DEVELOPMENT AND DISEASE

Hearing loss caused by progressive degeneration of cochlear hair cells in mice deficient for the *Barhl1* homeobox gene

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SUMMARY

The cochlea of the mammalian inner ear contains three rows of outer hair cells and a single row of inner hair cells. These hair cell receptors reside in the organ of Corti and function to transduce mechanical stimuli into electrical signals that mediate hearing. To date, the molecular mechanisms underlying the maintenance of these delicate sensory hair cells are unknown. We report that targeted disruption of *Barhl1*, a mouse homolog of the *Drosophila BarH* homeobox genes, results in severe to profound hearing loss, providing a unique model for the study of age-related human deafness disorders. *Barhl1* is expressed in all sensory hair cells during inner ear development, 2 days after the onset of hair cell generation. Loss of *Barhl1* function in mice results in age-related progressive degeneration of both outer and inner hair cells in the organ of Corti, following two reciprocal longitudinal gradients. Our data together indicate an essential role for *Barhl1* in the long-term maintenance of cochlear hair cells, but not in the determination or differentiation of these cells.

Key words: *Barhl1*, Homeobox, Transcription factor, Cochlea, Sensory hair cells, Organ of Corti, Deafness

INTRODUCTION

The mammalian inner ear consists of two sensory organs: the cochlea and vestibular apparatus. The hair cells in the sensory epithelia of each respective organ function as receptors for transducing mechanical stimuli into electrical signals that mediate hearing and balance. The organ of Corti in the cochlea contains three rows of outer hair cells and one row of inner hair cells spiraling along the longitudinal axis (Fig. 1H). These two types of hair cells have distinct morphologies, innervation patterns and physiological properties (Dallos, 1992; Hudspeth, 1989). The inner hair cells are the primary auditory sensory receptors that provide the large majority of afferent innervation. The outer hair cells function as sensors as well as mechanical feedback amplifiers that make only token projections to the central nervous system. Hair cells in the cochlea respond to different frequencies of sound in a region-specific manner. Thus, the apical, middle and basal turns of the organ of Corti are selectively but progressively tuned to low, medium and high frequencies of sound, respectively (Dallos, 1992; Hudspeth, 1989). The vestibular system of the inner ear is composed of saccule, utricle and semicircular canals, which together determine head positions by detecting angular and linear accelerations. During mouse development, classical birth-dating analyses have provided

evidence that all inner ear hair cells are produced in a period spanning from embryonic day 12 (E12) to postnatal day 1 (P1) (Ruben, 1967).

The development of inner ear hair cells involves multiple processes that include proliferation and fate commitment of hair cell progenitors, and initial differentiation, maturation and maintenance of the hair cell type (Fekete, 1996; Kelley et al., 1993). Recent molecular genetic approaches have begun to reveal some of the underlying molecular bases that control the determination and differentiation of inner ear hair cells. Notch signaling has been shown to play a key role in the initial selection of hair cell progenitors (Adam et al., 1998; Lanford et al., 1999; Zhang et al., 2000; Zine et al., 2000). In addition, as demonstrated by gene targeting and overexpression studies in rodents, fate commitment of hair cells are also positively controlled by the *Math1* basic helix-loop-helix (bHLH) transcription factor gene, as well as negatively regulated by the *Hes1* and *Hes5* bHLH genes (Bermingham et al., 1999; Zheng and Gao, 2000; Zheng et al., 2000; Zine et al., 2001). However, the POU domain transcription factor gene *Brn3c* (*Brn3.1/Pou4f3*) is required for the terminal differentiation and survival of inner ear hair cells, but not involved in their fate determination or initial differentiation (Erkman et al., 1996; Vahava et al., 1998; Xiang et al., 1997; Xiang et al., 1998). In the human, a mutation in the *Brn3c*-coding region has been

linked to autosomal dominant progressive hearing loss (Vahava et al., 1998).

Despite the rapid advances in our understanding of the molecular mechanisms governing early development of inner ear hair cells, relatively little is known about the molecular bases that maintain these delicate sensory cells. *Barhl1* is a mammalian homolog of the *Drosophila BarH1* and *BarH2* genes, which encode homeodomain proteins that are required for determination of external sensory organs and for normal eye morphogenesis (Higashijima et al., 1992a; Higashijima et al., 1992b; Kojima et al., 1991; Bulfone et al., 2000). In the developing central nervous system, *Barhl1* has been shown to be primarily expressed in migrating neurons that settle in specific domains within the diencephalon, rhombencephalon and spinal cord (Bulfone et al., 2000). In this study, we have analyzed the spatial and temporal expression patterns of *Barhl1* and investigated its role by gene targeting during development of the mouse inner ear. We found that in the inner ear, *Barhl1* was expressed in all hair cells, but more abundantly in the cochlear outer hair cells. Targeted deletion of *Barhl1* caused degeneration of cochlear hair cells that was progressive in space and time in the organ of Corti, resulting in severe to profound hearing loss. Our data indicate that *Barhl1* plays an essential role in the maintenance of cochlear hair cells, whereas it has no or little role in the specification and differentiation of these cells.

MATERIALS AND METHODS

Gene targeting

A human EST clone (GenBank accession no. AI367090) was used to screen a mouse E14.5 embryo cDNA library (Stratagene) to obtain a *Barhl1* full-length cDNA. λ phage clones covering the entire *Barhl1*-coding region were isolated by screening a 129Sv/J mouse genomic library (Stratagene) using the *Barhl1* cDNA as probe. To construct the targeting vector, a 3.8 kb *Bgl*II (artificial site)-*Kpn*I fragment was cloned into the *Bam*HI/*Kpn*I sites of *pPNTloxP* (Partanen et al., 1998; Tybulewicz et al., 1991) as the 3′ arm. The 5′ arm, a 3.7 kb *Hin*dIII-*Spe*I (artificial site) fragment, was inserted into the *pSDKlacZpA* plasmid (Shalaby et al., 1995), excised as a *Not*I/*Xho*I fragment, and cloned into the *Not*I/*Xho*I sites of *pPNTloxP* containing the 3′ arm. The linearized construct was electroporated into AB2.2 ES cells. All ES cell culture, transfection and screening were performed according to the manufacturer's protocol (Mouse Kit™, Stratagene). Targeted ES cell clones were identified by the presence of both a 7.8 kb wild-type band and a 4.5 kb disrupted band on *Sac*I-digested southern blot using a 3′ diagnostic probe. These clones were injected into blastocysts to derive chimeric mice, which were bred with C57BL/6J mice to produce heterozygotes.

