EBF1 limits the numbers of cochlear hair and supporting cells and forms the scala tympani and spiral limbus during the development of the inner ear

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Abstract

Early B-cell factor 1 (EBF1) is a basic helix-loop-helix transcription factor essential for the differentiation of various tissues. Our single-cell RNA-seq data suggest that *Ebf1* is expressed in the sensory epithelium of the inner ear. Here, we found that *Ebf1* and its protein are expressed in the inner ear prosensory domain, medial region of the cochlear floor, inner ear mesenchyme, and cochleo-vestibular ganglion. *Ebf1* deletion results in incomplete formation of the spiral limbus and scala tympani, an increase in the number of cells in the organ of Corti and Kölliker's organ, and deterioration of the running of the spiral ganglion axons. The inner ear-specific deletion of *Ebf1* revealed that scala tympani formation depended on *Ebf1* expressed in the otic mesenchyme. *Ebf1* deletion in the cochlear epithelia caused the proliferation of SOX2-positive cochlear cells at E13.5, indicating that EBF1 suppresses the proliferation of the prosensory domain and cells of Kölliker's organ to develop appropriate numbers of hair and supporting cells. Postnatal hearing function of cochlear epithelium-specific *Ebf1*-deleted mice was poor. Our results suggest that *Ebf1* is essential for normal auditory function in mammals.

Introduction

The inner ear is a unique and complex organ that consists of bony and membranous labyrinths. The membranous labyrinth contains multiple sensory organs including the cochlea, which is responsible for hearing. The cochlea comprises three compartments (scalae): the scala vestibuli, scala tympani, and scala media. Among them, the scala vestibuli and scala tympani develop from the mesenchyme surrounding the inner ear (Sher 1971). The scala media exists between the scala

vestibule and scala tympani, which are separated from the scala media by the tectorial and basilar membranes, respectively. The scala media contains sensory epithelia that transduce sound into electrical signals with specialized sensory cells: hair cells. Cochlear hair cells exist within the organ of Corti, the middle part of the scala tympani epithelium, and consist of one row of inner hair cells and three rows of outer hair cells. At least seven types of non-sensory supporting cells were found around these hair cells: inner boundary cells, inner and outer phalangeal cells, inner and outer pillar cells, Hensen's cells, and Claudius cells. The precise number and placement of mechanosensory hair cells and non-sensory supporting cells make it possible to correctly receive the mechanical stimulation of sound or body motion and convert it into neural signals.

Inner ear development in mice (Wu and Kelley 2012) begins with the formation of an ectodermal thickening called the otic placode, adjacent to the hindbrain. The otic placode invaginates to form a spherical structure called an otocyst at approximately E9.5. The ventral side of the otocyst forms the future sensory epithelium, where the sex-determining region Y-box transcription factor 2 (*Sox2*) is expressed (Kiernan et al. 2005). At E10.5, the cochlear duct and the endolymphatic duct and semicircular canals begin to form on the ventral and dorsal sides of the otocyst, respectively. After ventral cochlear duct formation becomes evident at E11 (Wu and Kelley 2012), the ventral side of the cochlear duct (cochlear duct floor) begins to develop into a future sensory domain by expressing *Sox2* and *Jagged1* at E11.5. *Sox2* positive region becomes limited to the middle part of the ventral cochlear duct and is recognized as a prosensory domain at E13.5 and E14.5 (Kiernan et al. 2005; Ohyama et al. 2010). This region is located between two molecularly distinct nonsensory areas. The region medial to the prosensory domain toward the axis of the cochlea (modiolus) is called the greater epithelial ridge (GER). The GER transiently contains Kölliker's organ composed of columnar supporting cells during the developmental stage,

and becomes the inner sulcus with cuboidal cells and the spiral limbus with interdental cells in the mature cochlea (Dayaratne et al. 2014). More importantly, GER is a source of sensory epithelia and has the potential to produce sensory cells after the establishment of hair cells (Kubota et al. 2021).

The complex cellular structure and developmental processes of the inner ear depend on the highly regulated expression patterns of signaling molecules and transcription factors. Although, several transcription factors have been found to have essential functions, the mechanisms underlying inner ear development are not fully understood. To elucidate this more comprehensively, we analyzed the single-cell RNA-seq data of the inner ear epithelial cells. In this study, we found that early B-cell factor 1 (*Ebf1*) was upregulated in clusters of sensory epithelial progenitors in the cochlea, macula, and crista, and confirmed that *Ebf1* was expressed on the medial side of the cochlear duct floor, the prosensory area of the macula and crista, and the spiral ganglion (Yamamoto et al. 2021).

Ebf1 belongs to the *Ebf* family of transcription factors and encodes four paralogous genes in mammals, including mice and humans (Liberg et al. 2002). EBF family proteins are composed of a functional DNA-binding domain, transcription factor immunoglobulin, helix-loop-helix (HLH) domain, and transactivation domain (Liberg et al. 2002), which are characteristic of bHLH transcription factors. Similar to other bHLH transcription factors, *Ebf1*is involved in various developmental processes, including cell fate determination and differentiation of B lymphocytes and olfactory epithelia (Liberg et al. 2002).

Considering the various developmental roles of *Ebf1* as a bHLH transcription factor, the importance of bHLH transcription factors such as ATOH1 in inner ear development, and the results of our previous study confirming *Ebf1* expression in the inner ear sensory epithelium, we

decided to analyze the function of *Ebf1* in the developmental process of the inner ear. In the present study, we confirmed the spatiotemporal expression of *Ebf1* during inner ear development and examined the effects of *Ebf1* deletion on inner ear development and hearing.

Results

Ebf1 is expressed in the developing mouse cochlea

As previously reported, *in silico* analysis of embryonic inner ear epithelia suggested that *Ebf1* is predominantly expressed in the inner ear sensory epithelium during early development (Yamamoto et al. 2021). To quantify the expression of *Ebf1* at each stage of inner ear development, we performed quantitative reverse transcription PCR (qRT-PCR) using whole embryonic inner ears from E9.5 to P0 (Fig. 1A). The expression of *Ebf1* mRNA transcripts started to increase at E10.5, and reached a maximum expression level at E13.5 (Fig. 1A). The relative expression level at E13.5 was approximately 10-fold higher than that at E9.5. The expression level then decreased but was still 7.5 times higher even at P0 than at E9.5.

To describe the spatiotemporal expression patterns of *Ebf1* during inner ear development, we performed *in situ* hybridization (ISH) and immunohistochemistry (IHC) on sections of the developing wild-type mouse inner ear from E9.5 to P0 (Fig.1 B–D, Supplemental Fig. S1). We stained *Sox2*, which is expressed in the sensory progenitor region of the inner ear from the early developmental stages (Kiernan et al. 2005), as well as *Ebf1* on adjacent sections to specify the location of *Ebf1* expression, and compared the expression of these two genes and their products.

First, we examined the expression of *Ebf1* at E13.5 (Fig. 1B) when the sensory epithelium of the inner ear forms and *Ebf1* expression level is maximized during inner ear development (Fig. 1A). *Ebf1* was expressed on the medial side of the cochlear floor, including the prosensory domain

(white arrows in Fig. 1B), spiral ganglion (white asterisks in Fig. 1B), otic mesenchyme (black asterisks in Fig. 1B), and parts of the prosensory regions of the vestibule and crista (black arrows in Fig. 1B). Compared with *Sox2*, *Ebf1* was expressed more medially within the cochlear floor, which developed into Kölliker's organ and the organ of Corti, and was more restricted in the vestibule (Fig. 1A).

Next, we checked the spatiotemporal expression of *Ebf1* throughout the inner ear development, including the onset of expression in the inner ear epithelium, using inner ear sections from E9.5 to P0 (Fig. 1C, Supplemental Fig. S1). At E9.5, *Ebf1* is not expressed in the otocyst but is expressed in the progenitor cells of the cochleo-vestibular ganglion (CVG), which delaminate from the ventral side of the otocyst into the otic mesenchyme (data not shown) (Wu and Kelley 2012). At E10.5, *Ebf1* expression was observed on the ventromedial side of the otocyst, which will develop into the cochlear duct, and in the ventrolateral epithelium of the otocyst, which will develop into the crista (black arrowheads in Fig. 1C). In addition, *Ebf1* expression was detected in the otic mesenchyme (black asterisk in Fig. 1C) and CVG, which persisted until later stages (Supplemental Fig. S1). At E11.5 and E12.5, *Ebf1* was expressed in the border region, where the cochlear duct begins to elongate at E11.5, and the medial side of the cochlear floor at E12.5, and in the future crista region in the vestibule (Supplemental Fig. S1). In the cochlea at E16.5 and E18.5, *Ebf1* was expressed throughout the organ of Corti and Kölliker's organ, whereas *Sox2* was expressed in the organ of Corti and in the lateral half of the Kölliker's organ (Fig. 1C and Supplemental Fig. S1), as previously reported (Urness et al. 2015). *Ebf1* was expressed in the spiral ligaments, tympanic border cells (Taniguchi et al. 2012), vestibules, and crista (Fig. 1B). *Ebf1* expression was maintained until P0 as far as we could confirm (Supplemental Fig. S1).

IHC showed that EBF1 was expressed throughout the Kölliker's organ and the prosensory domain, whereas SOX2 was expressed in part of the Kölliker's organ and the prosensory domain (upper panels of Fig. 1D), which is consistent with the ISH results. The disappearance of the EBF1 signal from the cochlear epithelia and mesenchyme in conventional *Ebf1* knockout (*Ebf1*$^{-/-}$) mice confirmed the specificity of the anti-EBF1 antibody used in this study (Fig. 1D).

Ebf1 deletion altered the structure of the cochlear duct

Our ISH and IHC experiments, showing the expression of *Ebf1* and its protein in both the developmental inner ear epithelia and mesenchyme, suggest that *Ebf1* is involved in the development of both the inner ear sensory epithelium and otic mesenchyme. To examine the roles of *Ebf1* in the developing inner ears, we used two different mutant mice. One is an *Ebf1* conventional knockout (*Ebf1*$^{-/-}$) mouse (Lin and Grosschedl 1995), and the other is a Foxg1-Cre mediated inner ear epithelia-specific conditional knockout mouse (*Foxg1Cre; Ebf1*$^{fl/fl}$) (Hébert and McConnell 2000; Gyory et al. 2012), in which *Ebf1* expression remains in the inner ear mesenchyme.

Comparing the gross morphology of the membranous labyrinth of the inner ear at E18.5, no difference was observed between *Ebf1*$^{+/+}$ and *Ebf1*$^{+/-}$ mice (Fig. 2A). However, after removing the lateral wall and roof of the cochlea to expose the cochlear floor, we found that *Ebf1*$^{-/-}$ mice had fewer turns in the cochlear duct than did *Ebf1*$^{+/+}$ mice (Fig. 2B).

Hematoxylin-eosin (HE) staining of the cochlea at E18.5 revealed incomplete formation of the scala tympani, especially in the middle and apical turns of the *Ebf1*$^{-/-}$ mice cochlea compared with control mice (arrowheads in Fig. 2C). Moreover, *Ebf1*$^{-/-}$ mice lacked a spiral limbus (sl; Fig.

2C). A lower number of cochlear turns was also observed in the cochlear sections of *Ebf1*$^{-/-}$ mice (arrows in the upper right panels in Fig. 2C), supporting the observation of gross morphology.

Ebf1 was expressed in the otic mesenchyme from early developmental stages, and its expression was maintained throughout inner ear development. To elucidate whether the hypoplastic scala tympani, fewer cochlear turns, and lack of spiral limbus are caused by the *Ebf1* deficient mesenchyme, we examined the cochlear morphology of *Foxg1Cre; Ebf1*$^{fl/fl}$ and *Foxg1Cre; Ebf1*$^{fl/+}$ mice using HE staining. In contrast to the hypoplastic scala tympani of *Ebf1*$^{-/-}$ mice, the scala tympani of *Foxg1Cre; Ebf1*$^{fl/fl}$ mice was completely formed throughout the cochlear turns (left panels of Fig. 2C). The number of cochlear turns in *Foxg1Cre; Ebf1*$^{fl/fl}$ mice was similar to that in the control mice (*Foxg1Cre; Ebf1*$^{fl/+}$ mice). However, *Foxg1Cre; Ebf1*$^{fl/fl}$ mice lacked a spiral limbus, as observed in *Ebf1*$^{-/-}$ mice. (Fig. 2C). These results suggest that *Ebf1* controls the formation of scala tympani and turning of the cochlear duct through the mesenchyme and the lack of the spiral limbus in Ebf1 deficient mice is secondarily caused by the EBF1 malfunction within the epithelia.

Ebf1 deletion caused an increase in the number of cochlear hair cells, supporting cells and Kölliker's organ

By carefully observing the cochlear epithelia in HE-stained samples, we found that both *Ebf1*$^{-/-}$ and *Foxg1Cre; Ebf1*$^{fl/fl}$ mice had deformed Kölliker's organs and organs of Corti (Ko and oC in Fig. 2C). To examine these phenotypes more precisely, we performed IHC on cochlear sections and cochlear whole-mount samples at E18.5 (Fig. 3 and Supplemental Figs. S2 and S3).

