# ATP8B1 is essential for maintaining normal hearing

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**ATP8B1 deficiency is caused by autosomal recessive mutations in *ATP8B1*, which encodes the putative phospatidylserine flippase ATP8B1 (formerly called FIC1). ATP8B1 deficiency is primarily characterized by cholestasis, but extrahepatic symptoms are also found. Because patients sometimes report reduced hearing capability, we investigated the role of ATP8B1 in auditory function. Here we show that ATP8B1/Atp8b1 deficiency, both in patients and in *Atp8b1*$^{G308V/G308V}$ mutant mice, causes hearing loss, associated with progressive degeneration of cochlear hair cells. Atp8b1 is specifically localized in the stereocilia of these hair cells. This indicates that the mechanosensory function and integrity of the cochlear hair cells is critically dependent on ATP8B1 activity, possibly through maintaining lipid asymmetry in the cellular membranes of stereocilia.**

ATP8B1 deficiency | extrahepatic symptoms | hearing impairment

P-type ATPases are essential for normal function of the human body (1). In general, this family of transport proteins maintains a cation gradient across cellular membranes. Of the five different subfamilies, the recently discovered P4 P-type ATPases (P4-ATPases) share a distinct function as phospholipid flippases (2–4). By translocation of aminophospholipids from the outer to the inner leaflet of cellular membranes, P4-ATPases are thought to be essential for maintaining membrane lipid asymmetry. This asymmetry is important for fundamental processes such as membrane transport, intracellular signaling and apoptosis (5–7).

ATP8B1 deficiency is a human disease known to be associated with mutations in the gene encoding the P4 P-type ATPase, ATP8B1 (formerly called FIC1) (8, 9). This disease presents with intrahepatic cholestasis either as benign recurrent intrahepatic cholestasis type 1 (BRIC type 1; MIM#243300) or progressive intrahepatic cholestasis type 1 (PFIC type 1; MIM#211600) (9–12). BRIC type 2 and PFIC type 2 are genetically distinct disorders caused by mutations in *ABCB11*, which encodes the main bile salt export pump (BSEP) that is exclusively expressed in the canalicular membrane of the liver (13, 14). Interestingly, ATP8B1 is similarly expressed in canalicular membranes of hepatocytes, but also in other epithelial tissues, which may explain some extrahepatic features exclusively observed in patients with ATP8B1 deficiency (9, 10, 15, 16). Pancreatitis, secretory diarrhea and growth retardation are well known extrahepatic features in ATP8B1 deficiency, that may persist after liver transplantation in PFIC type 1 patients (17–19).

Sporadically, hearing loss has been mentioned in patients with PFIC or BRIC of unknown genetic subtype (20). Given the widespread expression of ATP8B1 we hypothesized that these hearing problems comprise another extrahepatic feature in patients with ATP8B1 deficiency and consequently that the ATP8B1 protein is important for normal hearing. We therefore examined the role of ATP8B1 protein in auditory function in patients and mice with ATP8B1/Atp8b1 deficiency. We show here that ATP8B1 deficiency causes hearing loss, associated with progressive degeneration of the cochlear hair cells.

## Results

**Sensorineural Hearing Loss Is an Extrahepatic Feature in BRIC Type 1.** To investigate whether hearing loss is an extrahepatic feature in BRIC type 1, we tested hearing in ten BRIC type 1 patients. To control for secondary effects resulting from the cholestatic episodes in BRIC, we also included BRIC type 2 patients, who have episodic cholestasis, as occurs in BRIC type 1, but no extrahepatic symptoms (13). Patients with primary sclerosing cholangitis (PSC), affected by mild chronic cholestasis, formed a second control group. In total 10 patients with BRIC type 1, three BRIC type 2 patients, and seven patients with PSC were included. No confounders such as a family history of hearing disorders, noise-induced hearing loss or use of ototoxic medication were noted.

Retrospectively, nine of 10 patients with BRIC type 1 reported hearing problems, which were first noticed at a mean age of 20 years (range, 17–29 years). More importantly, on age-corrected pure tone audiometry, BRIC type 1 patients displayed significant hearing loss in both ears at all frequencies tested except for 500 Hz, compared with the group of PSC patients who displayed normal hearing. This hearing loss was more pronounced at higher frequencies (Fig. 1*A*). The transient evoked otoacoustic emissions (TEOAEs) were abnormal in all BRIC type 1 patients with hearing loss. In two BRIC type 2 patients, a normal hearing for age in both ears and at all frequencies tested was found. The third BRIC type 2 patient displayed a hearing loss of 65 dB in one ear at 4 and 8 kHz, but no hearing loss was found in the contralateral ear. Tympanometry results were normal in BRIC type 1 patients and all controls.