Auditory brainstem responses

Free field auditory brainstem responses were recorded from *Barhl1*^{+/+}, *Barhl1*^{+/−} and *Barhl1*^{−/−} mice in a double-walled, sound-isolation booth (Industrial Acoustics, Baltimore, MD). Acoustic stimuli and presentation were as follows. Broadband and pure tone stimuli were presented at a rate of 10 per second. These stimuli had a 5 mseconds duration with a 1 mseconds rise/fall time. Electrical activity was recorded for 20 ms after the stimulus presentation and 500 responses were averaged. Tone stimuli consisted of 4 kHz, 8 kHz, 16 kHz and 32 kHz pips. Clicks were produced by 2 V pulses of alternating polarity having 100 μseconds duration. Stimulus levels were varied randomly. All auditory stimuli were calibrated against a constant voltage delivered to the speaker and recorded with a free field

microphone (Bruel & Kjær, Norcross, GA). ABR stimuli were created with Tucker-Davis Technologies Real-Time Processor Visual Design Studio. The evoked potential was digitized with Tucker-Davis Technologies Real-Time Processors and thresholds were calculated with Matlab v.6.0 Release 12. Threshold responses were defined as the sound pressure level in which the peak amplitude of the evoked response (latency, 2.5-7.5 mseconds) was greater than two standard deviations above the averaged background activity prior to stimulus onset (15-20 mseconds). Threshold differences between mouse groups were statistically determined by ANOVA (StatView v5).

In situ hybridization and histochemistry

RNA in situ hybridization was performed as described (Sciavolino et al., 1997) using digoxigenin-labeled riboprobes prepared following the manufacturer's protocol (Roche Diagnostics). X-gal staining for β-galactosidase activity was conducted as previously described (Ben-Arie et al., 2000). All labeled sections were counterstained with Fast Red. Whole-mount organs of Corti were labeled with rhodamine-conjugated phalloidin following the manufacturer's protocol (Molecular Probes).

Semi-thin sections and scanning electron microscopy

Semi-thin sections of inner ears for light microscopy were prepared and stained with Toluidine Blue as described (Xiang et al., 1997). Scanning electron microscopy of the organs of Corti was carried out as described (O'Malley et al., 1995).

RESULTS

Patterns of *Barhl1* expression in the developing and adult inner ear

To isolate *BarH*-like genes that may be important for mammalian inner ear development, we identified a human EST clone from the dbEST database that shared significant homology with the *Drosophila BarH1* and *BarH2* in the homeodomain. Subsequent screening of a mouse E14.5 embryo cDNA library using the EST as a probe identified a *BarH*-like gene, designated *Barhl1* in a previous report (Bulfone et al., 2000). In the developing inner ear, we first detected *Barhl1* transcripts by RNA in situ hybridization at E14.5 in hair cells of both the cochlea and vestibular system (Fig. 1A). The expression of *Barhl1* remained strong in hair cells of the organ of Corti at late embryonic and postnatal stages (Fig. 1B,C). As the earliest cochlear hair cells are produced by E12 (Ruben, 1967), the late onset of *Barhl1* expression suggests a role for *Barhl1* in the differentiation and/or survival of inner ear hair cells.

To investigate in vivo the role of *Barhl1* during inner ear development, we generated a targeted *Barhl1* deletion in mice, by replacing the *Barhl1*-coding region with the *lacZ* reporter gene (Fig. 1I-K). The absence of an in situ hybridization signal for *Barhl1* in the inner ear of homozygous mutant mice confirmed that the mutation is a null allele (data not shown). In embryonic and postnatal mice heterozygous for the *Barhl1* deletion, *lacZ* expression was detected in hair cells of the organ of Corti as well as the vestibular apparatus, faithfully recapitulating the endogenous *Barhl1* expression pattern (Fig. 1D-G). In adult mice heterozygous or homozygous for the deletion, we observed continued *lacZ* expression in these hair cells (Fig. 3H,L,P and data not shown), suggesting that *Barhl1* expression is persistent in the adult inner ear. Interestingly, as visualized by β-galactosidase staining, *Barhl1* was highly