IHC of cochlear sections showed that *Ebf1* deletion increased the number of MYO7A-positive hair cells and SOX2-positive supporting and Kölliker's organ cells from the basal to the apical

region at E18.5 (Fig. 3A). An increase in SOX2-positive cells within the medial region of the cochlear floor was also observed at E14.5 (arrowhead in Fig. 1D). In contrast, *Ebf1* deletion had no morphological effects on the sensory epithelium of the crista or vestibule (Supplemental Fig. S2). In addition, the apical region of *Ebf1*$^{-/-}$ mouse cochleae contained multiple layers of SOX2-positive cells (asterisk in Fig. 3A). IHC of whole-mount cochlear samples showed that *Ebf1*$^{-/-}$ mice had an increased number of MYO7A-positive hair cells (Fig. 3B) and ectopic MYO7A-positive cells within the GER (arrows in Fig. 3B). Although the normal cochlea has one row of inner, and three rows of outer hair cells, the mutant cochlea has eight to nine hair cell rows. We found ectopic hair cells in seven of the 12 examined cochleae. These ectopic MYO7A-positive cells contained stereocillia-like structures, as indicated by phalloidin staining (arrows in Fig. 3C). Increased numbers of supporting cells were confirmed in whole-mount cochlear samples by IHC staining of SOX2 (Fig. 3D), a supporting and Kölliker's organ cell marker, and PROX1 (Fig. 3E), a pillar and Deiters' cell marker (Bermingham-McDonogh et al. 2006).

To quantify the number of hair and supporting cells, we counted the cells in three regions within the cochlea (basal, middle, and apical regions; Fig. 4A) and measured the number of MYO7A- or PROX1-positive cells per 200 μm at E18.5. For SOX2-positive cells, we counted the cell number per 100 μm only in the basal regions at E18.5. The results showed that the number of cochlear hair cells was significantly increased in *Ebf1*$^{-/-}$ mice compared to *Ebf1*$^{+/+}$ mice in all three regions (Fig. 4B). *Ebf1*$^{+/-}$ mice showed a significantly higher number of cochlear hair cells than *Ebf1*$^{+/+}$ mice in the middle and apical regions (Fig. 4B). The hair cell numbers per 200 μm of *Ebf1*$^{+/+}$, *Ebf1*$^{+/-}$, and *Ebf1*$^{-/-}$ mice are 142.3±9.0, 150.0±2.8, and 367.5±30.8 in the basal regions, 152.8±8.1, 190.3±7.0, and 363.0±22.4 in the middle regions, and 154.5±5.1, 183.5±6.8, and 440.3±23.8 in the apical regions, respectively.

SOX2-positive cells, constituting a part of Kölliker's organ cells and supporting cells within organs of Corti, also increased in number in *Ebf1*$^{-/-}$ mice by 1.7 times of *Ebf1*$^{+/+}$ mice SOX2-positive cell numbers (Fig. 4C). PROX1-positive cell numbers also significantly increased in *Ebf1*$^{-/-}$ mice compared to *Ebf1*$^{+/+}$ mice only in the basal and middle regions (Fig. 4D). In the apical region, the number of PROX1-positive cells was similar in *Ebf1*$^{-/-}$ and *Ebf1*$^{+/+}$ mice. The PROX1-positive cell numbers in *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice were 203.3±2.2 and 439.0±15.4 in the basal regions, 228.0±16.8 and 389.8±10.4 in the middle regions, and 196.0±27.3 and 204.5±19.6 in the apical regions.

We performed immunostaining for more specific markers to reveal which population of hair or supporting cells increased in number in *Ebf1*$^{-/-}$ mice (Supplemental Fig. S3). We immunostained the whole-mounted cochlea at E18.5 with anti-VGLUT3 and anti-p75 antibodies, which indicate inner hair cells (Li et al. 2018) and pillar cells (von Bartheld et al. 1991), respectively. VGLUT3-positive inner hair cells, which are arranged in one row in wild-type mice, increased in number in *Ebf1*$^{-/-}$ mice (Supplemental Fig. S3A), indicating an increase in both inner and outer hair cells. The number of p75-positive pillar cells, which separate inner and outer hair cells, did not increase in *Ebf1*$^{-/-}$ mice. Considering that PROX1-positive cells indicate pillar and Deiters' cells, the Deiters' cell number increased in *Ebf1*$^{-/-}$ mice. However, the arrangement of pillar cells was deteriorated in *Ebf1*$^{-/-}$ mice (arrows in Supplemental Fig. S3B), which was reflected in the appearance of VGLUT3 cells in the outer hair cell region of *Ebf1*$^{-/-}$ mice (arrowheads in Supplemental Fig. S3A).

The cochlear length was measured based on the length of MYO7A positive region (Supplemental Fig. S4A). The total cochlear length of *Ebf1*$^{-/-}$ mice was slightly, but significantly, shorter than that of *Ebf1*$^{+/+}$ and *Ebf1*$^{+/-}$ mice (Supplemental Fig. S4B).

Ebf1 deletion caused the aberrant spiral ganglion and nerve fibers.

Since *Ebf1* is expressed in the spiral ganglion during inner ear development and the number of hair cells, a target of the spiral ganglion cell axon, increased in *Ebf1*$^{-/-}$ mice, we examined the spiral ganglion morphology and innervation of cochlear hair cells in E18.5mice with *Ebf1* deletion (Supplemental Fig. S5).

Compared with *Ebf1*$^{+/+}$ mice with axons extending from the spiral ganglion cells to the cochlear hair cells, *Ebf1*$^{-/-}$ mice had spiral ganglion cells (sg in Supplemental Fig. S5A) under the organs of Corti (arrows in Supplemental Fig. S5A), as well as in their normal position. The axons, usually running parallel to the rows of outer hair cells, formed a mesh-like network in the area of the *Ebf1*$^{-/-}$ mouse cochlear hair cells (Supplemental Fig. S5B). Moreover, the innervation reached the Kölliker's organ (arrowheads and brackets in Supplemental Fig. S5A) in addition to the organs of Corti.

The Ebf1 deletion changed the distribution of JAG1-positive Kölliker's organ cells, the differentiation timing of a prosensory domain, and the proliferation of SOX2-positive cells.

Since *Ebf1*$^{-/-}$ mice had increased numbers of cochlear hair cells, which were differentiated from the prosensory domain, we investigated its specification, differentiation, proliferation, and cell death in the *Ebf1*$^{-/-}$ mouse cochlear duct floor.

First, to determine whether formation of the prosensory domain was affected by *Ebf1* deletion, we examined the formation of regions medial and lateral to the prosensory domain. Since these two regions express FGF10 and BMP4 to induce non-sensory or sensory epithelia in the cochlear floor (Ohyama et al. 2010; Urness et al. 2015), respectively, we performed ISH for *Fgf10* and

Bmp4 in the basal region *Ebf1*$^{+/+}$ *and Ebf1*$^{-/-}$ mice of the cochlear duct at E13.5 (Fig. 5A). The formation of both regions was similar in *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice, suggesting that *Ebf1* is not involved in the development of cell populations expressing *Fgf10* or *Bmp4*. To verify the medial cell population more precisely, we immunostained E13.5 and E14.5 cochleae with an anti-JAG1 antibody (Fig. 5A), which is exclusively expressed in the Kölliker's organ, a part of the medial region, at this stage (Ohyama et al. 2010). The JAG1-positive cells in the *Ebf1*$^{-/-}$ mouse cochlea expanded to the more medial region compared with those in the *Ebf1*$^{+/+}$ mouse cochlea at E13.5 and E14.5 (arrowheads in Fig. 5A).

Next, we examined the expression of *Atoh1* within the prosensory domain using ISH at E14.5 and E15.5 (Fig. 5B) because *Atoh1*is necessary for hair cells to differentiate from the prosensory cell population (Bermingham et al. 1999) and its expression indicates the initiation of hair cell development from the prosensory domain. Compared to *Ebf1*$^{+/+}$ mice that express *Atoh1* within the prosensory domain from E14.5, we could not detect *Atoh1* mRNA in the basal to middle region of the E14.5 *Ebf1*$^{-/-}$ mouse cochlea, although the vestibular organs expressed *Atoh1* within the prosensory epithelia. However, E15.5 *Ebf1*$^{-/-}$ mice exhibited an *Atoh1* signal within the prosensory domain of the cochlea. This result suggests that the cell fate sepcification of sensory epithelia in the cochlea is not affected, but its timing is delayed by *Ebf1* deletion. Considering that the differentiation of cochlear sensory epithelia promotes the transition from the basal to apical turns of the cochlea (Sher 1971), the expression of hair and supporting cell markers at later stage, E18.5, also indicated delayed differentiation of *Ebf1*$^{-/-}$ mouse cochleae (Supplemental Fig. S3). These markers were detected in more basal cochlear regions in *Ebf1*$^{-/-}$ mice than in *Ebf1*$^{+/+}$ mice.

The expansion of the JAG1-positive cell area and the increased numbers of hair and supporting cells suggest that the enhancement of proliferation or suppression of cell death occurs within the prosensory domain and/or Kölliker's organ of the *Ebf1*$^{-/-}$ mouse cochlea. To identify the mechanisms that correlate with the functions of EBF1 within the cochlea, we tested the proliferation and apoptotic status in the *Ebf1*$^{-/-}$ mice cochlea. To evaluate the proliferation status of the prosensory domain and/or Kölliker's organ, we immunostained cochlear sections with SOX2, a marker of the prosensory domain and/or a part of the Kölliker's organ, and EdU at E12.5, 13.5, 14.5, and 16.5 after administering to pregnant mice (Fig. 6A). Quantification of SOX2-positive cells showed that their number decreased in *Ebf1*$^{+/+}$ mice from E12.5, onward (Fig. 6B), and their location was limited to the prosensory domain (brackets in Fig. 6A). In contrast, SOX2-positive cells in the *Ebf1*$^{-/-}$ mouse cochlea were found both in the prosensory domain and in the medial region, even at E13.5, which was consistent with the results of JAG1 immunostaining (Fig. 5A). The number of SOX2-positive cells in the *Ebf1*$^{-/-}$ mice was similar with that in the *Ebf1*$^{+/+}$ mice at E12.5, but increased at E13.5 and returned to E12.5 level at E14.5. As a result, SOX2-positive cell number in the *Ebf1*$^{-/-}$ mice was significantly higher at E13.5 and E14.5 (Fig. 6B). Regarding EdU-positive proliferating cells within SOX2-positive cells, their number in the *Ebf1*$^{-/-}$ mice was significantly higher than in *Ebf1*$^{+/+}$ mice at E13.5 and E14.5 (Fig. 6C). Since SOX2 positive cell number increased in *Ebf1*$^{-/-}$ mice from E13.5 (Fig. 6B), normalization of SOX2-positive cell numbers was necessary to correctly evaluate the proliferation status of SOX2-positive cells. We calculated the proportion of EdU-positive cells among SOX2-positive cells and found that the proliferation was enhanced in SOX2-positive cells of the *Ebf1*$^{-/-}$ mice cochlea only at E13.5 (49.8±4.3%) compared with the *Ebf1*$^{+/+}$ mice (37.8±4.2%) (Fig. 6D). These results suggested that EBF1 suppressed the proliferation of

SOX2-positive cells within a limited time window. Morphologically, a difference in proliferation was observed in the prosensory domain, as indicated by EdU immunostaining (brackets at E13.5; Fig. 6A). EdU staining was observed in the Kölliker's organs of *Ebf1*$^{-/-}$ mice, even at E16.5, which was not observed in *Ebf1*$^{+/+}$ mice (E16.5 of Fig. 6A).

The loss of proliferation within the prosensory domain around E13.5 (bracket in the *Ebf1*$^{+/+}$ sample at E13.5, Fig. 6A), has been well documented in previous studies (Chen et al. 2002). The post-mitotic domain is called the zone of non-proliferating cells (ZNPC), which is characterized by the expression of the cyclin-dependent kinase inhibitor p27Kip1. To confirm the EdU immunostaining results, we performed p27Kip1 immunostaining on E13.5 and E14.5 (Fig. 6E). Although p27Kip1 was detected in the prosensory domain of *Ebf1*$^{+/+}$ mice at E13.5, the *Ebf1*$^{-/-}$ mouse cochlea did not express it at this stage (arrows in Fig. 6E), which was consistent with the results of EdU detection. At E14.5, p27Kip1 was detected in the middle part of *Ebf1*$^{-/-}$ mouse cochlear floors in a broader area than in *Ebf1*$^{+/+}$ mice.

In contrast, apoptosis within the inner ear or cochlear duct did not increase in *Ebf1*$^{-/-}$ mice at E11.5 and E13.5, compared with *Ebf1*$^{+/+}$ mice (Supplemental Fig. S6).

Ebf1 deletion impairs auditory function.

The aberrant cochlear sensory epithelia observed in *Ebf1* deletion mice suggest that hearing ability is impaired in these mice. To evaluate the effect of *Ebf1* deletion on auditory function, we measured the auditory brainstem response (ABR) (Fig. 7A) and distortion product of otoacoustic emissions (DPOAE) (Fig. 7B) in P21 *Foxg1Cre; Ebf1*$^{fl/fl}$ mice. We did not use conventional *Ebf1* knockout mice (*Ebf1*$^{-/-}$ mice) for this analysis to avoid embryonic lethality and to eliminate the effects of the hypoplastic scala tympani observed in *Ebf1*$^{-/-}$mice on auditory function. The

phenotype of *Foxg1Cre; Ebf1*$^{fl/fl}$ mice was evaluated using whole-mount cochlear samples collected at P23 (Fig. 7C). We observed a marked increase in the number of cochlear hair cells in *Foxg1Cre; Ebf1*$^{fl/fl}$ mice, comparable to the morphology of E18.5 *Ebf1*$^{-/-}$ mice (Fig. 3B and Fig. 7C).

