pax2*$^{-/-}$ (L) embryos. *Bmp4* is not expressed in *Eya1*$^{-/-}$ embryos. Scale bars, 100 μm.

Table 1

Inner ear defects in *Pax2*$^{+/-}$, *Pax2*$^{+/-}$;*Eya1*$^{+/-}$, *Pax2*$^{+/-}$;*Six1*$^{+/-}$ and *Pax2*$^{+/-}$;*Eya1*$^{+/-}$;*Six1*$^{+/-}$ heterozygous embryos at E17.5

Abnormalities	***Pax2*$^{+/-}$ *n*=24 (12) [a]**	***Six1*$^{+/-}$ *n*=20 (10)**	***Eya1*$^{+/-}$ *n*=20 (10)**	***P2*$^{+/-}$;*S1*$^{+/-}$ *n*=20 (10) [b]**	***P2*$^{+/-}$;*E1*$^{+/-}$ *n*=24 (12) [c]**	***E1*$^{+/-}$;*S1*$^{+/-}$ *n*=20 (10) [d]**	***P2*$^{+/-}$;*E1*$^{+/-}$;*S1*$^{+/-}$ *n*=8 (4) [e]**
Endolymphatic duct/sac (truncated)	0	2 (2)	2 (2)	0	2 (2)	3 (2)	2 (1)
Saccule (malformed)	0	1 (1)	0	0	18 (10)	2 (1)	8 (4)
Ampullae (absent—^{A}; small—^{S})							
Posterior	0	0	0	0	2^{A} (1), 12^{S} (7)	2^{A} (1), 9^{S} (5)	4^{A} (3), 4^{S} (3)
Anterior	0	0	0	0	12^{S} (6)	11^{S} (6)	8^{s} (4)
Lateral	0	0	0	0	12^{S} (6)	11^{S} (6)	8^{s} (4)
Semicircular canal (small—^{S}; truncated—^{T})							
Posterior	0	0	0	0	2^{T} (1), 5^{S} (3)	2^{T} (1)	4^{T} (3), 4^{S} (3)
Anterior	0	0	0	0	5^{S} (3)	2^{S} (1)	8^{S} (4)
Lateral	0	0	0	0	5^{S} (3)	2^{S} (1)	8^{s} (4)
Cochlea-shortened							
<1.75 turn~≥1.5 turn	16 (9)	6 (4)	17 (9)	10 (6)	12 (8)	6 (4)	0
<1.5 turn~≥1.25 turn	3 (2)	0	3 (2)	0	0	4 (3)	0
<1.25 turn~≥1.0 turn	2 (2)	0	1 (1)	1 (1)	6 (4)	5 (4)	1 (1)
<1.0 turn	0	0	0	0	0	4 (3)	7 (4)
Mal-shaped	0	0	0	0	4 (2)	4 (4)	8 (4)

n, number of ears (the numbers shown in parentheses are the numbers of embryos).

[a] 13 of 24 *Pax2*$^{+/-}$ ears (7 of 12 embryos) in a mixed background of 129 and C57BL/6J showed a slight reduction in their overall volume but were structurally normal (Fig. 5D). Among the 13 ears, 6 (4 embryos) showed slightly shortened cochlea but completed 1.5 turns.

[b] 11 of 20 *Pax2*$^{+/-}$;*Six1*$^{+/-}$ ear (6 of 10 embryos) in the same background also showed a reduction in their overall volume but were structurally normal (Fig. 5E). 10 of 20 *Pax2*$^{+/-}$;*Six1*$^{+/-}$ ears (6 embryos) showed slightly shortened cochlea but reached 1.5 turns. Only one *Pax2*$^{+/-}$;*Six1*$^{+/-}$ ear coiled between 1 and 1.25 turns.

[c] 18 of 24 *Pax2*$^{+/-}$;*Eya1*$^{+/-}$ ears (10 of 12 embryos) in the same background showed smaller or malformed saccule and significantly smaller or morphologically unidentifiable ampullae (Fig. 5F). 2 of 24 ears (1 of 12 embryos) showed absence of the posterior ampullae and truncation of the posterior semicircular canals. The cochlea of 6 ears (4 embryos) only reached 1 and 1.25 turns and 4 (2 embryos) of them exhibited malformed distal tips (Fig. 5F).

[d] The inner ear phenotype was enhanced in *Eya1*$^{+/-}$;*Six1*$^{+/-}$ animals than in each single heterozygous animals in the same background, similar to previous observation from 129 strain (Zheng et al., 2003).

[e] All *Pax2*$^{+/-}$;*Eya1*$^{+/-}$;*Six1*$^{+/-}$ triple heterozygous ears showed small or mal-shaped saccule and significantly smaller or morphologically unidentifiable ampullae (Figs. 5G–I). 4 of 8 *Pax2*$^{+/-}$;*Eya1*$^{+/-}$;*Six1*$^{+/-}$ ears (3 of 4 embryos) showed large truncation of the posterior semicircular canals and absence of the posterior ampullae (Fig. 5H). All *Pax2*$^{+/-}$;*Eya1*$^{+/-}$;*Six1*$^{+/-}$ ears showed severely affected cochlea with severely malformed distal tips and only one ear coiled between 1 and 1.25 turns.