Fig. 1. Expression of *Barhl1* in the developing inner ear and targeted disruption of the *Barhl1* locus. (A-C) RNA in situ hybridization analysis of coronal ear sections shows signals in the utricular maculae, cristae of semicircular canals and cochlear hair cells (arrows). (D,E) β-galactosidase staining (counterstained with Fast Red) of *Barhl1*+/− inner ear sections labels cochlear outer and inner hair cells, and saccular hair cells. (F,G) β-galactosidase staining of *Barhl1*+/− whole-mount organs of Corti and cristae at P5: note the much more intense signals in cochlear outer hair cells compared with inner hair cells and hair cells of the crista. (H) Schematic of the inner ear, indicating the expression levels of *Barhl1* in the cochlea and vestibule. (I) Homologous recombination between the wild-type allele and the targeting vector results in the replacement of *Barhl1*-coding exons (black boxes) with the *lacZ* marker DNA and a *PGK-Neo* cassette flanked by two *loxp* sites (hatched boxes). E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; ScI, *Sac*I; ScII, *Sac*II; Xb, *Xba*I; Xh, *Xho*I. (J) Southern blot analysis of SacI-digested DNA from wild-type, heterozygous and homozygous mutant mice. The 3′ probe identifies *Sac*I fragments of 7.8 kb (wild-type allele) and 4.5 kb (targeted allele). (K) PCR analysis of DNA from wild-type, heterozygous and homozygous mutant mice. The wild-type and targeted alleles yield a product of 350 bp and 730 bp (derived from *lacZ*), respectively. AVC, anterior vertical canal; Co, cochlea; Cr, crista; HC, horizontal canal; IHC, inner hair cell; OHC, outer hair cell; PVC, posterior vertical canal; S, saccule; U, utricule. Scale bars: 50 μm.
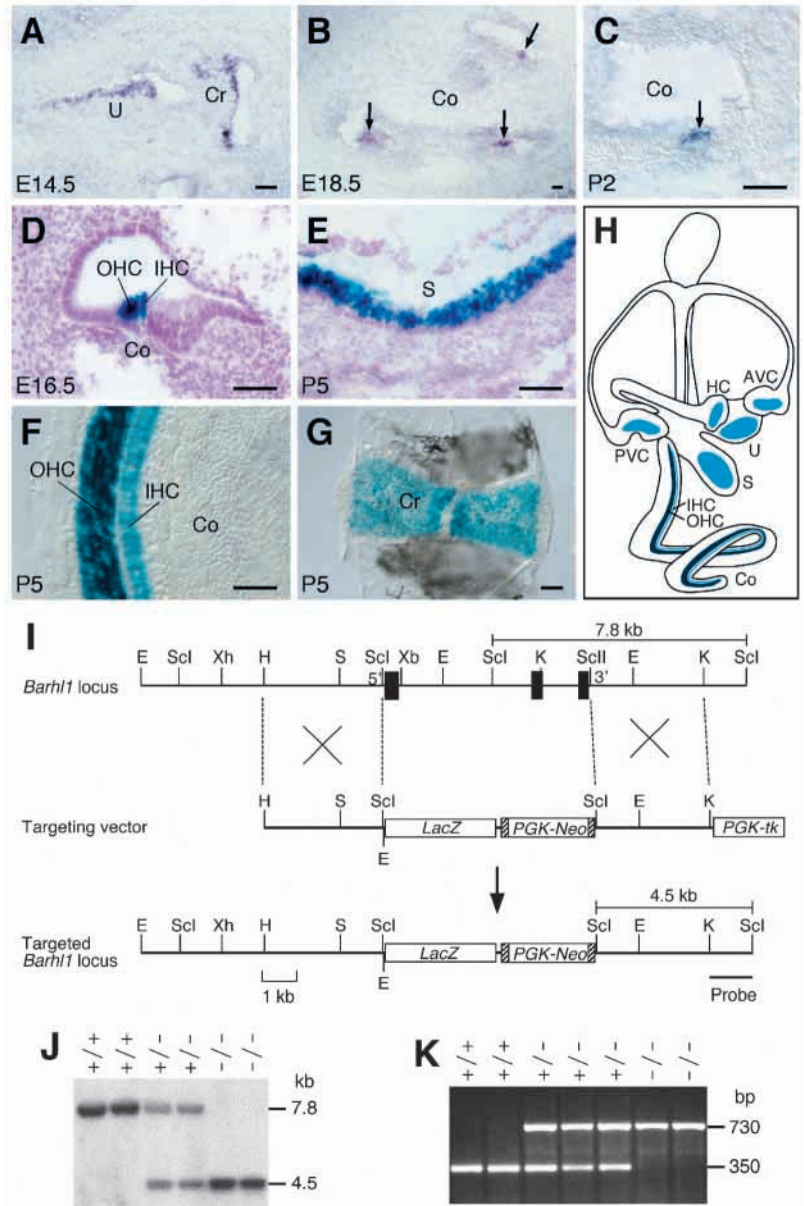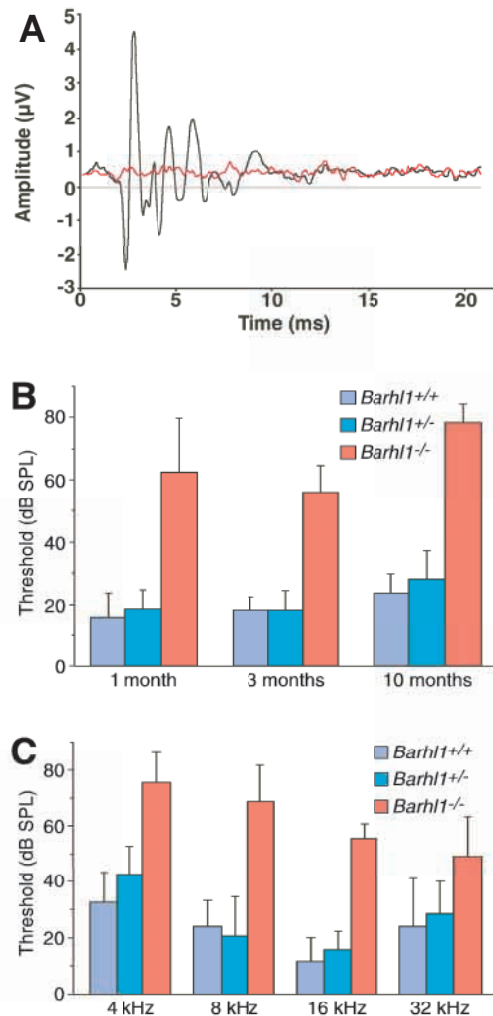

expressed in the cochlear outer hair cells but exhibited a much weaker expression level in the cochlear inner hair cells and vestibular hair cells (Fig. 1F-H).

Progressive hearing loss in *Barhl1* null mice

Barhl1−/− mice were born at the expected Mendelian ratio in crosses between *Barhl1*+/− animals, and resembled wild-type and heterozygous littermates in viability, growth rate, fertility and gait. However, *Barhl1*-null mutants exhibited reduced startle reflexes as early as 2 weeks of age, indicating a deficit in the auditory system, yet their vestibular function appeared to be normal. These mice lacked any phenotypes indicative of a vestibular dysfunction, such as circling, hyperactivity and head tossing. They were also indistinguishable from their wild-type and heterozygous littermates in their ability to right themselves when laid on their backs, or in their effectiveness in swimming when placed in a tub of water.

To quantitatively assess hearing loss in *Barhl1* null mutants, we measured evoked auditory brainstem responses (ABRs) in 1-, 3- and 10-month-old *Barhl1*−/− and control mice (Fig. 2; Table 1). As shown in Fig. 2A, *Barhl1*+/+ mice showed ABR potentials consistent in sensitivity, form and latency with many strains of mice with normal hearing (Zheng et al., 1999). No significant difference was observed between *Barhl1*+/+ and *Barhl1*+/− mice in ABR thresholds (Fig. 2B,C). By contrast, *Barhl1*−/− mice had elevated thresholds at all frequencies tested (Fig. 2C and data not shown), and some mice did not respond

to any auditory stimuli. The number of *Barhl1*−/− mice that did not respond to broadband clicks showed a marked increase with age (three out of 10 at 3 months but seven out of 10 at 10 months; Table 1), indicating that hearing loss in *Barhl1*−/− mice was progressive. When *Barhl1*−/− mice responded to sound, there were threshold shifts ranging from 20-55 dB SPL, depending on the auditory stimulus and age (Fig. 2B,C). The threshold values of *Barhl1*+/+ and *Barhl1*+/− mice were significantly lower than those of the responding null mutants (*P*<0.02). Interestingly, at one month of age, we found that eight out of 10 *Barhl1*−/− mice did not respond to the 4 kHz stimulus, whereas nearly all responded to 8, 16 and 32 kHz stimuli, albeit with higher thresholds (Table 1). Thus, young *Barhl1*−/− mice exhibited a preferential loss of low-frequency hearing. Consistent with progressive hearing loss, up to half of the null mice also lost responsiveness to higher frequency stimuli by 3 months of age (Table 1).