ABR measurement showed significant elevations of thresholds of the response to sound in *Foxg1Cre; Ebf1*$^{fl/fl}$ mice at all frequencies examined (93.3±2.5; 10kHz, 86.7±3.8; 20kHz, 105.0±0.0; 40kHz) (Fig. 7A) compared with control mice, indicating severe hearing loss of *Ebf1*-deleted mice. We then performed DPOAE tests to assess the function of the increased outer hair cells caused by *Ebf1* deletion because DPOAE detects nonlinear responses of outer hair cells to sound. The DPOAE responses in *Foxg1Cre; Ebf1*$^{fl/fl}$ mice were significantly lower than those in control mice (Fig. 7B). The decreased DPOAE response in *Foxg1Cre; Ebf1*$^{fl/fl}$ mice also suggests that the increased number of hair cells caused by *Ebf1* deletion did not function as outer hair cells.

Discussion

The results presented in this study indicate a new and interesting role for *Ebf1* in the development of the cochlear epithelia and otic mesenchyme. *Ebf1* is involved in the formation of appropriate numbers of hair and supporting cells within the cochlear sensory epithelia. Moreover, *Ebf1* is important for the development of scala tympani, which is formed through *Ebf1* expression within the mesenchyme, and spiral ganglion cells. Owing to the indispensable role of *Ebf1* in the cochlear epithelia, its deficiency resulted in impaired auditory function, suggesting that *Ebf1* is required for establishing normal hearing.

Ebf1 was originally found from two different sources in mice. One source is a peptide that binds

to the promoter region of the mb-1 gene, whose product is expressed specifically in the early stages of B cell differentiation (Hagman et al. 1991). The other is a transcriptional activator that binds to olfactory-specific genes (Wang and Reed 1993). Since then, the expression patterns and functions of EBF1 have been studied in many organs, including B lymphocytes (Lin and Grosschedl 1995), olfactory epithelia (Wang et al. 1997), striatum neurons (Garel et al. 1999), retina (Jin et al. 2010; Jin and Xiang 2011), kidney (Fretz et al. 2014; Nelson et al. 2019), bone marrow stromal cells (Derecka et al. 2020), osteoblasts (Hesslein et al. 2009), and adipose tissue (Jimenez et al. 2007). The expression of *Ebf1* and its protein product has been reported in all three germinal layers, and the function of EBF1 varies (Liberg et al. 2002). EBF1 is involved in the cell fate specification (Jin et al. 2010; Nechanitzky et al. 2013), differentiation (Lin and Grosschedl 1995; Wang et al. 1997; Garel et al. 1999; Jimenez et al. 2007; Jin and Xiang 2011; Nieminen-Pihala et al. 2021), maturation (Fretz et al. 2014; Nelson et al. 2019), migration (Derecka et al. 2020), and path finding by neuron (Garel et al. 2000; Jin and Xiang 2011), mainly during the developmental stage in mammals.

The present study revealed that *Ebf1* is expressed not only in the inner ear epithelium and spiral ganglion but also in the otic mesenchyme. Since the inner ear epithelium and spiral ganglion cells are derived from the ectoderm (Wu and Kelley 2012), *Ebf1* is expressed in both ectodermal and mesenchymal tissues within the inner ear. Its expression in the inner ear began at approximately E10.5, as confirmed by qRT-PCR and ISH (Fig. 1A and C). Within the inner ear epithelia, *Ebf1* showed different expression patterns in the cochlear and vestibular organs from E13.5 to E18.5. In the vestibule and crista, *Ebf1* was expressed in a part of *Sox2*-positive prosensory regions (Fig. 1B, arrows and C). However, its expression in the cochlea was not limited to a part of *Sox2*-positive prosensory domain (arrow in Fig. 1B) but expanded toward a

more medial region in the cochlear floor, where Kölliker's organ exists (Fig. 1B–D). Thus, the *Ebf1* expression area contained most of the GER. This difference suggests different functions of *Ebf1* in the development of the cochlear and vestibular organs.

To elucidate the function of Ebf1 in inner ear development, we examined the inner ear morphology of *Ebf1* conventional and inner ear-specific conventional knockout mice. As suggested by the expression study, morphological phenotypes differed between the cochlear and vestibular organs. We did not observe any differences in $Ebf1^{+/+}$ and $Ebf1^{-/-}$ mice vestibular morphology (Supplemental Fig. S2), despite *Ebf1* expression. As observed in the olfactory epithelia and retina (Wang and Reed 1993; Wang et al. 1997; Jin et al. 2010), the deletion of *Ebf1* may be compensated for by other *Ebf* family genes, and we need to observe other mutant mice, including double- or triple-knockout mice and mice with the dominant negative form of the EBF protein, to elucidate the function of EBF1 within the vestibular organ.

In contrast, the cochlea of *Ebf1*–deleted mice showed various phenotypes, indicating that other *Ebf* subtypes do not have redundant functions with *Ebf1* in the cochlea as in B cells, osteoblasts, and the striatum (Lin and Grosschedl 1995; Garel et al. 1999; Nieminen-Pihala et al. 2021). HE staining revealed loss of the scala tympani and spiral limbus (Fig. 2C). Because both structures are derived from mesenchymal tissues (Sher 1971; Phippard et al. 1999), we hypothesized that these phenotypes reflect the roles of EBF1 in cochlear mesenchymal tissue. To confirm this, we compared the formation of the scala tympani and spiral limbus in two different *Ebf1* deletion mice, conventional ($Ebf1^{-/-}$ mice) and epithelia-specific conditional (*Foxg1Cre;* $Ebf1^{fl/fl}$ mice) *Ebf1* knockout mice (Fig. 2C). Although the scala tympani developed normally in *Foxg1Cre;* $Ebf1^{fl/fl}$ mice, the spiral limbus was hypoplastic in *Foxg1Cre;* $Ebf1^{fl/fl}$ mice, as observed in $Ebf1^{-/-}$ mice. These results clearly indicate that EBF1 in the cochlear mesenchyme is involved in the

formation of the scala tympani. In contrast, spiral limbus formation depends on both mesenchymal and epithelial or epithelial expression of EBF1. Since EBF1 was expressed in both the epithelia and mesenchyme (Fig. 1B and Supplemental Fig. S1), we could not determine which was the case in this study; however, mesenchyme-specific deletion of *Ebf1* will confirm the tissue responsible for inducing the spiral limbus. Gross morphological observation (Fig 2B) and MYO7A staining of the whole-mount cochlea (Supplemental Fig. S4) showed that the length of the cochlear duct was shorter in *Ebf1*$^{-/-}$ mice. Since the number of cochlear turns in the cochlear section was lower in *Ebf1*$^{-/-}$ mice than in *Foxg1Cre; Ebf1*$^{fl/fl}$ mice, this phenotype reflects the function of mesenchymal EBF1 in regulating the length of the cochlear duct.

In addition to the mesenchyme, more prominent roles for *Ebf1* have been found in the cochlear epithelia. In both *Ebf1*$^{-/-}$ and *Foxg1Cre; Ebf1*$^{fl/fl}$ mice, both hair and supporting cells increased in number throughout the cochlear turns at E18.5 (Fig. 3 and 4). Regarding hair cells, we found that both inner and outer hair cell numbers increased (Supplemental Fig. S3A). However, more detailed IHC for supporting cells showed that the increased cells were Deiters' cells among PROX1-positive and SOX2-positive cells located medial to the organs of Corti, and the pillar cell number did not increase (Supplemental Fig. S3B). This phenotype suggests that EBF1 is involved in the regulation of hair and supporting cell number during cochlear development. To determine the mechanisms of hair and supporting cell number increase in *Ebf1*-deletion, we evaluated the specifications and differentiation of the cochlear prosensory domain and its proliferation and cell death status. We evaluated the formation of cochlear non-sensory regions medial and lateral to the prosensory domain using ISH of *Fgf10* and *Bmp4* and found that these regions were normal in *Ebf1*$^{-/-}$ mice (Fig. 5A). Even though the lateral region marker BMP4 is reported to affect the development of the prosensory domain (Ohyama et al. 2010), the normal

formation of this region indicates that the phenotypes observed in *Ebf1*$^{-/-}$ mouse cochlear sensory epithelia were not caused by aberrant BMP4 expression, but by factors within the prosensory domain. In contrast to markers outside the prosensory domain, molecules expressed in the prosensory domain and/or Kölliker's organs, JAG1 and SOX2, showed abnormal expression patterns (Fig. 1D, Fig. 3A, and Fig. 5A). These two molecules were expressed in a more medial region of the *Ebf1*$^{-/-}$ mouse cochlear floor at E14.5 and E18.5. Considering that EBF1 was expressed in a more medial region than SOX2 in wild-type mice, EBF1 plays a role in suppressing the localization of JAG1- and SOX2-positive cells in the most medial region. The study of proliferation status within the SOX2-positive cells showed that *Ebf1* deletion caused the enhanced proliferation of SOX2-positive cells specifically at E13.5 (Fig. 6 D), which was supported by the loss of p27Kip1 expression in the possible prosensory domain of the *Ebf1*$^{-/-}$ mice at E13.5 (Fig. 6E). The highest *Ebf1* expression level at E13.5 (Fig. 1A) may be related to these phenotypes in *Ebf1*$^{-/-}$ mice. This aberrant proliferation within SOX2-positive cells was suggested to increase the numbers of hair and supporting cells at later stages (Fig. 3A, B, D, and E). Evaluation of hearing ability at the postnatal stage showed that an increase in hair and supporting cell numbers caused a deteriorated hearing threshold (Fig. 7). These results indicate that EBF1 suppresses the proliferation of SOX2-positive cells to develop proper numbers of hair and supporting cells, resulting in the development of normal hearing. Since the SOX2-positive cells within the E13–14 cochlea become Kölliker's organ and lateral and medial prosensory domains at a later stage (Kolla et al. 2020) and the GER has the potential to form cochlear sensory epithelia (Kubota et al. 2021), EBF1 expressed in SOX2-positive cells regulates the number of hair and supporting cells. Although the role of EBF1 in suppressing cell proliferation has not been reported during the development of animal organs, several lines of evidence support

this function of EBF1. First, human EBF1 has been reported to suppress the proliferation of malignant tumors (Shen et al. 2020). Second, the deletion of *Rb1*, a known tumor suppressor and cell cycle regulator , results in the same morphology in the cochlea as *Ebf1* deletion (Sage et al. 2005).

The expression of mature cochlear cell markers, including MYO7A, VGULT3, and p75, in *Ebf1*$^{-/-}$ mice indicated that each cell type developed with normal cell fate specification. Although some GER cells expressed the hair cell marker MYO7A in *Ebf1*$^{-/-}$ mice (Fig. 3B and C) and cell fate specification appeared to deteriorate, the penetrance of this phenotype was low. A small level of cell fate may be regulated by EBF1 in the cochlea; however, cell specification does not play a prominent role in cochlear EBF1 as in other tissues, including B lymphocytes (Nechanitzky et al. 2013). In contrast, several results from our study indicate that the speed of differentiation appears to deteriorate in the *Ebf1*$^{-/-}$ mouse cochlea. The expression of *Atoh1* within the cochlear prosensory domain, which was observed at E13.5, in wild-type mice, was detected as late as E14.5, in the *Ebf1*$^{-/-}$ mice (Fig. 5B); The regions where VGLUT3- and p75-positive cells were detected were more basal regions in the *Ebf1*$^{-/-}$ mice cochlea than in the *Ebf1*$^{+/+}$ mice cochlea at E18.5 (Supplemental Fig. S3). The delay in differentiation may be caused by the aberrant proliferation of the prosensory domain in *Ebf1*$^{-/-}$ mice, because the deterioration of proliferation affects the differentiation of cells (Ruijtenberg and van den Heuvel 2016).

In addition to the cochlear epithelia and mesenchyme, deletion of *Ebf1* resulted in an altered neuroaxonal composition of spiral ganglion neuronal cells in the organ of Corti, suggesting that EBF1 may also affect the pathfinding of spiral ganglion cells within the cochlea, as observed in the aberrant pathfinding of facial branchiomotor neurons and retina (Garel et al. 2000; Jin and Xiang 2011). The present study was limited to the IHC analysis of TUJ1, and further studies are

needed to elucidate the role of *Ebf1* in spiral ganglion differentiation and development. Spiral ganglion axons synapse with hair cells form within the otic mesenchyme, and axons extend through the mesenchyme to innervate the organ of Corti (Coate and Kelley 2013). Deletion of *Ebf1* in the mesenchyme may also contribute to the phenotype observed in the spiral ganglion cells.

The expression *Ebf1* in the medial region of the cochlear floor (Fig. 1B, C, and D), spiral limbus loss (Fig. 2C), and ectopic MYO7A-positive cells within the GER (Fig. 3B and C) in *Ebf1* deletion mice suggest that EBF1 has functions within the GER or Kölliker's organ. Recently, PRDM16 was reported to be a marker of Kölliker's organ and an important molecule in its development (Ebeid et al. 2022). *Prdm16* null mutant mice showed phenotypes similar to *Ebf1* deletion mice, including shortened cochlear length, spiral limbus loss, and ectopic hair cells within the GER. Moreover, the *Prdm16* null mutant mice cochlear duct expressed approximately half the amount of *Ebf1* compared to the wild-type mouse cochlear duct (Ebeid et al. 2022), suggesting that EBF1 is involved in the development of the Kölliker's organ. However, if PRDM16 is the direct upstream regulator of EBF1, the *Ebf1* expression level should be much less in *Prdm16* null mutant mice, and these mice should have increased hair and supporting cells and the loss of scala tympani that were observed in *Ebf1*$^{-/-}$ mice. Thus, PRDM16 does not control the expression of EBF1.