***Atp8b1*$^{G308V/G308V}$ Mutant Mice Show Significant Hearing Loss.** To further investigate the role of the ATP8B1 protein in auditory function, *Atp8b1*$^{G308V/G308V}$ mutant mice were tested for auditory brainstem responses (ABRs) as described previously (21). These mutant mice harbor the mutation G308V (NP_005594), similar to the mutation in Amish PFIC type 1 patients (10, 22). This mutation causes a marked decreased expression of Atp8b1 in mice (22). However, in contrast to the Amish patients, these mutant mice display only a mild hepatic phenotype when on normal diet, and cholestasis only develops when high amounts of bile acids are added to the diet (22). In this work al mice were kept on normal diet. Using ABRs, the sensitivity of the auditory system was tested in 16 days, 1, 3 and 6-month-old mutant mice versus wild-type mice. Fig. 1*B* displays the mean auditory brainstem thresholds for three different tone burst stimuli and the click stimulus. These data revealed that

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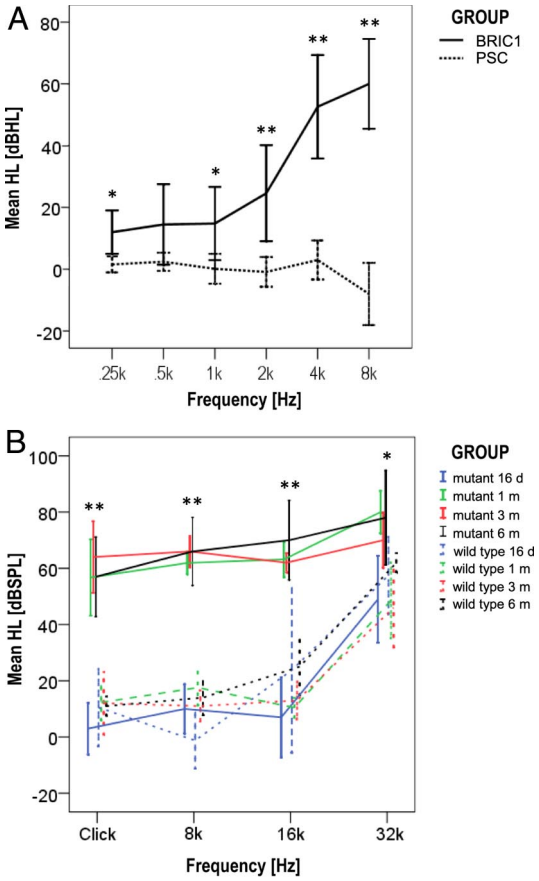


**Fig. 1.** Hearing loss in BRIC type 1 patients and *Atp8b1*$^{G308V/G308V}$ mutant mice. (*A*) Mean age-corrected hearing loss expressed in decibel hearing loss (dBHL) for six octave frequencies (250 to 8000 Hz) of the BRIC type 1 patient group ($n = 10$) and PSC patient group ($n = 7$). (*B*) Mean auditory brainstem thresholds expressed in decibel sound pressure level (dBSPL) for wild-type and *Atp8b1*$^{G308V/G308V}$ mutant mice at different ages (16 days, 1, 3 and 6 months) at three octave frequencies (8–32 kHz) and a click stimulus. d, days; m, months; BRIC, benign recurrent intrahepatic cholestasis; PSC, primary sclerosing cholangitis. Asterisks indicate significant differences between BRIC type 1 versus PSC patients or mutant mice of 1, 3, and 6 months versus age-matched wild-type mice (*$P < 0.05$; **$P < 0.01$). The 16-day-old mutant mice display a normal hearing just like the age-matched wild-type mice. Bars indicate 2× standard error (SE).

hearing function of mutant mice at 16 days was equal to that in wild-type mice. In contrast, significant auditory brainstem threshold shifts were present for all stimuli evoked in 1, 3 and 6-month-old mutants. To exclude any retrocochlear pathology, the interwave intervals (IWI) of ABR peaks were calculated at 1 and 6 months for both mutant and wild-type mice. Since a prolonged IWI between the first (originating from the cochlear nucleus) and the later fourth ABR peak would suggest any retrocochlear pathology, IWIs of these peaks were calculated additionally and tested for statistical differences. The results revealed no statistically significant differences between IWIs of all stimuli found in wild-type and mutant mice at the age of 1 and 6 month (t-tests, two-tailed, $P > 0.05$), suggesting that the hearing losses measured at these ages do not have a retrocochlear origin.