Fig. 2. Anomalous auditory brainstem responses (ABRs) in *Barhl1*-null mice. (A) Representative ABR evoked by 50 dB SPL click stimuli is illustrated for *Barhl1*$^{+/+}$ (black line) and *Barhl1*$^{-/-}$ (red line) mice. The sensitivity, latency and waveform of the ABR response were essentially identical for *Barhl1*$^{+/+}$ and *Barhl1*$^{+/-}$ mice. The auditory stimulus was presented at 0 mseconds. All *Barhl1*$^{-/-}$ mice either had elevated thresholds or were unresponsive to sound. (B) Mean (±s.d.) click ABR thresholds are shown for *Barhl1*$^{+/+}$, *Barhl1*$^{+/-}$ and responsive *Barhl1*$^{-/-}$ mice at different ages. (C) Mean (±s.d.) ABR thresholds at different frequencies are shown for *Barhl1*$^{+/+}$, *Barhl1*$^{+/-}$ and responsive *Barhl1*$^{-/-}$ mice at 3 months of age. Because *Barhl1*$^{-/-}$ mice did not always respond to even the loudest of the auditory stimuli presented (>70 dB at 4 kHz and >95 dB SPL for all other stimuli; Table 1), thresholds were calculated only for mice with ABR responses (clicks: n=10 out of 10 at one month, seven out 10 at 3 months, and three out of 10 at 10 months; 4 kHz: n=2 out of 10; 8 kHz: n=7 out of 10; 16 kHz: n=5 out of 10; and 32 kHz: n=5 out of 10). Every *Barhl1*$^{+/+}$ and *Barhl1*$^{+/-}$ mouse exhibited responses to acoustic stimulation.

(Erkman et al., 1996; Xiang et al., 1997; Xiang et al., 1998), and myosin VI and myosin VIIa (Avraham et al., 1995; Xiang et al., 1998) in the *Barhl1*$^{-/-}$ inner ear at P0-P6. All these markers were found to be expressed at a similar level in cochlear and vestibular hair cells of *Barhl1*$^{+/+}$, *Barhl1*$^{+/-}$ and *Barhl1*$^{-/-}$ mice (Fig. 3A-D and data not shown). Thus, all inner ear hair cells appeared to form and at least partially differentiate in the absence of *Barhl1*, in agreement with its late onset of inner ear expression (Fig. 1), indicating little role, if any, for *Barhl1* in the initial generation of hair cells.

We next followed the fate of cochlear hair cells in *Barhl1*$^{-/-}$ and control *Barhl1*$^{+/-}$ mice by staining whole-mount organs of Corti with X-gal and with phalloidin, a toxin that binds to the actin-rich stereocilia bundles of hair cells. From P0 to P5, no obvious abnormalities were observed in cochlear hair cells of the null mice. At P6 in the mutant, however, the outer hair cells in the apical turn of the organ of Corti started to show overt misalignment and disorganization (Fig. 3E,F). This phenomenon spread into the middle turn by P16-P19, when many of the outer hair cells from the apical and middle turns degenerate in the mutant (Fig. 3G,K; Fig. 4A-C,E-G). By about two months of age, only some residual outer hair cells remained in the apical and middle turns in the mutant, whereas the basal turn of the organ of Corti was still largely intact, except for the loss of occasional outer hair cells (Fig. 3H,L,P; Fig. 4I-L). This apical-to-basal progression of outer hair cell degeneration correlates with the observation that *Barhl1*$^{-/-}$

Gradient degeneration of cochlear outer hair cells in *Barhl1* null mice

To understand the mechanisms underlying the severe deafness phenotype of *Barhl1*$^{-/-}$ mice, we first investigated whether *Barhl1* played a role in the specification of inner ear hair cells. We examined by immunostaining or in situ hybridization the expression patterns of several hair cell markers, including *Math1* (*Atoh1* – Mouse Genome Informatics) (Bermingham et al., 1999), *Brn3c* (*Pou4f3* – Mouse Genome Informatics)

Table 1. Percent of *Barhl1*$^{+/+}$, *Barhl1*$^{+/-}$ and *Barhl1*$^{-/-}$ mice that displayed any evoked auditory brainstem responses (ABRs)

| Age	Genotype	Click (%)	4 kHz (%)	8 kHz (%)	16 kHz (%)	32 kHz (%)
1 month	*Barhl1*$^{+/+}$	100 (n=6)	100 (n=6)	100 (n=6)	100 (n=6)	100 (n=6)
	Barhl1$^{+/-}$	100 (n=5)	100 (n=5)	100 (n=5)	100 (n=5)	100 (n=5)
	Barhl1$^{-/-}$	100 (n=10)	20 (n=10)	90 (n=10)	100 (n=10)	90 (n=10)
3 months	*Barhl1*$^{+/+}$	100 (n=8)	100 (n=8)	100 (n=7)	100 (n=7)	100 (n=7)
	Barhl1$^{+/-}$	100 (n=8)	100 (n=8)	100 (n=7)	100 (n=8)	100 (n=8)
	Barhl1$^{-/-}$	70 (n=10)	20 (n=10)	70 (n=10)	50 (n=10)	50 (n=10)
10 months	*Barhl1*$^{+/+}$	100 (n=9)	NA	NA	NA	NA
	Barhl1$^{+/-}$	100 (n=10)	NA	NA	NA	NA
	Barhl1$^{-/-}$	30 (n=10)	NA	NA	NA	NA