The phenotypes of the increased numbers of hair and supporting cells suggest the involvement of previously reported molecules crucial for the development of cochlear sensory epithelia, including Notch signal-related molecules (Yamamoto et al. 2011) and SOX2 (Kiernan et al. 2005), in the regulation of *Ebf1* expression. However, Foxg1Cre-mediated *Notch 1*- or *Sox2*-deleted mice do not show lower expression level of *Ebf1* in striatal neurons (Mason et al. 2005;

Ferri et al. 2013), suggesting that Notch signaling and SOX2 do not control EBF1 expression. Identification of the molecules upstream and downstream of EBF1 will be the next step in revealing the precise function of EBF1 in the cochlea and the grand scheme of inner ear development.

In conclusion, our study confirms that *Ebf1* and its protein are expressed in the epithelia of the inner ear prosensory domain and the cochlear Kölliker's organ, mesenchyme, and CVG cells within the cochlea, and play important roles in the formation of each structure. Most importantly, epithelial EBF1 regulates the number of cochlear hair and supporting cells by suppressing the proliferation of the prosensory domain and Kölliker's organ cells, mainly at E13.5, while mesenchymal EBF1 contributes to scala tympani formation. Epithelial EBF1 is crucial for normal hearing in mammals. We believe that the search for genes that regulate *Ebf1* and those that are regulated by *Ebf1* will further our understanding of inner ear development.

Material and Methods

Animals

The Slc: ICR mice were purchased from Japan SLC (Hamamatsu, Japan). *Ebf1*$^{-/-}$ mice (Lin and Grosschedl 1995) and *Ebf1*$^{fl/fl}$ (Gyory et al. 2012) were used in this study. *Ebf1*$^{fl/+}$ mice were crossed with *Foxg1*$^{Cre/+}$ *mice* (*Foxg1Cre*) (Hébert and McConnell 2000). *Ebf1*$^{-/-}$ or *Foxg1Cre; Ebf1*$^{fl/fl}$ mice were used as experimental animals and *Ebf1*$^{+/+}$, *Ebf1*$^{+/-}$ or *Foxg1Cre; Ebf1*$^{fl/+}$ were used as control animals.

Ebf1$^{+/-}$, *Foxg1Cre* and *Ebf1*$^{fl/fl}$ mice were maintained on a C57BL/6 background. All experimental protocols were approved by the Animal Research Committee of Kyoto University (Med Kyo 20132, Kyoto, Japan). All animal experiments were performed according to the

National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All the animals used in this study were maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. The mice were mated in the evening, and vaginal plugs were checked early in the morning. The day a vaginal plug was detected was defined as embryonic day 0.5 (E0.5).

qRT-PCR

The inner ears were dissected from E9.5, E10.5, E11.5, E12.5, E13.5, E14.5, E16.5, E18.5, and P0 ICR mice. After surrounding tissue was removed from these inner ears, at least four samples were immersed in TRIzol Reagent (Thermo Fischer Scientific, Waltham, MA, USA) and preserved at -80°C until RNA extraction. Total RNA was extracted using the RNeasy ™ Mini Kit (QIAGEN, Venlo, Netherlands) and reverse transcribed using the ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). The cDNA was subjected to real-time PCR quantification using a StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) after mixing with PowerUp SYBR Green Master Mix (Applied Biosystems) and various sets of gene-specific forward and reverse primers. The primer sequences were as follows: *Ebf1* forward, AACTCCAAGCACGGGCGGAG; *Ebf1* reverse, CGGGCTGATGGCTTTGATACAGG; *Rplp0* forward, CACTGGTCTAGGACCCGAGAAG; *Rplp0* reverse, GGTGCCTCTGGAGATTTTCG. Relative mRNA expression levels were calculated using the standard curve method, with the mouse housekeeping gene *Rplp0* as an invariant control.

In situ hybridization (ISH)

Whole embryos (E9.5-E11.5) and whole heads (E12.5-P0) were fixed in 4% paraformaldehyde (PFA, Nacalai Tesque, Kyoto, Japan) in 0.1M phosphate buffer saline (PBS) at 4°C overnight. Samples were cryoprotected in 30% sucrose/PBS (Nacalai Tesque), embedded in Tissue-Tek® O.C.T.™ compound (Sakura Finetek Japan, Tokyo, Japan), and sectioned at 10μm thickness using a cryostat (CryoStar™ NX70, Thermo Fisher Scientific). The sections were mounted on silane-coated glass slides (Matsunami Glass, Osaka, Japan).

cDNA fragments were generated by PCR from E13.5 inner ear cDNA of Slc:ICR mouse and then cloned into the pCR®-Blunt II-TOPO® vector (Invitrogen, New York, USA) to prepare the templates of RNA probes. We synthesized digoxigenin (DIG)-labeled sense and antisense RNA probes using a DIG RNA Labeling Kit (Roche, Basel, Switzerland) after digestion with the appropriate restriction enzymes *Bam*HI-HF, *Hind*III, *Not*I-HF, *Sac*I, or *Xho*I (New England Biolabs, Massachusetts, USA). The probes used for in situ hybridization were as follows: *Ebf1* (NM_001,290,709, nucleotides 1436–2269), *Sox2* (IMAGE clone: 6413283), *Bmp4* (NM_007554.3, nucleotides 1013-1876), *Atoh1* (NM_007500.5, nucleotides 13-2111), *Fgf10* (NM_008002.5, nucleotides 571-1027). Each corresponding sense probe was used as a negative control.

Sections were fixed with 4% PFA and 0.2% glutaraldehyde (Nacalai Tesque, Kyoto, Japan) in PBS at room temperature (RT) for 10 min, bleached with 6% hydrogen peroxidase (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in PBST at RT for 10 min, treated with proteinase K (20μg/μL, Roche) for 5 min, and re-fixed with 4% PFA and 0.2% glutaraldehyde in PBS at RT for 10 min.

The prehybridization was performed in hybridization solution containing 50% formamide (Nacalai Tesque), 5 × Saline Sodium Citrate Buffer (SSC, Nacalai Tesque; adjusted to pH 4.5

with citrate), 1% sodium dodecyl sulfate (SDS, Sigma-Aldrich), 50 μg/mL yeast RNA (Invitrogen), and 50μg/mL heparin (Sigma-Aldrich) at 70°C for 1h. For hybridization, we incubated the sections in hybridization solution with a 0.2μg/mL DIG-labelled RNA probe at 70°C for 16h in sealed plastic bags.

Sections were rinsed first in 50% formamide with 6 × SSC and 1% SDS at 70°C, then in 50% formamide with 2.4 × SSC at 65°C, and finally in 1 × Tris-buffered saline (TBS, Nacalai Tesque) with 0.1% Tween 20 (TBST) at RT. Sections were blocked with 5% sheep serum (Sigma-Aldrich) and incubated with a 1:4000 dilution of Anti-Digoxigenin-AP, Fab fragments (Roche) at 4°C overnight.

After rinsing with TBST and NTMT containing 100mM NaCl (Nacalai Tesque), 100 mM Tris-HCl pH9.5, 10 mM $MgCl_2$ (FUJIFILM Wako Pure Chemical Corporation), 0.1% Tween-20, and 480μg/mL levamisole (Sigma-Aldrich), sections were incubated with nitro-blue tetrazolium chloride (Roche) and 5-bromo-4-chloro-3-indolyl phosphate solution (Roche). Images were captured using a BX-50 microscope (Olympus Corp., Tokyo, Japan).

Immunohistochemistry (IHC)

IHC sections were prepared in a manner similar to that used for ISH.

After washing with PBS, all samples were incubated with Blocking One Histo (Nacalai Tesque) for 10 min at RT and 10% normal donkey serum (Sigma Aldrich, St. Louis, MO, USA) in PBS/0.5% Triton X- 100 with 5% Blocking One Histo for 30 min at RT.

The samples were stained with primary antibodies at 4°C overnight or RT for 1h. After washing with 0.1% Tween20 in PBS (PBST), the samples were incubated with Alexa Fluor secondary antibodies. F-actin (actin filaments) was stained with phalloidin 647 (1:500, Thermo Fisher

Scientific) at RT for 1h. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific).

The primary antibodies used in this study were as follows: rabbit anti-EBF1 antibody (1:1000, Millipore, Darmstadt, Germany, AB10523), mouse anti-MYO7A antibody (1:1000, Developmental Studies Hybridoma Bank[DSHB], Iowa City, IA, USA, 138-1), rabbit anti-MYO7A antibody (1:1000, Proteus BioSciences, Waltham, MA, USA, 25-6790), goat anti-SOX2 antibody (1:250, R&D Systems, Minneapolis, MN, USA, AF2018), rabbit anti-SOX2 antibody (1:100, Proteintech, Manchester, UK, 11064-1-AP), rabbit anti-VGLUT3 antibody (1:500, Synaptic systems, Goettingen, Germany, 135 203), goat anti-JAG1 antibody (1:500, Santa cruz, Dallas, Texas, USA, sc-6011), mouse anti-p27Kip1 antibody (1:200, BD Transduction Laboratories, 610242), rabbit anti-Tubulin β 3 (TUJ1) antibody (1:1000, Biolegend, San Diego, CA, USA, PRB-435P), rabbit anti-PROX1 antibody (1:500, Millipore, AB5475), rabbit anti-p75 antibody (1:500, Millipore, AB1554), and rabbit anti Cleaved caspase3 (CC3) antibody (1:400, Cell Signaling Technology, Danvers, MA, USA, Asp175).

Antigen retrieval was performed for p27Kip1 staining, by heating sections in HistoVT one (Nacalai Tesque) at 90℃ for 10 min prior to the addition of the primary antibodies.

The secondary antibodies used were Alexa Flour 488 donkey anti-rabbit IgG, Alexa Flour 488 donkey anti-goat IgG, Alexa Flour 488 donkey anti-mouse IgG, Alexa Flour 568 donkey anti-rabbit IgG, Alexa Flour 568 donkey anti-goat IgG, Alexa Flour 633 donkey anti-rabbit IgG, Alexa Flour 633 donkey anti-goat IgG, Alexa Flour 633 donkey anti-mouse IgG (1:500, Thermo Fisher Scientific).

The sections were mounted using Fluoromount-G® Anti-Fade (Southern Biotechnology Associates Inc., Birmingham, AL, USA).

Images of the specimens were captured using an Olympus BX50 microscope (Olympus), an Olympus DP70 digital camera (Olympus), and a Zeiss LSM900 with Airyscan2 (Carl Zeiss AG, Oberkochen, Germany).

Hematoxlylin and Eosin staining

Frozen sections with 10 μm thickness were immersed in Mayer's Hematoxylin Solution (FUJIFILM Wako Pure Chemical Corporation) at RT for 3 min and in Eosin Alcohol solution (FUJIFILM Wako Pure Chemical Corporation) at RT for 1 min. Dehydration was performed using graded ethanol solutions (70%, 80%, 90%, and 100%), and clearing was performed three times using xylene.

Cochlea whole-mount preparation

The inner ears were dissected from the mouse head at E18.5 and fixed in 4% PFA in PBS at RT for 1h. After fixation and before primary antibody staining, the outer membrane, including the tectorial membrane, was removed to expose the organs of Corti. After staining with a secondary antibody, the organ of Corti was dissected and mounted on a glass slide for imaging.

Proliferation and apoptosis assays

Cell proliferation in the cochlea was assessed by detecting the incorporated 5-ethynyl-2'-deoxyuridine (EdU, Thermo Fisher Scientific) on frozen sections. EdU was detected using the Click-iT Plus EdU Cell Proliferation Kit for Imaging Alexa 555 Dye (Thermo Fisher Scientific), according to the manufacturer's instructions. Pregnant mice were injected with 5-ethynyl-2'-deoxyuridine (EdU, Thermo Fisher Scientific) at E12.5, E13.5, and E14.5 (three injections at 50

μg/g at 2-hour intervals) and at E16.5 (a single injection at 100 μg/g). Embryos were collected 8 h after the first injection for E12.5, E13.5, and E14.5 embryos. E16.5 embryos were collected 4h after the injection. The basal or basal-to-middle regions of the cochlear duct were observed at E12.5 or at E13.5, E14.5, and E16.5, respectively.

Apoptotic cells were detected by identifying the expression of cleaved caspase 3 in frozen sections by IHC.

Auditory brainstem response (ABR) and Distortion product otoacoustic emissions (DPOAE)

ABR measurements were performed under general anesthesia, as previously described (Kada et al. 2009) at P21 (n = 3 for each genotype). The thresholds for 10, 20, and 40 kHz were determined from the responses at different intensities with 5 dB sound pressure level (SPL) intervals. DPOAE recordings were performed as described previously (Hamaguchi et al. 2012) at P21 (n = 4 for each genotype). Two primary tones (f1, f2, f1<f2) were used as input signals, with f2 set at eight frequency points (4, 6, 8, 12, 16, 24, 32, and 40 kHz), maintaining a frequency ratio of f2/f1 = 1.2. The intensity levels of the stimulatory sounds were 65 dB and 55 dB SPL for f1 and f2, respectively. DPOAE was detected as a peak at 2f1-f2 in the spectrum.

Quantification

Cell quantification and measurements were performed at E18.5 using the Cell Counter plugin of ImageJ (author: Kurt De Vos). The total length of the cochlea was measured based on the region with MYO7A-positve hair cells from the basal to apical turns. Cochlear hair cells were identified by phalloidin and MYO7A labeling. For the quantification of supporting cells of the cochlea, we counted two types of cells, PROX1 and SOX2 positive cells. The cochlear duct was divided into

three regions: basal, middle, and apical, and we counted the number of cells per 200 μm in each region for MYO7A- and PROX1-positive cells or 100 μm in the basal region for SOX2-positive cells.

Statistical analysis

For all statistical analyses, at least three samples from each experimental group were analyzed. Student's *t*-test was used to determine the differences between two experimental groups. One-way or two-way ANOVA was used to assess the differences between more than two experimental groups. *p*-values less than 0.05 were considered statistically significant. Statistical analyses were performed using the R version 4.2.2 (2022-10-31). All the statistical details are provided in the figures and legends.