**Atp8b1 Is Localized in the Hair Cells of the Organ of Corti in the Cochlea.** The abnormal TEOAEs in BRIC type 1 patients and the decreased ABR in 1 month and onwards aged *Atp8b1*$^{G308V/G30}$ mutant mice, as well as the lack of a prolonged IWI in these mutant mice, pointed to a cochlear origin for the effect of ATP8B1 deficiency on hearing. We therefore used immunohistochemistry to investigate the expression and localization of the Atp8b1 protein in the murine inner ear. Immunostaining with the anti-ATP8B1-C6 antibody in paraffin-embedded sections of the inner ear of 4-day-old wild-type mice revealed localization of Atp8b1 in the inner and outer hair cells of the cochlea, and more specifically in the apical regions of these cells (Fig. 2 *B–Bii*). These cells also proved to be positive for calbindin-$D_{28K}$, a marker for the inner and outer hair cells of the organ of Corti (oc; Fig. 2 *A–Aii*) and for spiral ganglion cells (sg; Fig. 2 *A and Ai*). However, neither the spiral ganglion cells nor any other cell type in the cochlea displayed Atp8b1 staining, indicating that Atp8b1 was exclusively localized in the hair cells. No signal was observed in cochlear sections incubated with preimmune serum (Fig. 2 *C–Cii*).

To investigate the localization of Atp8b1 in the hair cells in more detail we performed staining of 3 month old wild-type murine whole mount cochleae. Confocal images through the hair cells showed Atp8b1 staining exclusively in the stereocilia of inner as well as outer hair cells (Fig. 3 *A–E*). We therefore conclude that Atp8b1 is expressed in the apical region of cochlear hair cells, and more specifically in the stereocilia.

**Hair Cells Degenerate in Atp8b1 Deficient Mice.** To assess the consequences of Atp8b1 deficiency on cochlea structure, we compared sections from wild-type (Fig. 4 *A–D*) and *Atp8b1*$^{G308V/G30}$ mutant (Fig. 4 *E–N*) mice. The results show a morphologically normal development of hair cells in 4-day-old mutant mice (Fig. 4*E*). Already in 16-day-old mutant mice degeneration had initiated in some outer hair cells. At this age degeneration was more prominent in the basal turn of the cochlea (Fig. 4*I*) than in the other turns (Fig. 4*G*). The spiral ganglion neurons appear unaffected in all turns (Fig. 4 *F*, *H*, and *J*) in comparison to age-matched wild-type mice (Fig. 4 *B* and *D*). In 1-month-old mutant mice this analysis revealed a clear loss of outer hair cells and degeneration of the surrounding supporting cells (Fig. 4*K*) as well as a markedly decreased number of spiral ganglion cells and nerve fibers in all turns (Fig. 4*L*). At ultrastructural level, features of degeneration in inner hair cells were also present (Fig. 5 *B–G*), which is in agreement with the hearing loss that we could demonstrate in this age group. Progression to complete degeneration of organ of Corti's was evident at 6 months of age (Fig. 4*M*) accompanied by a nearly complete loss of all spiral ganglion neurons (Fig. 4*N*).

For a more detailed view of the consequences of Atp8b1 deficiency in the stereocilia we performed scanning electron microscopy (Fig. 6 *A–F*). This analysis revealed normal development of stereocilia in 4-day-old mutant mice (Fig. 6*B*); an initial loss of stereocilia was seen in some outer hair cells in 16-day-old mutant mice (Fig. 6*D*) which progressed to complete degeneration, whereby stereocilia on inner hair cells still look intact at the age of 1 month (Fig. 6*E*). Finally, at 6 months of age, organs of Corti have degenerated in the mutant mice, remaining a simple epithelium (Fig. 6*F*).