NA, not analyzed.
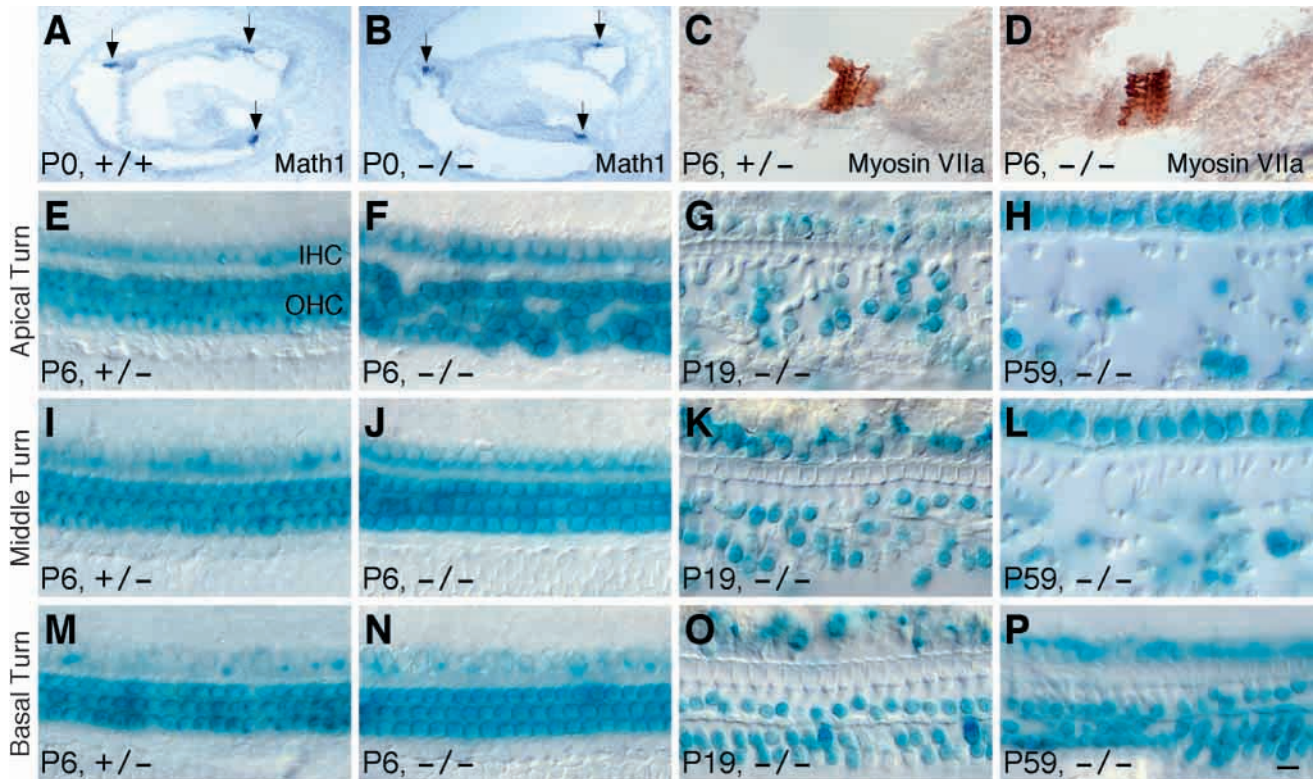
Fig. 3. Generation and progressive degeneration of hair cells in the organ of Corti of *Barhl1*-null mice. (A,B) RNA in situ hybridization analysis of coronal ear sections shows similar levels of *Math1* expression in cochlear hair cells (arrows) between *Barhl1*$^{+/+}$ and *Barhl1*$^{-/-}$ mice at P0. (C,D) Similar levels of myosin VIIa immunoreactivity are seen in cochlear hair cells between *Barhl1*$^{+/-}$ and *Barhl1*$^{-/-}$ mice at P6. (E-P) β-galactosidase staining of *Barhl1*$^{+/-}$ and *Barhl1*$^{-/-}$ whole-mount organs of Corti: note the disorganization of outer hair cells (OHC) in the apex of the mutant at P6 (F), followed by the loss of many of these cells from the apical and middle turns by P19 (G,K) and the degeneration of the majority of these cells by P59 (H,L). The outer hair cells in the basal turn, however, show no obvious degeneration by P59 in the mutant (M-P). Similarly, the inner hair cells (IHC) remain normal up to P59 (E-P). Scale bar in P: 100 μm in A,B; 25 μm in C,D; 18.75 μm in E-P.

mice display preferential low-frequency hearing loss at early ages (Table 1). In spite of the progressive loss of outer hair cells, no obvious loss of cochlear inner hair cells and vestibular hair cells was observed in the null mice by two months of age (Fig. 3E-P; Figs 4, 5). Therefore, the absence of *Barhl1* does not appear to affect the initial production and differentiation of inner ear hair cells, instead, it can cause progressive degeneration of cochlear outer hair cells, following an apical-to-basal gradient.

To analyze cytoarchitectural defects in the cochlea of *Barhl1*$^{-/-}$ mice, we examined the organs of Corti of *Barhl1*$^{-/-}$ and control mice by scanning electron microscopy (SEM) and their semi-thin cross sections by light microscopy (LM). In 2-4 months old *Barhl1*$^{+/+}$ and *Barhl1*$^{+/-}$ mice, three rows of outer hair cells and one row of inner hair cells are orderly arrayed in the entire organ of Corti (Fig. 5A,E). In the null mutant, however, only a few stereocilia bundles of outer hair cells could be observed in the apical and middle turns of the organ of Corti (Fig. 5B,C). Although these residual bundles were totally disorganized in the apical turn, some showed the intact V-shape in the middle turn (Fig. 5B,C). As *Barhl1* expression is restricted to hair cells in the inner ear, the loss of outer hair cells appeared to cause a secondary degeneration of Deiters' cells, which are normally located underneath the outer hair cells (Fig. 5E-H). During degeneration, many of the Deiters'

cells shrink into an outer pillar cell-like morphology with their nuclei lowered to just slightly above the basilar membrane (Fig. 5F,G). Other supporting cells in the organ of Corti, including the outer and inner pillar cells that line the tunnel of Corti, did not appear to be affected in the mutant (Fig. 5E-H). At the age of 2-4 months, SEM and LM analyses confirmed that all inner hair cells in the *Barhl1*$^{-/-}$ organ of Corti were still morphologically intact and outer hair cells in the basal turn were essentially normal (Fig. 5A-H). Similarly, hair cells remained normal in the maculae of saccule and utricle and ampullae of semicircular canals in the vestibular system of *Barhl1*$^{-/-}$ mice (Fig. 5I,J and data not shown), consistent with the absence of a vestibular phenotype in the mutants.

Gradient degeneration of cochlear inner hair cells in *Barhl1* null mice

While the gradual loss of cochlear outer hair cells can partly explain the elevated ABR thresholds in *Barhl1*$^{-/-}$ mice (Fig. 2), it fails to explain the profound deafness in some of the null mice (Table 1). As cochlear inner hair cells are the primary auditory sensors and they express *Barhl1* (Fig. 1), we investigated in older animals whether the absence of *Barhl1* also resulted in degeneration of inner hair cells. In *Barhl1*$^{-/-}$ mice at the age of 6 months, phalloidin labeling of whole-mount organs of Corti revealed a near complete loss of outer