Competing Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Author Contributions

.N.Y. and H.O. designed the study. H.K. A.Y. and H.O. performed the experiments with advice from N.Y., Y. T., T. N., and K.O.. R.Y., H.K., Y.T. and T.N. analyzed the data and performed the statistics. H.K. and N.Y. wrote the manuscript with contribution from all authors.

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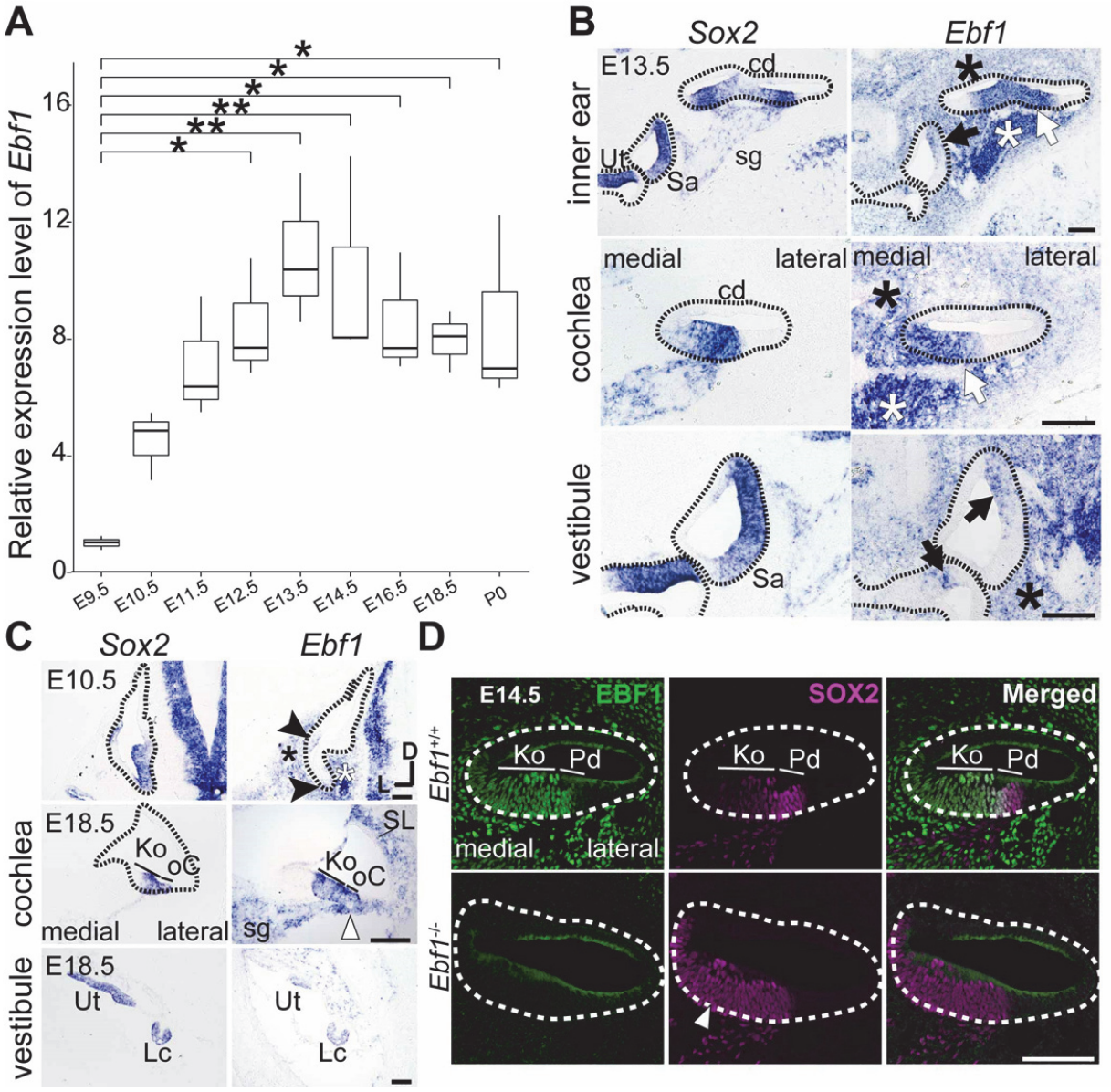
Figures and Figure Legends

Fig. 1. Quantitative and spatiotemporal expression of *Ebf1* during inner ear development.



A. Results of qRT-PCR analysis for *Ebf1* in the inner ear of wild-type mice from E9.5 to P0. The value of each date is normalized to the value of E9.5. Box plot representing medians and interquartile ranges of relative mRNA expression of *Ebf1*.

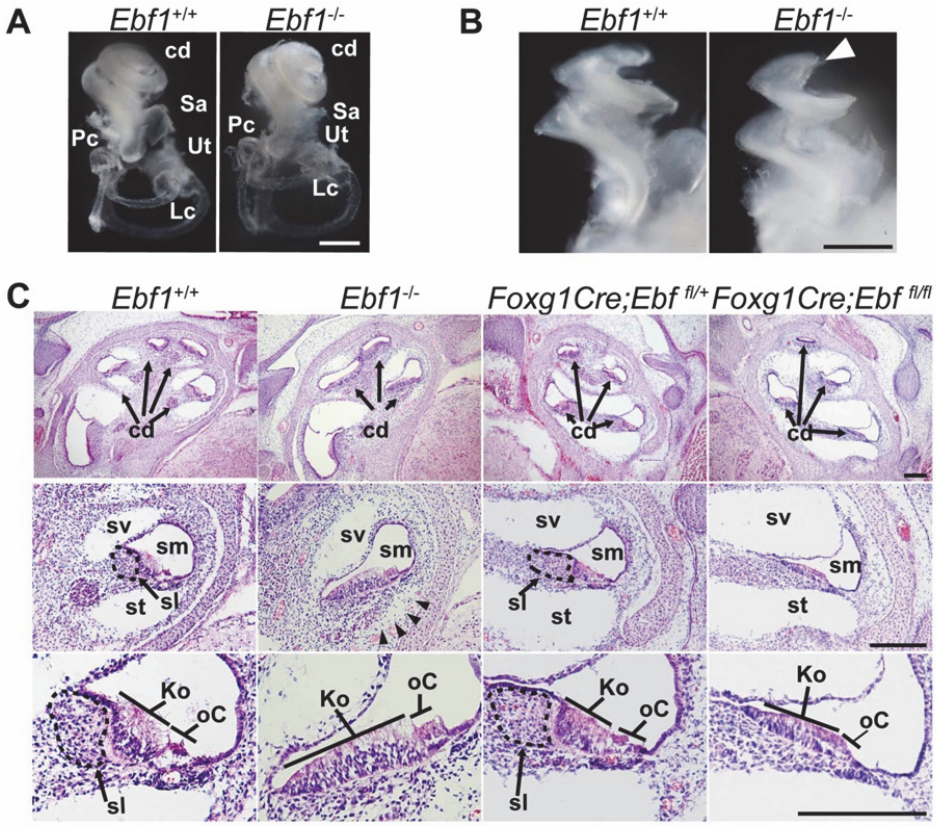
B and **C**. *in situ* hybridization (ISH) for *Ebf1* and *Sox2* in cross-sections of the inner ear of wild-type mice at E13.5 (B) and E10.5 and E18.5 (C). Areas enclosed by dashed lines indicate the

inner ear epithelium. Low-magnification images of the cochlear basal turn and vestibule are presented in the uppermost panels of **B**. High-magnification images of the apical turn of the cochlear duct and vestibules are presented in the middle and lower panels of **B**, respectively. *Ebf1* was expressed in the prosensory domain of the cochlea (white arrows) and medial side of the cochlear floor, a part of Sox2 positive region within the vestibule (black arrows), spiral ganglion (white asterisks), and surrounding mesenchymal tissues (asterisks). Images of E10.5 otocysts, E18.5 cochleae, and E18.5 vestibules are presented in the uppermost, middle, and lowermost images of **C**, respectively. *Ebf1* was expressed on the lateral and ventromedial sides of the otocyst at E10.5 (arrowheads). *Ebf1* was also expressed in the cochleo-vestibular ganglion (white asterisk). In the inner ear of E18.5, *Ebf1* was expressed in Kölliker's organ, the organ of Corti, the macula, and the crista. *Ebf1* was also expressed in the spiral ligament and tympanic border cells (white arrowhead).

D. Immunohistochemical images of E14.5 $Ebf1^{+/+}$ and $Ebf1^{-/-}$ mice cochlear ducts. In $Ebf1^{+/+}$mice, EBF1 is expressed throughout Kölliker's organ and in the medial part of prosensory domain, whereas SOX2 was expressed in the lateral part of Kölliker's organ and in the prosensory domain. In $Ebf1^{-/-}$ mice, EBF1 expression was absent in both the epithelia and mesenchyme, and the region of the SOX2-positive Kölliker's organ was expanded (arrowhead).

One-way analysis of variance (ANOVA) with Tukey–Kramer post hoc tests was performed. $*p < 0.05$, $**p < 0.01$. D, dorsal; L, lateral. cd, cochlear duct; sg, spiral ganglion; Ut, utricle; Sa, saccule; Lc, lateral crista; SL, spiral ligament; Ko, Kölliker's organ; oC, organ of Corti. Pd, prosensory domain. Scale bars: 100μm.

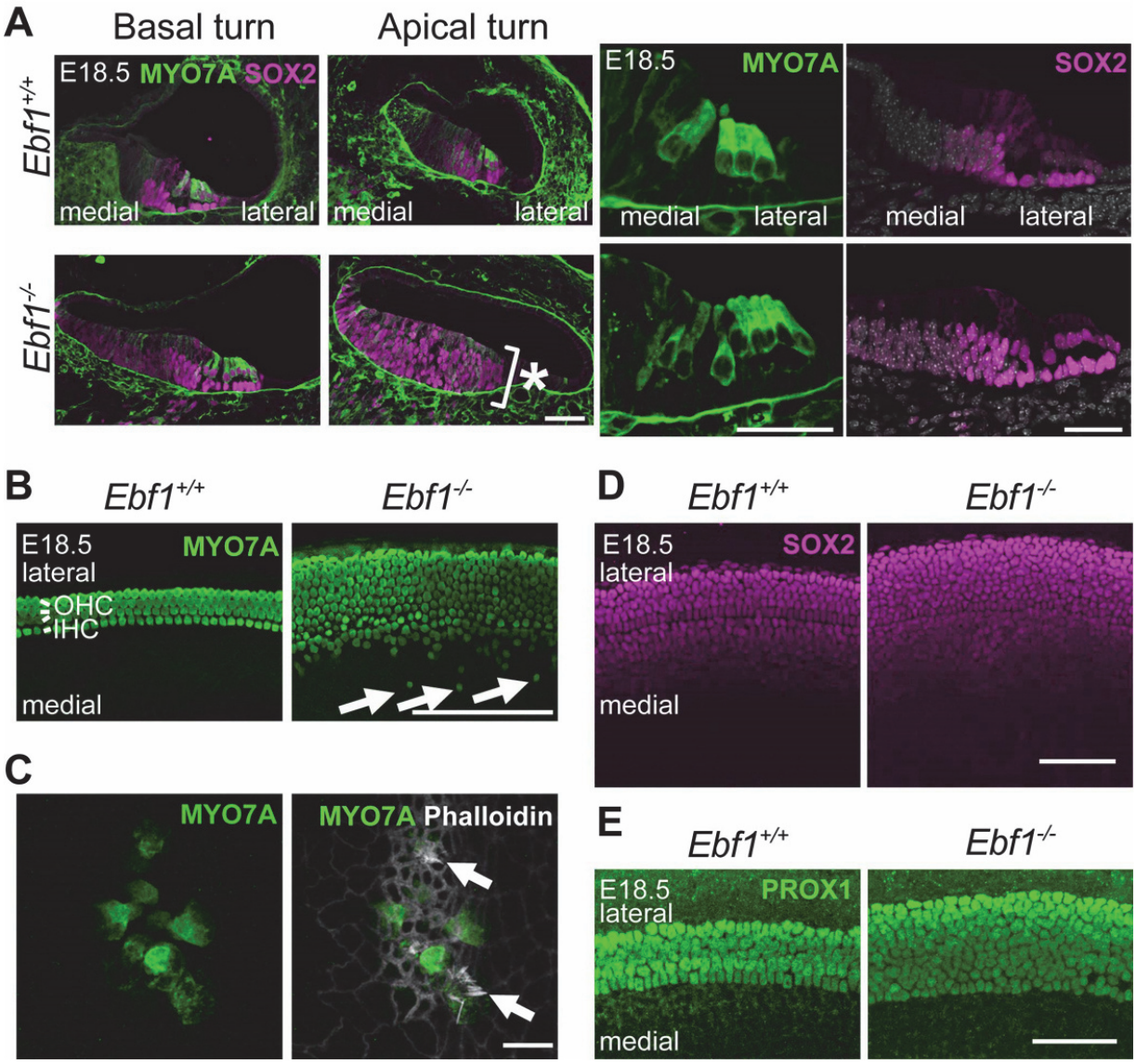
Fig. 2 Development of the cochlear duct is deteriorated in *Ebf1*$^{-/-}$ mice.