## Discussion

In the present study we show that adequate Atp8b1 function in the stereocilia is essential for maintaining viable hair cells within the cochlea. Mice with Atp8b1 deficiency developed complete degeneration of these hair cells and hearing loss. Severe hearing loss in both ears, requiring hearing support, was also observed in BRIC type 1 patients. Our data strongly suggest that hearing loss is caused by a compromised ATP8B1 function and is not the consequence of cholestasis. This notion is underscored by the finding that PSC patients and BRIC type 2 patients had normal hearing. BRIC type 2 patients have the same cholestatic episodes as the BRIC type 1 patients tested here (but due to bile salt export pump (BSEP) deficiency). In addition, Atp8b1 mutant mice displayed a significant hearing loss compared to wild-type mice, but do not show any symptoms of cholestasis when on a normal diet, as during our experiments (22). We conclude that the primary consequence of

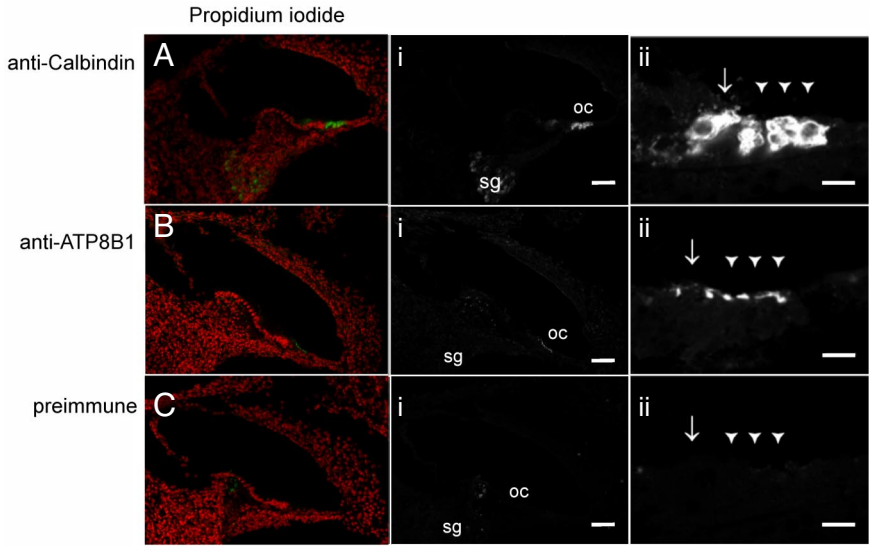


**Fig. 2.** Localization of Atp8b1 in cochlea. (*A–C*) Fluorescence images of paraffin sections of 4-day-old wild-type murine inner ear (basal turn) incubated with anti-ATP8B1-C6 or anti-calbindin-$D_{28k}$ antibody (green or white signal) and counterstained with propidium iodide (red signal). (*A–Aii*) For orientation anti-calbindin-$D_{28K}$ was used as a marker for hair cells in the organ of Corti. (*B–Bii*) Atp8b1 expression was seen in apical region of these hair cells. (*C–Cii*) Serial sections were incubated with preimmune serum as a negative control. Arrow points to row of inner hair cells; arrowhead points to three rows of outer hair cells. sg, spiral ganglion; oc, organ of Corti. Scale bar, (*Ai–Ci*) 50 $\mu$m, (*Aii–Cii*) 10 $\mu$m.

the Atp8b1$^{G308V/G308V}$ mutation is cochlear hair cell degeneration during early postnatal development of the organ of Corti. The observed secondary loss of surrounding supporting cells and spiral ganglion neurons is a well known consequence of deprivation from input signals originating from cochlear hair cells, as has been showed in other genetically modified animal models with degenerated cochlear hair cells (23–31).