Fig. 4. Progressive loss of outer hair cells in the organ of Corti of *Barhl1*-null mice. (A-L) Labeling of *Barhl1*$^{+/-}$ and *Barhl1*$^{-/-}$ whole-mount organs of Corti with rhodamine-conjugated phalloidin. Compared to the heterozygote (A-C), many outer hair cells degenerate in the apical and middle turns by P19 (E-G) and only a few residual outer hair cells (arrows) remain by P63 (I-K) in the mutant. By contrast, the basal turn of the mutant is essentially normal except for occasional missing outer hair cells (arrowheads) by P63 (D,H,L). Background staining in mutants presumably results from degenerating and degenerated supporting cells (E-G,I-K). Scale bars: 18.75 μm.
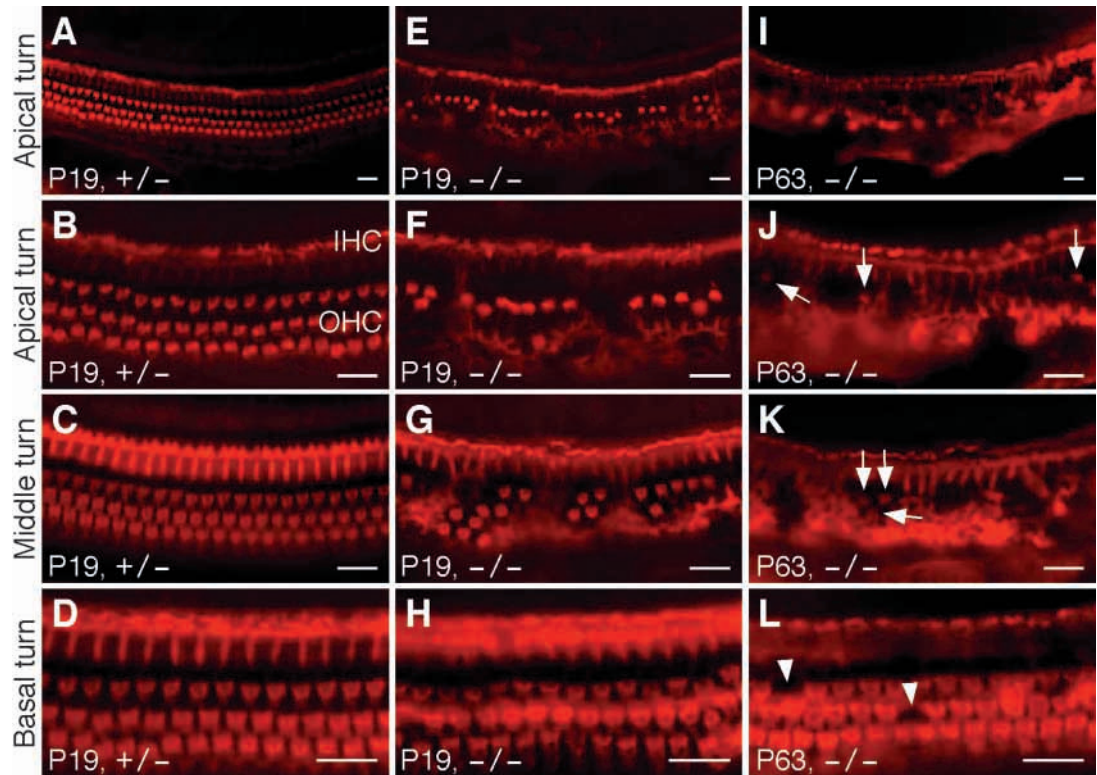
hair cells in the apical and middle turns, but similar to those at early ages (Figs 3, 4, 5), inner hair cells as well as most of the basal outer hair cells still remained (data not shown). By contrast, at 10 months of age, inner hair cells in the basal turn of the mutant organ of Corti were completely absent (Fig. 6E,F) and many of them in the middle turn were missing (Fig. 6C,D), even though the apical turn still retained most of the inner hair cells (Fig. 6A,B). Thus, inner hair cells in the mutant organ of Corti also display progressive degeneration, albeit a delayed one relative to that of outer hair cells. Notably, the loss of inner hair cells follows a basal to apical gradient, which is opposite to that of outer hair cells. The progressive degeneration of outer hair cells in the null cochlea appeared to be complete by 10 months of age, as these cells in the entire organ of Corti, including the most persistent ones in the basal turn all disappeared (Fig. 6). Therefore, the extensive loss of both outer and inner hair cells in the cochlea well correlates with our observation that all *Barhl1*-null mice become profoundly or severely deaf by 10 months of age (Fig. 2B; Table 1).

DISCUSSION

We show in this report that *Barhl1* is expressed in all sensory hair cells during inner ear development, beginning at E14.5 when many hair cells are already generated. Targeted *Barhl1* disruption causes severe to profound hearing loss as a result of a gradual degeneration of both outer and inner hair cells in the cochlea, but does not affect the initial production or differentiation of inner ear hair cells. Interestingly, the loss of outer and inner hair cells are progressive with aging, following

two opposite longitudinal gradients in the mutant organ of Corti (Fig. 7). Together, our data suggest that *Barhl1* is specifically required for the maintenance and perhaps terminal differentiation of cochlear hair cells, but is not involved in their fate commitment or initial differentiation.

Hearing loss and gradient degeneration of cochlear hair cells in *Barhl1* null mice

Our time course analyses of the organ of Corti in *Barhl1*$^{-/-}$ mice revealed an apical-to-basal gradient of outer hair cell degeneration, as well as a basal-to-apical gradient of inner hair cell degeneration (Fig. 7). These pathological changes provide the cellular bases for the observed elevation of ABR thresholds or complete lack of ABR responses in the null mice. A simple prediction can be made that all null mice will eventually become completely deaf when aged mice lose all their cochlear hair cells. At approximately 3 months of age, we found that three out of 10 *Barhl1*$^{-/-}$ mice did not respond to any auditory stimuli, but our SEM analyses revealed that in the mutant organ of Corti all inner hair cells as well as outer hair cells in the base were still morphologically intact (Fig. 5A-D). There are at least two possible explanations for this observation. First, prior to any obvious morphological degeneration, cochlear hair cells in the null mutant may have undergone molecular and physiological changes that render them malfunctional. This is a likely scenario as other changes do occur before visible degeneration takes place in mutant hair cells. For example, cochlear outer hair cells in the mutant were found to undergo disorganization first (Fig. 3E,F). Second, *Barhl1* may play a role in the terminal differentiation of cochlear hair cells. In the null mice, these cells may look normal morphologically but may not be completely mature to be fully functional.
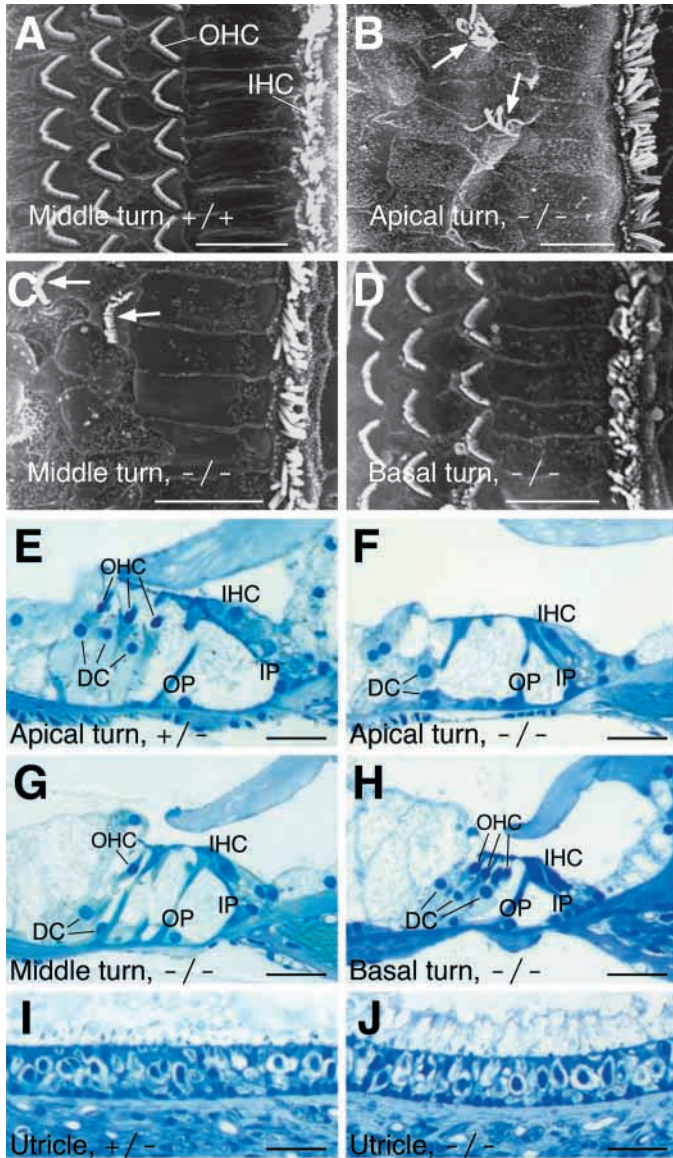
Fig. 5. Cytoarchitectural abnormalities in the organ of Corti of *Barhl1*-null mice. (A-D) Scanning electron micrographs of the organs of Corti from 3.5-month-old *Barhl1*⁺/⁺ and *Barhl1*⁻/⁻ mice. In the apical and middle turns, in contrast to the three rows of organized outer hair cells in the wild type (A), only a few residual outer hair cells remain with disorganized or V-shaped stereocilia in the mutant (B,C, arrows). The outer hair cells in the basal turn and all inner hair cells in the mutant organ of Corti appear to be normal (A-D). (E-J) Light micrographs of semi-thin transverse sections of inner ear sensory epithelia in P66 *Barhl1*⁺/⁻ and *Barhl1*⁻/⁻ mice. Although the cellular structure of the mutant organ of Corti at the basal turn is similar to the control (E,H), most of the outer hair cells in the apical and middle turns are missing and many Deiters' cells become lost or irregular in morphology (F,G). The maculae of the utricle are similar between the mutant and control (I,J). DC, Deiters' cell; IHC, inner hair cell; IP, inner pillar cell; OHC, outer hair cell; OP, outer pillar cell. Scale bars: 10 μm in A-D; 25 μm in E-J.