A. Gross morphology of membranous labyrinth of the inner ear of *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$mice at E18.5. In *Ebf1*$^{-/-}$, the gross appearance of the cochlea, vestibule, and semicircular canals was normal. **B.** Gross morphology of the cochlear floor after removing the lateral wall and roof of the cochlea at E18.5. Cochlear epithelium of *Ebf1*$^{-/-}$ mice had slightly smaller numbers of cochlear epithelial turn (white arrowhead). **C.** Hematoxylin and Eosin (HE) staining for cochlear cross-sections of E18.5 *Ebf1*$^{+/+}$, *Ebf1*$^{-/-}$, *Foxg1Cre; Ebf1*$^{fl/+}$, and *Foxg1Cre; Ebf1*$^{fl/fl}$ mice. The upper panels are low-magnification images, and the middle and lower panels are high-magnification images. Areas enclosed by dashed lines indicate spiral limbus. Spiral limbus and scala tympani were absent in *Ebf1*$^{-/-}$ mice (arrowheads). In *Foxg1Cre; Ebf1*$^{fl/fl}$, scala tympani was present, but

spiral limbus was absent. Both *Ebf1*$^{-/-}$ and *Foxg1Cre; Ebf1*$^{fl/fl}$ mice had an expanded floor of the cochlea. sv, scala vestibuli; sm, scala media; st, scala tympani; sl, spiral limbus. Scale bars: 0.5mm in **A** and **B** and 200μm in **C**.

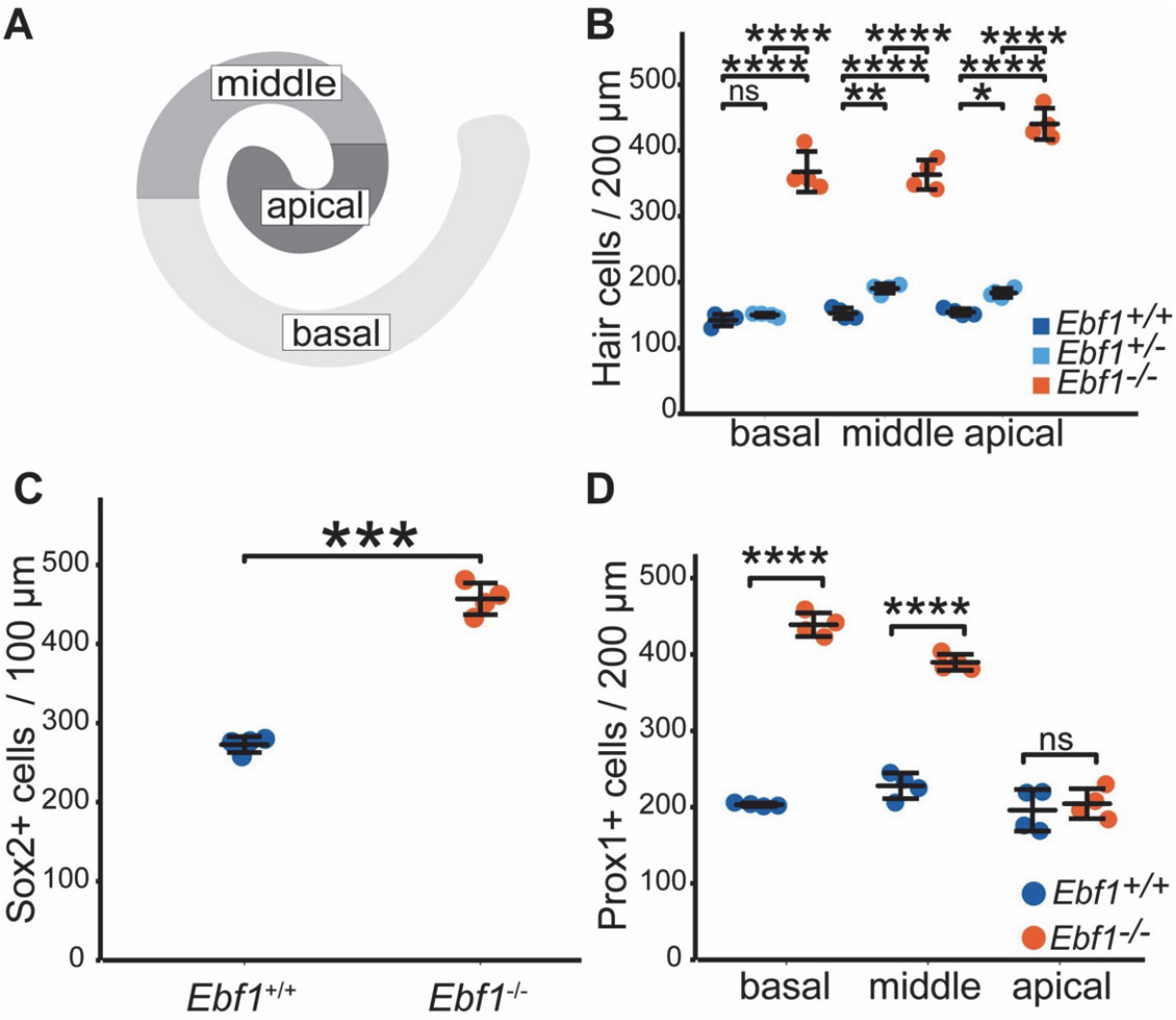
Fig. 3. *Ebf1* deletion increases the number of MYO7A-, SOX2-, and PROX1-positive cells in the cochlea



A. Cross sections of basal and apical turns of the cochlea at E18.5 from *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice labeled with MYO7A (green) and SOX2 (magenta). Magnified images of organ of Corti (oC) and Kölliker's organ (Ko) are presented in the right four panels. MYO7A- and SOX2-positive cell numbers increase in *Ebf1*$^{-/-}$ mice. The apical turn of *Ebf1-/-* mice had multiple layers of SOX2-positive cells (asterisk). **B**–**E**. Whole mount cochlear images at E18.5 from *Ebf1*$^{+/+}$ and

Ebf1$^{-/-}$ mice labeled with MYO7A (green, **B** and **C**), phalloidin (white, **C**), SOX2 (magenta, **D**), and PROX1 (green, **E**). In *Ebf1*$^{-/-}$ mice, MYO7a-positive hair cells increased in number (**B**) and ectopic MYO7A-positive cells (**B**, white arrows) were found in GER (medial region of the cochlear floor). Magnified images of ectopic MYO7A-positive cells (**C**) show stereocilia-like structures stained with phalloidin (arrows in **C**), suggesting that these cells are hair-cell like cells. Scale bars: 50 μm in **A**, **D**, and **E**, 100 μm in **B**, 10 μm in **C**.

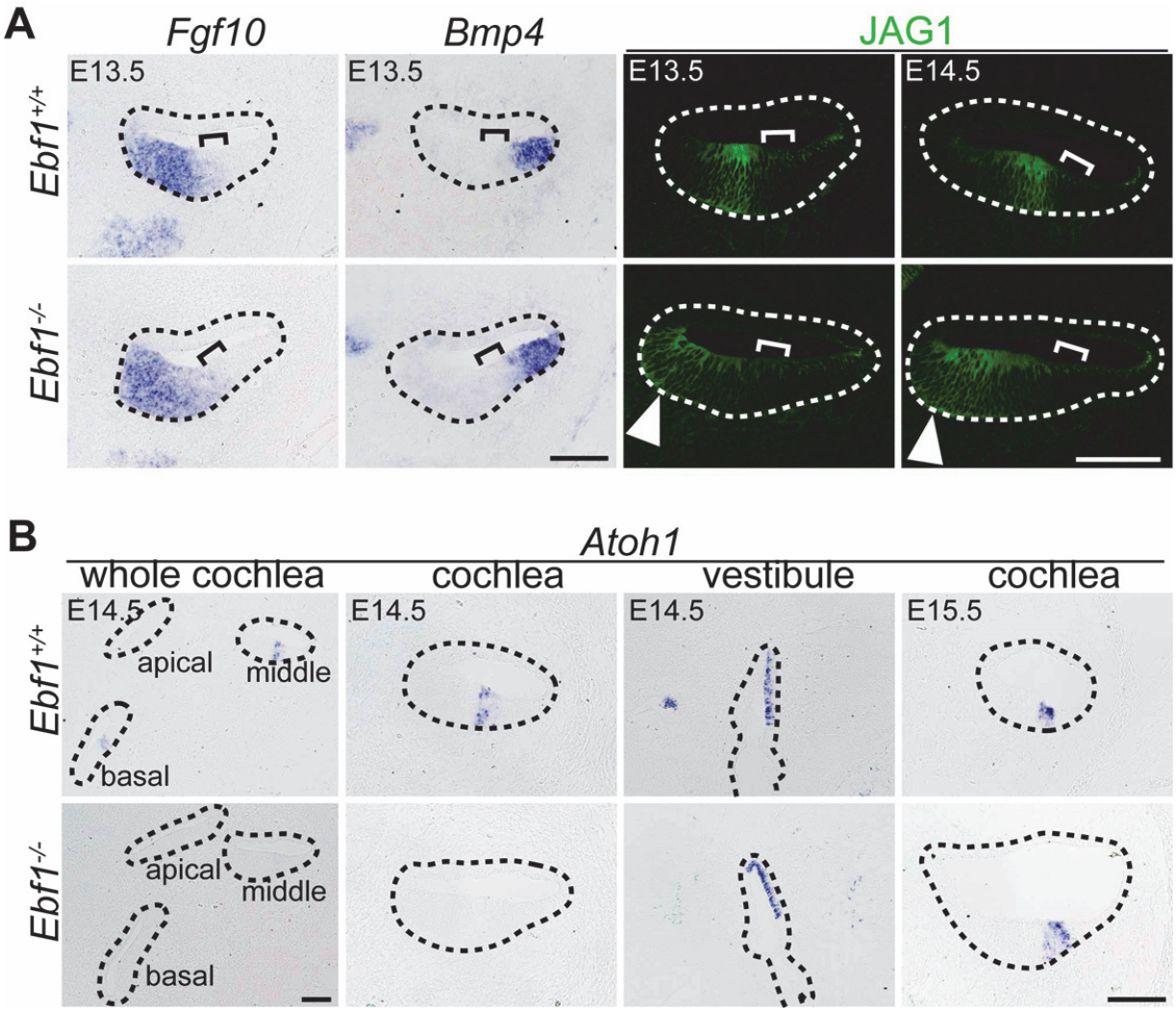
Fig. 4. Quantifiation of the hair and supporting cell increase.



A. Schematic drawings of the cochlea duct showing the positions of basal, middle, and apical region of the cochlea. **B**. Total hair cell numbers per 200µm of E18.5 *Ebf1*$^{+/+}$, *Ebf1*$^{+/-}$, and *Ebf1*$^{-/-}$ mouse cochlear ducts at the basal, middle, and apical region. *Ebf1*$^{-/-}$ mice showed significantly smaller numbers of hair cells through the whole cochlea. Even *Ebf1*$^{+/-}$ mice showed a significantly higher number of cochlear hair cells than *Ebf1*$^{+/+}$ mice in the middle and apical regions **C**. The numbers of SOX2-positive cells per 100µm at the basal region of the cochlea. SOX2-positive cell numbers significantly increased in *Ebf1*$^{-/-}$ mice. **D**. The numbers of PROX1-

positive cells per 200μm at the basal, middle, and apical region of the cochlea. PROX1-positive cell numbers significantly increased in *Ebf1*$^{-/-}$ mice at basal and middle region. two-way ANOVA test with Bonferroni post hoc tests (**B** and **D**) and Student's *t*-test (**C**) were performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$; ns, not significant. Error bars represent mean ± SD. n = 4.

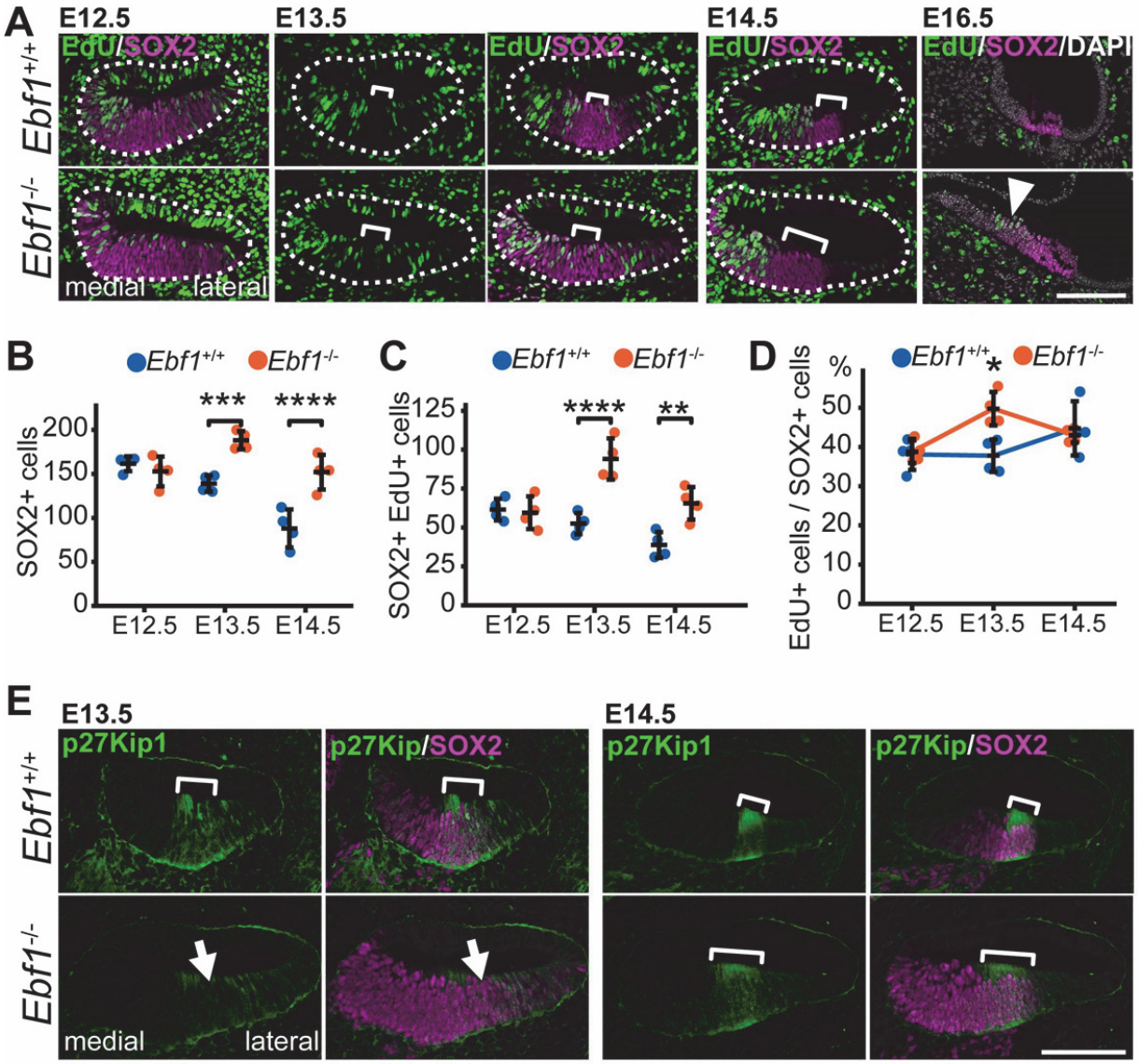
Fig. 5. *Ebf1* deletion causes JAG1 expression to spread inward, delaying *Atoh1* expression in the cochlea development.