We propose several possible pathogenic mechanisms that link ATP8B1 deficiency to hearing loss. ATP8B1 is thought to be important for maintaining phospholipid asymmetry of the cellular membranes, most likely by translocating phosphatidylserine from the outer leaflet of the membrane to the inner leaflet (3, 4, 32). Asymmetric distribution of phospholipids in the cell membrane improves the mechanical stability of the red cell membrane (33). Similarly, the canalicular membrane of patients and mice affected by ATP8B1/Atp8b1 deficiency is unstable, causing extraction of lipids and ectoenzymes into the bile as a consequence of the continuous chemical stress by the detergent action of bile salts. Eventually this results in the formation of coarsely granular bile that contains remnants of extruded cell membranes (34, 35). We here showed that Atp8b1 is abundantly expressed at the stereociliar membrane. Presumably, a deficiency of this protein would result in an impaired phosphatidylserine translocation, and alter the composition of the stereociliar membrane, resulting in a reduced mechanical stability, with devastating effects for hair bundle stereocilia that are under constant undulating mechanical stress forces (36, 37).

Proper membrane lipid composition has also been found to be essential for ATP-dependent $Ca^{2+}$-transporter activity (38). $Ca^{2+}$-ATPase isoform type 2 (PMCA2) is highly expressed in stereociliar membranes where it is pivotal for normalizing intracellular $Ca^{2+}$ concentration in the hair cells after mechanical deflection of the

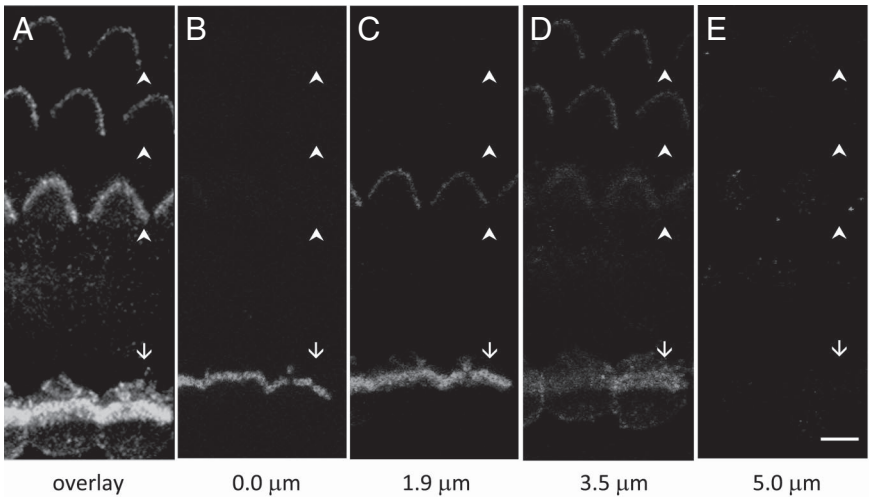


**Fig. 3.** Localization of Atp8b1 in cochlear hair cell stereocilia. Confocal images of cochlear whole mounts of 3-month-old wild-type mice incubated with anti-ATP8B1. Confocal images overlay (*A*) or at level 0.0 $\mu$m (*B*), 1.9 $\mu$m (*C*), 3.5 $\mu$m (*D*), and 5.0 $\mu$m (*E*) showing Atp8b1 staining from top to base in the stereocilia of the cochlear basal turn. The arrow points to the row of inner hair cells, the arrowhead points to the (three) rows of outer hair cells. Scale bar, 6 $\mu$m.