Although all inner ear hair cells are mechanoreceptors, different hair cell types in the inner ear have different functions with distinct morphologies, cellular structures, innervation
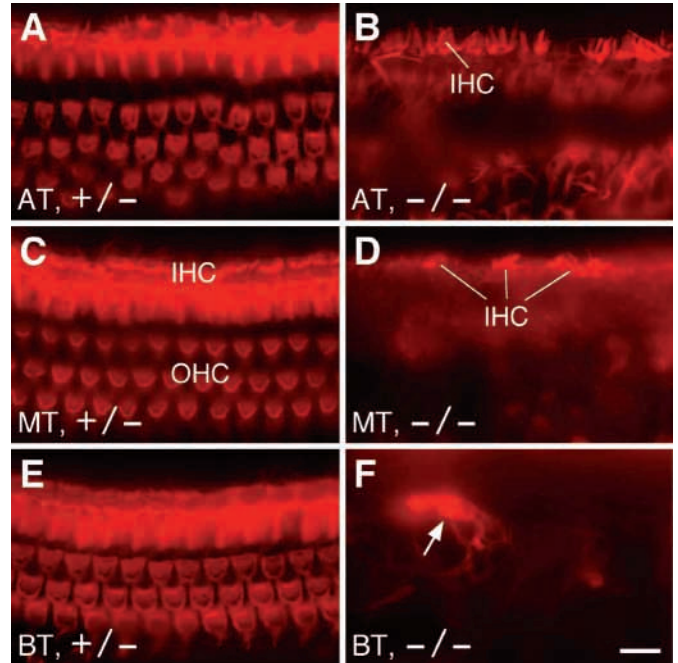

Fig. 6. Region-specific loss of inner hair cells in the organ of Corti of 10-month-old *Barhl1*-null mice. Whole-mount organs of Corti from *Barhl1*⁺/⁻ (A,C,E) and *Barhl1*⁻/⁻ (B,D,F) mice were labeled with rhodamine-conjugated phalloidin. Compared with the heterozygote, no inner hair cells are present within the basal turn of the null mutant (E,F), many of them are missing from the middle turn (C,D), but the large majority of them still remain in the apical turn (A,B). The outer hair cells, however, appear to be entirely missing from the mutant organ of Corti (B,D,F). The arrow points to cell debris presumably resulting from degenerated supporting cells (F). AT, apical turn; BT, basal turn; IHC, inner hair cell; MT, middle turn; OHC, outer hair cell. Scale bar: 18.75 μm.

patterns and physiological properties. For example, the cochlear hair cells are auditory sensors while the vestibular hair cells mediate balance by detecting head movement and position. Moreover, the cochlear and vestibular hair cells are further divided into different subtypes with distinct functions. In the cochlea, the inner hair cells are sensory receptors that provide the principal afferent innervation, while the outer hair cells function as both sensors and mechanical feedback amplifiers (Dallos, 1992; Hudspeth, 1989). There is evidence to suggest that the differentiation and maintenance of inner and outer hair cells are differentially regulated. For example, the Bronx Waltzer mice and Beethoven homozygous mutants exhibit a preferential loss of inner hair cells (Whitlon et al., 1996; Vreugde et al., 2002). In *Barhl1*⁻/⁻ mice, we found that the onset of degeneration of cochlear inner hair cells was delayed for more than half a year relative to that of the outer hair cells, further lending support for the notion that inner and outer hair cells are differentially maintained. In fact, these two hair cell types appear to require different levels of *Barhl1* for their maintenance as a significantly higher level of *Barhl1* expression is seen in the outer hair cells (Fig. 1).

In the cochlea, hair cells also display region specificity and frequency selectivity. Along the longitudinal axis of the organ of Corti, ciliary bundles of hair cells display a gradation in
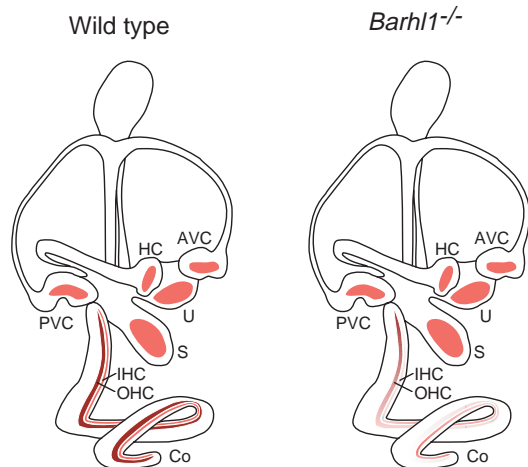
Fig. 7. Progressive loss of cochlear hair cells along the longitudinal axis of the *Barhl1*$^{-/-}$ organ of Corti. In the null mice, degeneration of cochlear hair cells follows two reciprocal longitudinal gradients – apical-to-basal for outer hair cells but basal-to-apical for inner hair cells. AVC, anterior vertical canal; Co, cochlea; HC, horizontal canal; IHC, inner hair cell; OHC, outer hair cell; PVC, posterior vertical canal; S, saccule; U, utricule.