A. *in situ* hybridization of *Fgf10* and *Bmp4* and immunostaining of JAG1 (green) on cross sections of E13.5 and E14.5 *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice cochlear basal-to-middle region. *Fgf10* and *Bmp4* expression in the *Ebf1*$^{-/-}$ mice cochlea was similar to that in *Ebf1*$^{+/+}$ mice cochlea. In contrast, JAG1 expression area of the *Ebf1*$^{-/-}$ mice cochlea was expanded toward the more medial side (A, arrowheads) compared with that of *Ebf1*$^{+/+}$ mice cochlea. The prosensory domain is shown in brackets. **B**. *in situ* hybridization for *Atoh1* on cross sections of E14.5 and E15.5

Ebf1$^{+/+}$ and *Ebf1*$^{-/-}$ mice cochlear basal-to-middle region. In the inner ear of E14.5, *Atoh1* expression was observed in the cochlea and vestibule in *Ebf1*$^{+/+}$ mice, while in *Ebf1*$^{-/-}$ mice, *Atoh1* expression was observed in the vestibule, but not in the cochlea. In the cochlea of E15.5, *Atoh1* was expressed in both *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice. Areas enclosed by dashed lines indicate cochlear ducts and vestibules. Scale bars: 100μm.

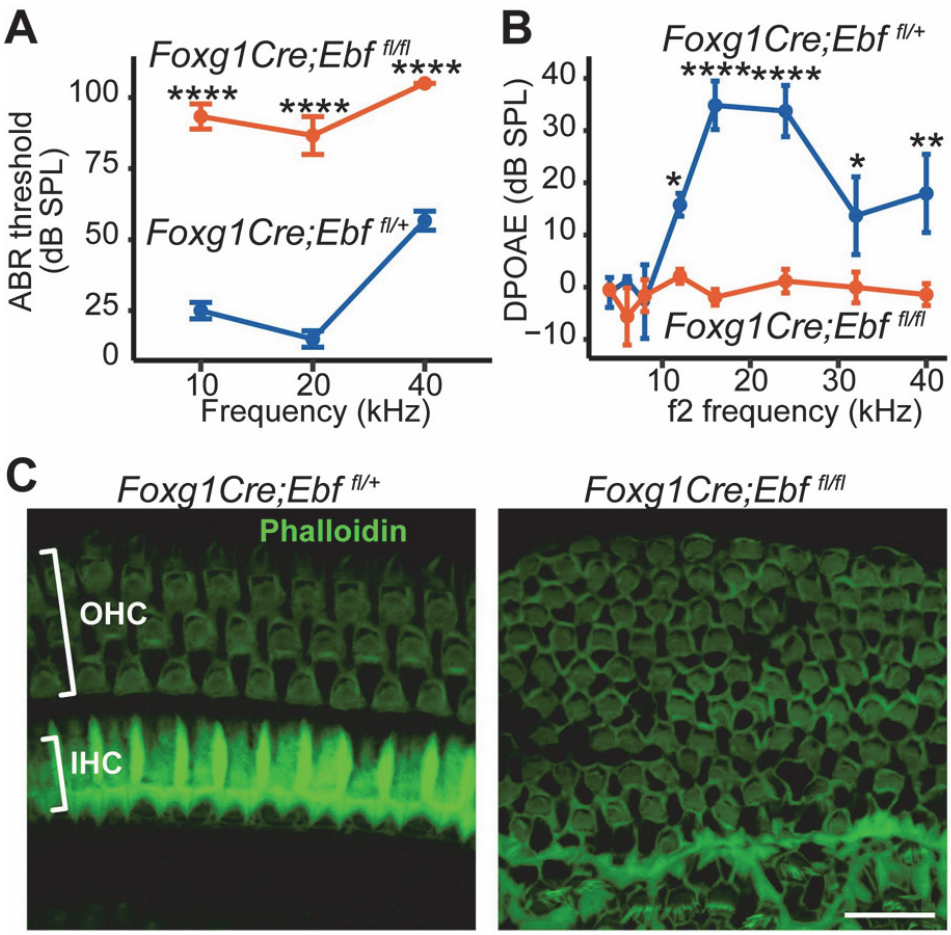
Fig. 6. Effect of *Ebf1* deletion on cell proliferation during inner ear development.



A. Cross-sections of the cochlear basal regions at E12.5, E13.5, E14.5, and E16.5 from *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice. Sections were immunostained with EdU (green) and SOX2 (magenta). E16.5 sections were counter-stained with DAPI (gray). Areas enclosed by dashed lines indicate the cochlear ducts and brackets indicate prosensory domains. Although the *Ebf1*$^{+/+}$ mice cochlear floor had EdU negative region (bracket), EdU was detected in the corresponding area of the

Ebf1$^{-/-}$ mice at E13.5 (bracket). ZNPC appeared in the *Ebf1*$^{-/-}$ mice cochlear floor at E14.5 (bracket). Kölliker's organ at E16.5 loses EdU-positive cells in the *Ebf1*$^{+/+}$ mice. In contrast, EdU positive cells were still present in Kölliker's organ of the *Ebf1*$^{-/-}$ mice at E16.5 (arrowhead). **B**–**D**. Quantitative assessment of the SOX2 positive region in the cochlea epithelia. The numbers of SOX2-positive cells (**B**) and EdU- and SOX2- double positive cells (**C**) were counted, and the percentage of EdU-positive cells among SOX2-positive cells (**D**) was calculated. In *Ebf1*$^{-/-}$ mice, the number of SOX2-positive and EdU- and SOX2-double positive cells significantly increased in the cochlear duct at E13.5 and E14.5. The percentage of EdU-positive cells among SOX2-positive cells was significantly higher in *Ebf1*$^{-/-}$ mice than in *Ebf1*$^{+/+}$ mice only at E13.5. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. Error bars represent mean ± SD. n = 4.. **E**. Cross-sections of the cochlear basal regions at E13.5 and E14.5 from *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice immunostained with p27Kip1 (green) and SOX2 (magenta). In *Ebf1*$^{-/-}$ mice, p27Kip1 expression (brackets) was absent in the cochlear duct at E13.5 (arrows), although it was present at E14.5. Scale bars: 100μm.

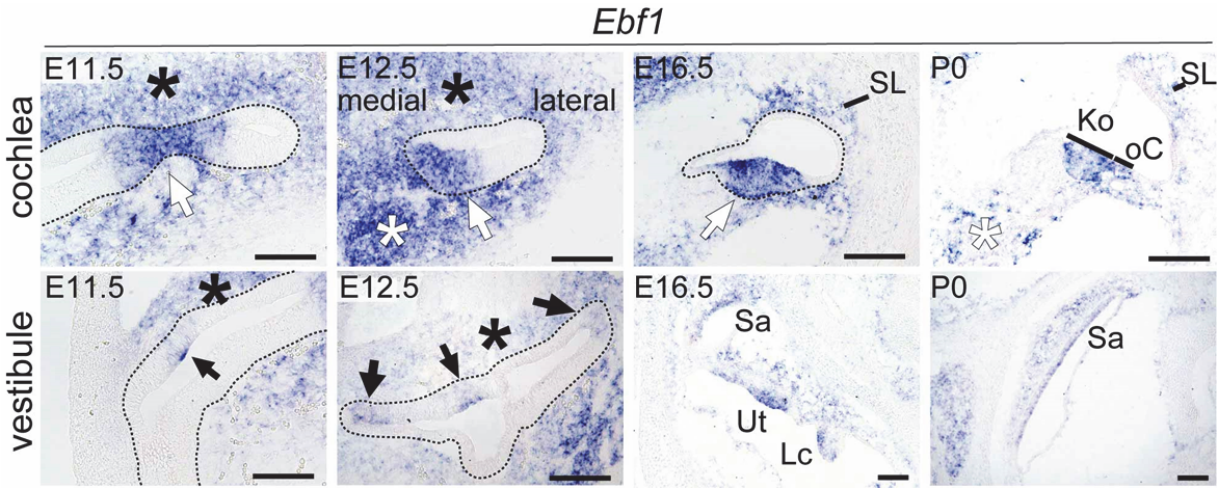
Fig. 7. *Ebf1* deletion impairs auditory function.



A. Auditory brainstem response (ABR) thresholds of P21 *Foxg1Cre; Ebf1*$^{fl/fl}$ (red) and *Foxg1Cre; Ebf1*$^{fl/+}$ (blue, control) mice. *Foxg1Cre; Ebf1*$^{fl/fl}$ mice showed significant higher ABR thresholds at all frequencies than control mice. **B**. Distortion product optoacoustic emissions (DPOAE) were measured at P21 from *Foxg1Cre; Ebf1*$^{fl/fl}$ (red) and control (blue) mice. At f2 frequencies from 12 to 40 kHz, the DPOAE response of *Foxg1Cre; Ebf1*$^{fl/fl}$ was significantly lower than that of control mice. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ from Tukey's HSD (one-way ANOVA post hoc); Error bars represent mean ± SEM. **C**. The whole mount images of the cochlear basal regions from *Foxg1Cre; Ebf1*$^{fl/+}$ and *Foxg1Cre; Ebf1*$^{fl/fl}$ mice at P23 labeled with phalloidin (green). Scale bar: 20μm.

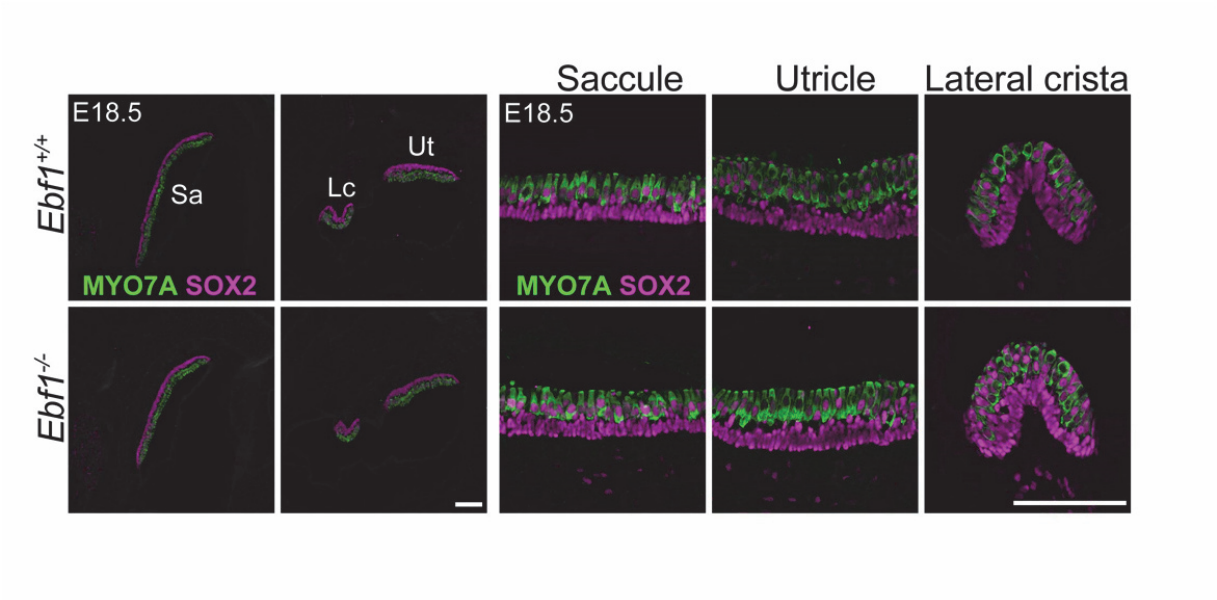
Supplemental Figures and Legends for Supplemental Figures

Supplemental Fig. S1. Spatiotemporal expression of *Ebf1* during inner ear development.