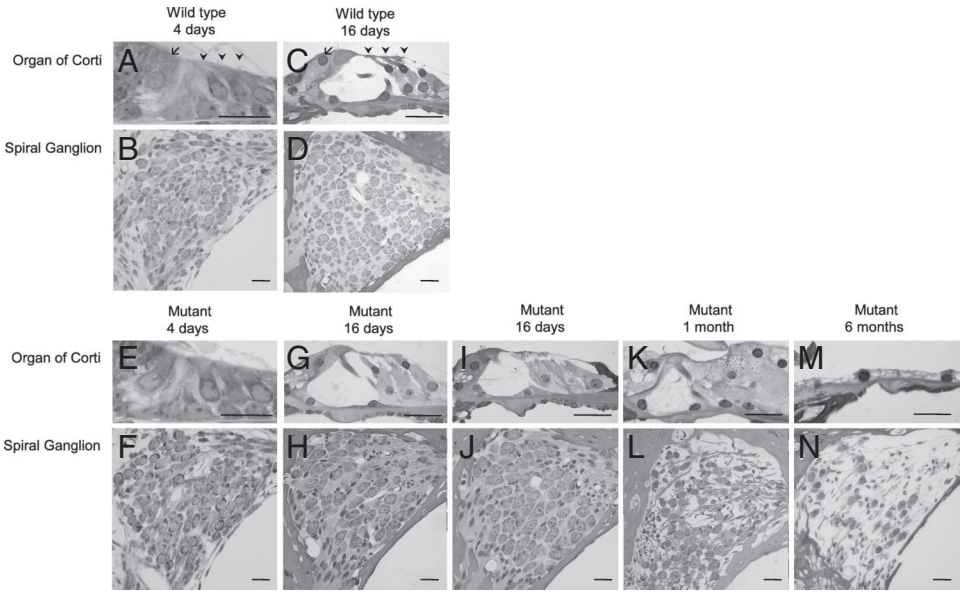


**Fig. 4.** Degeneration of hair cells and spiral ganglion neurons in *Atp8b1*$^{G308V/G308V}$ mutant mice. Light micrographs of cochlear morphology of the organ of Corti (*A*, *C*, *E*, *G*, *I*, *K*, *M*) and spiral ganglion (*B*, *D*, *F*, *H*, *J*, *L*, *N*) in wild-type (*A–D*) and mutant (*E–N*) mice at 4 days (*A*, *B*, *E*, *F*), 16 days (*C*, *D*, *G–J*), 1 month (*K*, *L*) and 6 months (*M*, *N*) of age. All sections are from the basal turn except for (*G*, *H*), which represent the medial turn. Although morphologically intact looking inner hair cells could be seen in the mutant mice till the age of 1 month (*E*, *G*, *I*, *K*), the outer hair cells show signs of degeneration from 16 days on (*I*, *K*, *M*), and the number of neurons in the spiral ganglion is decreased from 1 month of age (*L*). Complete degeneration of the organ of Corti and nearly all connected spiral ganglion cells and nerve fibers is observed in 6-month-old mutant mice (*M*, *N*). Scale bar, 24 $\mu$m.

stereocilia (37, 39, 40). Cochlear hair cells of Pmca2 mutant mice degenerate and cause severe hearing loss early in life, demonstrating that Pmca2 activity is essential for the preservation of the structural integrity of these cells (25, 30, 41). Consequently ATP8B1 deficiency, with a secondary disturbance of membrane lipid asymmetry, likely inhibits PMCA2 activity and affects the efficiency of mechanotransduction. Hence, the similarities in histopathological and physiological findings between Atp8b1 deficient and Pmca2 deficient mice provide a likely explanation for the inadequate hearing associated with Atp8b1 deficiency. Interestingly, compensatory Pmca1 and 3 isoforms are present in the inner hair cells bodies, but not in outer hair cells, which is consistent with our observation that outer hair cells degenerate before inner hair cells (39).