length and their electrical properties also vary (Dallos, 1992; Hudspeth, 1989). Thus, there is an apical-to-basal gradient of sensitivity toward low to high frequencies of sound (Dallos, 1992; Hudspeth, 1989). Given these region-specific properties, the observed degeneration gradients of inner and outer hair cells in *Barhl1*$^{-/-}$ mice may simply manifest a differential requirement for *Barhl1* in different hair cells distributed along the longitudinal axis of the organ of Corti. Because the expression of *Barhl1* itself does not display a gradient in cochlear hair cells, it is possible that *Barhl1* may control the expression of a molecule(s) that is expressed in a gradient in cochlear hair cells and is required for their maintenance. It has recently been shown that there are indeed molecules whose expression exhibits gradients in cochlear hair cells. For example, both the potassium channel KCNQ4 and neurotrophin BDNF display developmental gradient expression patterns in cochlear hair cells along the longitudinal axis (Beisel et al., 2000; Farinas et al., 2001). KCNQ4, in particular, shows a basal-to-apical expression gradient in inner hair cells and an apical-to-basal expression gradient in outer hair cells (Beisel et al., 2000), corresponding to the two reciprocal degeneration gradients of inner and outer hair cells in the *Barhl1* null cochlea. However, mutations in the human *KCNQ4* gene often lead to a preferential high-frequency hearing loss, whereas *Barhl1*$^{-/-}$ mice exhibit a preferential low-frequency hearing loss at early ages (Beisel et al., 2000; Kubisch et al., 1999; Marres et al., 1997; Talebizadeh et al., 1999) (Table 1).

Essential role for *Barhl1* in the maintenance of cochlear hair cells

In the developing mouse inner ear, *Barhl1* commences its expression in hair cells at E14.5, at least 2 days after the onset of hair cell production (Ruben, 1967). Thus, it is unlikely that *Barhl1* is involved in the initial specification and generation of inner ear hair cells. Using a series of hair cell markers, we have

demonstrated in *Barhl1*-null mice that this is indeed the case. First, all cochlear hair cells appear to be present in *Barhl1*-null mice at early postnatal stages, as revealed by β-galactosidase staining (Fig. 3F,J,N). Second, these hair cells in *Barhl1*-null mice can express several other maker molecules, including Math1, Brn3c, myosin VI and myosin VIIa (Fig. 3A-D and data not shown). Because in the inner ear, Brn3c and myosins VI and VIIa have been shown to be expressed only by differentiating and differentiated hair cells and not by their progenitors (Xiang et al., 1998), *lacZ*-positive cells in the cochlea of *Barhl1*-null mice must be able to differentiate in the absence of *Barhl1*. In fact, we observed by SEM and phalloidin labeling that both inner and outer hair cells in the null organ of Corti can undergo extensive differentiation to acquire ciliary bundles (Figs 4, 5, 6). The ensuing progressive loss of cochlear hair cells in *Barhl1*-null mice indicates a crucial role for *Barhl1* in the maintenance of these sensory cells. However, we cannot exclude the possibility that it may also have some limited role in their terminal differentiation.

Recent studies have identified Math1 and Brn3c as two positive transcriptional regulators that control the development of inner ear hair cells. The absence of *Math1* in mice causes a failure to generate both cochlear and vestibular hair cells and *Brn3c* deletion leads to the formation of immature hair cells (Bermingham et al., 1999; Erkman et al., 1996; Xiang et al., 1997; Xiang et al., 1998). Thus, the late onset of hair cell defects in the *Barhl1*$^{-/-}$ inner ear suggests that *Barhl1* is likely to act genetically downstream of *Math1* and *Brn3c* during inner ear development. Notably, *Brn3c* appears to be required primarily for the maintenance of newly generated hair cells, as *Brn3c*-null mice quickly lose the majority of their inner ear hair cells during late embryonic and perinatal stages (Erkman et al., 1996; Xiang et al., 1997; Xiang et al., 1998). Whereas in *Barhl1*-null mice, cochlear hair cells degenerate over a time course of many months, suggesting an essential role for *Barhl1* in the long-term survival of these cells. It is interesting to note that both *Barhl1* and its *Drosophila BarH* homologs play a functionally conserved role in sensorineural development (Higashijima et al., 1992a; Higashijima et al., 1992b; Kojima et al., 1991). However, *Barhl1* is primarily involved in the maintenance of sensory cells whereas *BarH* genes play a principal role at the stages of determination and differentiation in *Drosophila* (Higashijima et al., 1992a; Higashijima et al., 1992b; Kojima et al., 1991).

Barhl1-null mice as a unique deafness model

Hearing impairments represent the most prevalent category of clinically important childhood sensory disorders, affecting approx. one in 1000 children (Brown and Steel, 1994; Steel, 1995; Steel and Kros, 2001). Although no human deafness locus has yet been mapped to the region of the *Barhl1* gene on chromosome 9q34 (Bulfone et al., 2000; Lander et al., 2001; Venter et al., 2001), our studies identify a candidate gene for human deafness disorders. While a number of deafness genes have been identified in the mouse and human in the past few years (Avraham, 1998; Fekete, 1999; Kelsell et al., 1997; Kubisch et al., 1999; Steel and Bussoli, 1999; Steel and Kros, 2001), *Barhl1*-null mice represent a new class of mouse models for non-syndromic, autosomal recessive forms of deafness, which are usually sensorineural and congenital (Brown and Steel, 1994; Fekete, 1999; Steel, 1995; Steel and Bussoli, 1999;

Steel and Kros, 2001). Because *Barhl1*-null mice exhibit region-specific progressive loss of cochlear hair cells, they may also offer a novel model for studying mechanisms leading to the differential frequency hearing loss associated with some deafness disorders. For example, mutations in the *KCNQ4* gene often cause a preferential loss of high-frequency hearing in humans (Beisel et al., 2000; Kubisch et al., 1999; Marres et al., 1997; Talebizadeh et al., 1999). Similarly, the gene responsible for age-related hearing loss (*Ahl*) in C57BL/6J mice has a preferential effect on high-frequency hearing (Johnson et al., 1997; White et al., 2000).

As mammalian inner ear hair cells normally do not regenerate, sustained hearing may crucially depend on a mechanism of preventive maintenance and damage repair of cochlear hair cells. Such a mechanism may gradually fail with aging, as age-related deafness affects more than 60% of the population aged over 70 (Davis, 1989). The *Barhl1* mutant mice display unique features as a model for age-related progressive hearing loss, including late-onset and region-specific degeneration. These characteristics may reflect a crucial requirement for *Barhl1* in the lifelong maintenance of cochlear hair cells. Conceivably, a molecular understanding of this requirement will provide significant insights into the pathology of age-related deafness.

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