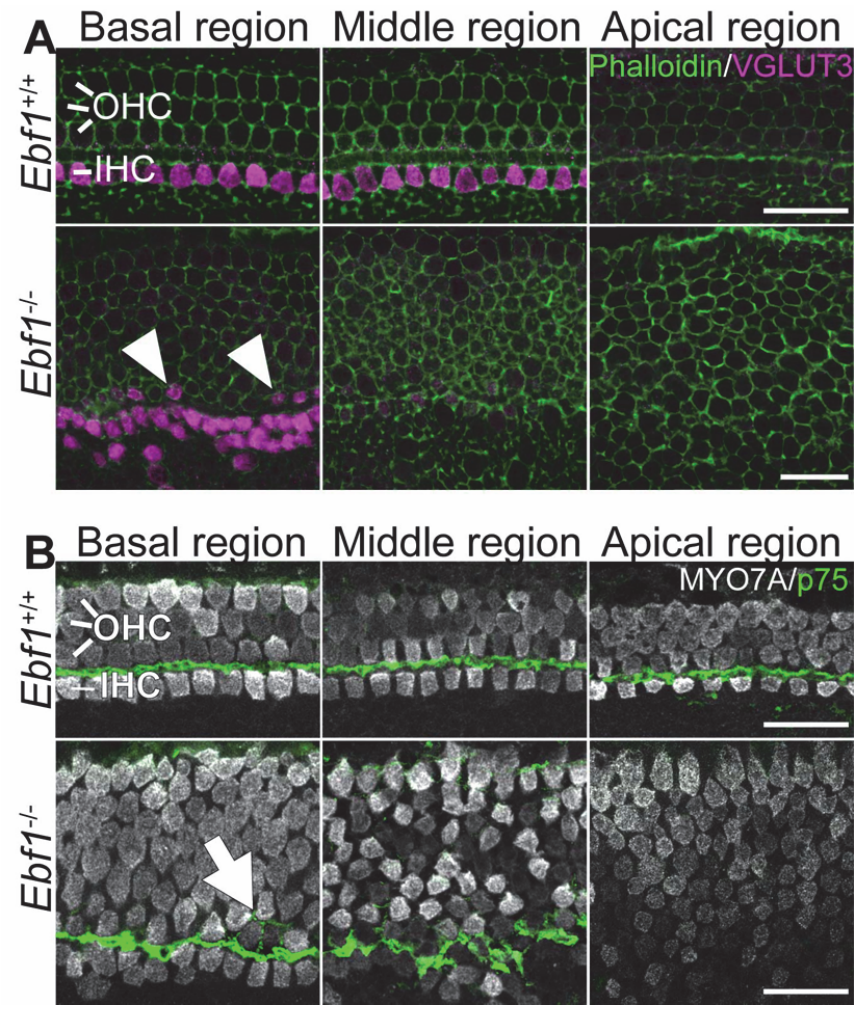
in situ hybridization (ISH) for *Ebf1* and *Sox2* on cross-sections of the inner ear of wild-type mice at E11.5, E12.5, E16.5, and P0. Areas enclosed by dashed lines indicate the inner ear epithelium. From E11.5 to P0, *Ebf1* is expressed in the sensory epithelium of the cochlea (white arrows), the vestibular and semicircular canals (arrows), the spiral ganglion (white asterisks), and the surrounding mesenchymal tissues (asterisks). sg, spiral ganglion; Ut, utricle; Sa, saccule; Lc, lateral crista; SL, spiral ligament; Ko, Kölliker's organ; oC, organ of Corti. Scale bars: 100μm.

Supplemental Fig. S2. *Ebf1* deletion does not affect the hair and supporting cells in the vestibular macula and crista.



Cross sections of the E18.5 vestibular region immunostained for MYO7A (green) and SOX2 (magenta). Low-magnification (left four panels) and high-magnification (right panels) images of the saccule, lateral crista, and utricle are presented. Ut, utricle; Sa, saccule; Lc, lateral crista. Scale bars: 100μm.

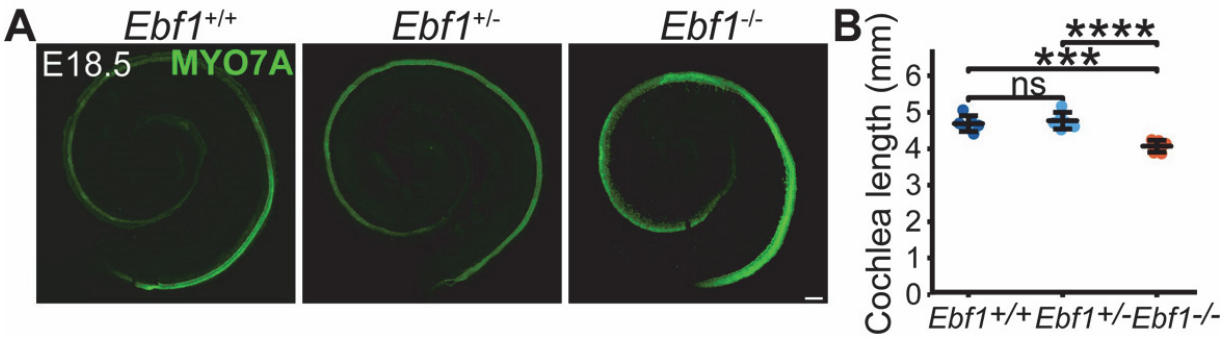
Supplemental Fig. S3. *Ebf1* deletion causes inner hair cell number increase and the delay of hair and supporting cell differentiation



High-magnification images of the basal, middle, and apical regions of whole-mount cochlear samples from E18.5 *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice. **A**. Immunostaining with phalloidin (green) and an anti-VGLUT3 antibody (magenta). In the cochlea of *Ebf1*$^{-/-}$ mice, the row number of VGLUT3-positive cells was two to three, whereas this number was only one in wild-type mice. VGLUT3-positive cells were observed only in the basal region of the cochlea but not in the middle and apical regions of *Ebf1*$^{-/-}$ mice. Several VGLUT3-positive cells were observed in the outer hair cell area (arrowheads). **B**. Immunostaining with anti-MYO7A (gray) and p75 (green) antibodies.

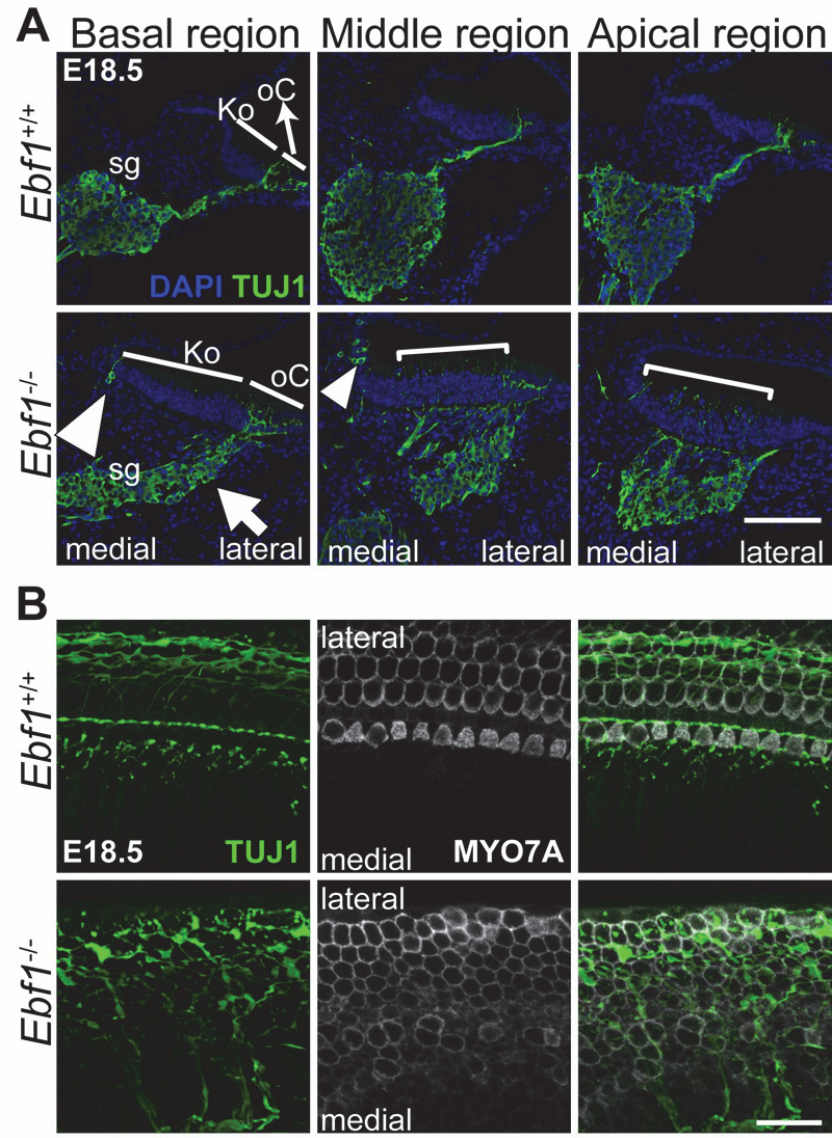
In the cochlea of *Ebf1*$^{-/-}$ mice, the row number of p75-positive cells was similar to *Ebf1*$^{+/+}$ mice, although their arrangement was deteriorated in *Ebf1*$^{-/-}$ mice (arrow**)**. p75-positive cells are observed only in the basal and middle region in the *Ebf1*$^{-/-}$ mice. Scale bars: 20μm.

Supplemental Fig. S4. Quantification of the cochlear length



A. Whole mount images of the cochlea of E18.5 *Ebf1*$^{+/+}$, *Ebf1*$^{+/-}$ and *Ebf1*$^{-/-}$ mice labeled with MYO7A (green). **B**. Quantification of cochlear duct length of E18.5 *Ebf1*$^{+/+}$, *Ebf1*$^{+/-}$, and *Ebf1*$^{-/-}$ mice. The cochlear length of *Ebf1*$^{-/-}$ mice was significantly shorter than those of *Ebf1*$^{+/+}$and *Ebf1*$^{+/-}$ mice. Two-way ANOVA test with Bonferroni post hoc tests was performed. ***$p < 0.001$, and ****$p < 0.0001$; ns, not significant. Error bars represent mean ± SD. n = 6

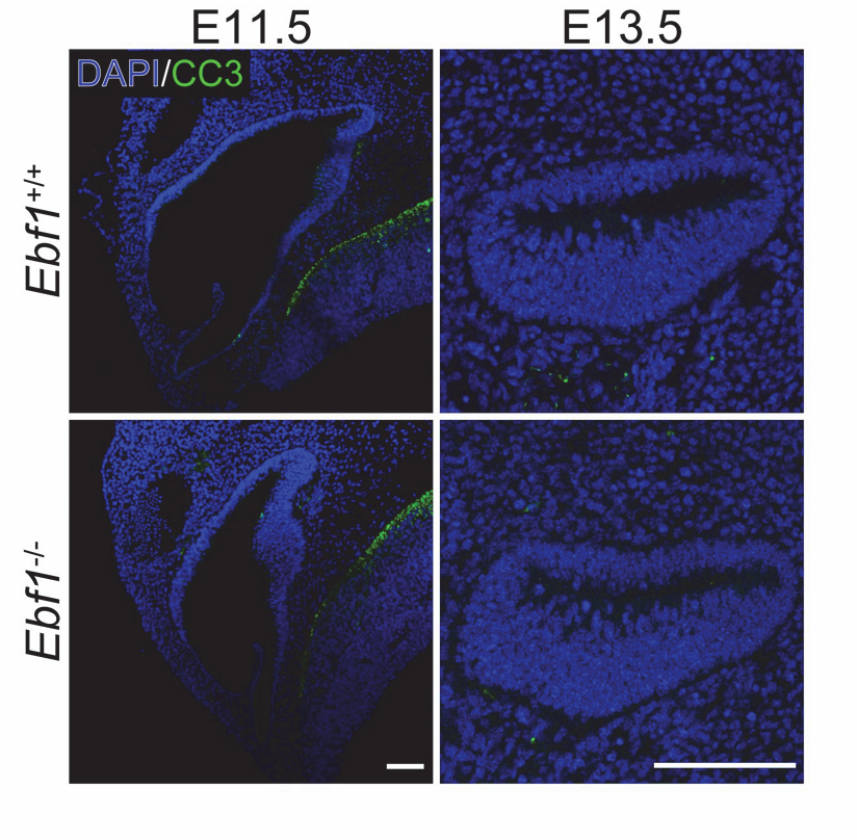
Supplemental Fig. S5. *Ebf1* deletion causes aberrant spiral ganglion development and axon outgrowth to cochlear hair cells.



A. Cross sections of the basal, middle, and apical region of the cochlea and spiral ganglion in E18.5 *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice stained with TUJ1 (green) and DAPI (gray). In *Ebf1*$^{-/-}$ mice, the mophology of the spiral ganglion is different from *Ebf1*$^{+/+}$ mice. TUJ1-positive cell bodies were observed below the organ of Corti (arrow) as well as at their normal site. In addition, innervation from the spiral ganglion was observed in the hair cell part and in the Kölliker's organ in the

middle and apical of the cochlea (arrowheads and brackets). **B**. High magnification view of the basal region of the whole mount cochlear image in E18.5 *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice labeled with TUJ1 (green) and MYO7A (gray). In *Ebf1*$^{+/+}$ mice, the neurons ran parallel to the outer hair cells, whereas in *Ebf1*$^{-/-}$ mice, the neurons formed a reticulation in the cochlear hair cells. Scale bars: 100μm (**A**) and 20μm **(B**).

Supplemental Fig. S6. *Ebf1* deletion did not affect apoptosis during inner ear development.



Cross-sections of the E11.5 inner ear and E13.5 cochlear basal region from *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ mice. Sections were labeled with cleaved caspase 3 (CC3, green) and (DAPI, blue). The number of CC3-positive cells was similar between *Ebf1*$^{-/-}$ and *Ebf1*$^{+/+}$ mice at E11.5 and E13.5. Scale bars: 100μm.