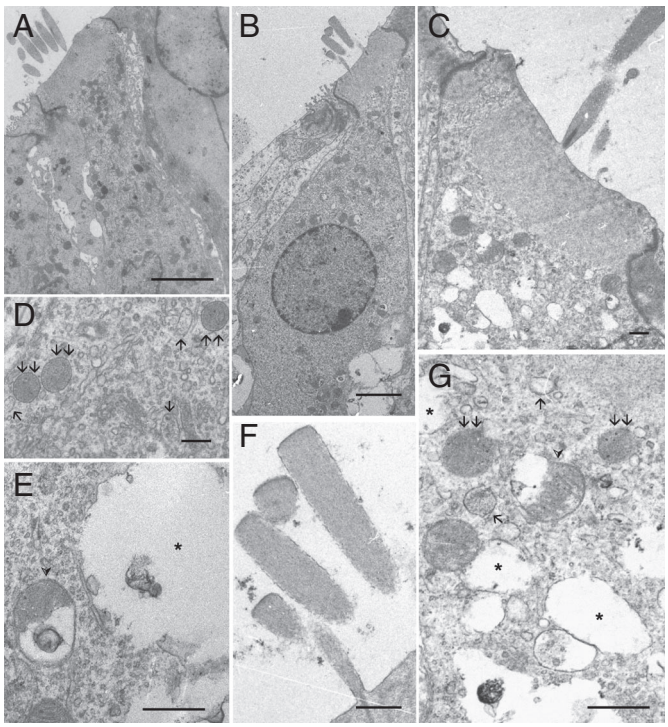


**Fig. 5.** Ultrastructural features of inner hair cell degeneration in *Atp8b1*$^{G308V/G308V}$ mutant mice. Transmission electron micrographs of inner hair cells in the upper basal turn of the cochlea of wild-type (*A*) and mutant (*B–G*) mice at 1 month of age. The inner hair cells of the mutant mice display a normal cell shape and nucleus (*B*) and largely intact stereocilia (*B*, *F*). However, signs of degeneration are already observed, such as condensed cytoplasm (*B*), membrane-bound vesicles enclosing cellular debris (*D*, arrow), multivesicular bodies (*D*, double arrow), degenerating mitochondria (*E*, arrowhead) and vacuoles with cellular debris (*E*, asterisk). More severe degeneration aspects can also be seen in hair cells of the same turn (*C*) especially using higher magnification (*G*). Scale bars: *A*, *B*, 2 $\mu$m; *C–G*, 400 nm.

Finally, ATP8B1 deficiency might alter the phospholipid composition of the inner membrane leaflet, thereby impairing the generation of phosphoinositides (42, 43) that are important for adequate mechanotransduction and maintenance of the hair bundle structure (23, 44). Phosphoinositide signaling was recently found to be involved in age-related hearing loss, raising the possibility that *ATP8B1* may reflect a modifier locus for presbycusis (45).

Our study included only patients with the recurrent form of ATP8B1 deficiency (BRIC type 1) because, until recently, patients with progressive ATP8B1 deficiency (PFIC type 1) did not survive beyond childhood. In addition, this permitted us to study adult patients from different age groups, and will allow future follow up studies to monitor the progression of ATP8B1-induced hearing loss. Consequently, all BRIC type 1 patients studied here have mutations in the *ATP8B1* gene with relatively mild consequences, and supposedly have some residual function of ATP8B1. In contrast, the Atp8b1$^{G308V/G308V}$ mutant mice have severely compromised Atp8b1 function and expression, thus providing a rationale for the relatively mild hearing loss in ATP8B1 deficient patients as opposed to mice. Conversely, the liver phenotype of *Atp8b1* mutant mice is relatively mild. This phenomenon is generally explained by the presence of large amounts of hydrophilic bile salts in murine bile. The murine bile salt pool is less detergent and therefore less efficient in destabilizing canalicular membranes with altered phospholipid asymmetry (22, 35). Collectively, these findings indicate that the severity of hepatic and extrahepatic features of ATP8B1 deficiency are both dependent on the extent by which the ATP8B1 protein function is compromised, as well as tissue specific additional factors that are relevant for the pathogenesis of the disorder (22, 35).

In conclusion, we show that ATP8B1/Atp8b1 deficiency causes hearing loss, associated with progressive degeneration of cochlear hair cells. These data indicate that the preservation of the cochlear hair cells in the inner ear, required for adequate hearing, is critically dependent on normal ATP8B1 function, analogous to the preser-

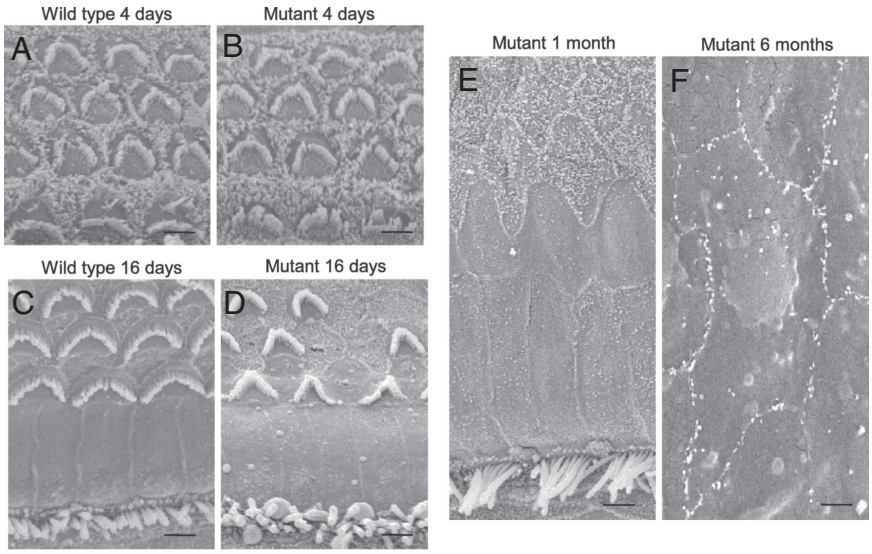


**Fig. 6.** Degeneration of hair cells and stereocilia in *Atp8b1*$^{G308V/G308V}$ mutant mice. Scanning electron microscopy images of the organ of Corti in wild-type (*A*, *C*) and mutant mice (*B*, *D*–*F*) at 4 days (*A*, *B*), 16 days (*C*, *D*), 1 month (*E*) and 6 months (*F*) of age, all in the basal turn. There is normal development of stereocilia in the 4-day-old mutant mice (*B*). Loss of the stereocilia starts in some outer hair cells in 16-day-old mutant mice (*D*) and progresses to complete absence, whereas stereocilia on inner hair cells do not show detectable morphological aberrations in 1-month-old mutant mice (*E*). The total organ of Corti has degenerated at 6 months in mutant mice, resembling merely a simple nonciliated epithelial layer (*F*). Scale bar, 1 $\mu$m.

vation of structural integrity of the bile canaliculus, necessary for adequate bile salt excretory function.

## Materials and Methods

**Patients.** The 10 adult Dutch BRIC type 1 patients investigated had at least 1 episode of cholestasis with normal serum concentration of gamma-glutamyl transpeptidase (GGT) in combination with known mutations on both alleles of *ATP8B1*. To control for secondary effects of cholestasis on hearing, three BRIC type 2 and seven PSC patients were tested. The BRIC type 2 patients also had at least one episode of low GGT cholestasis, in combination with known *ABCB11* mutations on both alleles. All patients with PSC were previously diagnosed with inflammatory bowel disease and did have elevated transaminases and GGT. The diagnosis was confirmed with magnetic resonance cholangiopancreatography and/or a liver biopsy. At the time of audiometry the mean age of the patients with BRIC type 1 was 37 years (range, 18–60 years), in the BRIC type 2 this was 36 years (range, 23–43 years), whereas the mean age of the patients with PSC was 47 years (range, 35–57 years). This human study was conducted under approved protocols and after written informed consent was obtained for all patients, in accordance with the guidelines of the medical ethical committee of the University Medical Center Utrecht.

**Audiometry.** For detection of hearing loss, all patients were tested with standard pure tone audiometry for six octave frequencies (250–8000 Hz), both for air and bone conduction. To exclude possible effects of age-related hearing loss (presbycusis) audiometrical thresholds were individually corrected for age according to ISO 7029 (1984) values based on normative data by Robinson and Sutton (46). Although hearing losses were generally symmetrical, in cases of small asymmetries thresholds of the best ear were used for statistical analysis. Middle ear pressure and tympanic compliance was measured by tympanometry. The normal range for middle ear pressure was taken as between −100 and +100 daPa and normal compliance was defined as 0.3–1.5 ml of equivalent air volume. To test the cochlear sensory function, TEOAEs were recorded. The TEOAEs were classified as normal or abnormal based on the criteria described by Welzl-Muller and Stephan (47). Only with a normal tympanogram do abnormal emissions indicate cochlear dysfunction.

**Mice.** *Atp8b1*$^{G308V/G308V}$ mutant mice (129/Sv strain) are used as a murine model for PFIC1 disease (22). For control experiments, wild-type mice of the same strain as the mutants were used. All mice were maintained on a standard 12-hour light,12-hour dark cycle and fed *ad libitum* with standard rodent chow. Animal experiments were approved by the institutional animal care and use committees.

**Electrophysiological Testing: Auditory Brainstem Responses.** For ABR comparison 16 days, 1, 3 and 6-month-old *Atp8b1*$^{G308V/G308V}$ mutant ($n$ = 5, 12, 5, 5) and wild-type ($n$ = 5, 7, 5, 5) mice were measured. In both groups auditory brainstem responses (ABRs) were evoked with four different stimuli: i.e., 8, 16, and 32 kHz tone burst with 1 millisecond rise/fall times, 3 millisecond plateau and with a monophasic click stimulus (100 microsecond duration) according to standard audiometric procedures. Stimuli were presented with a stimulation rate of 30 pulses per second in a sound field setup by a high-frequency loudspeaker (Elac Jet III) 6 cm in front of the ear. All stimulus intensity levels were calibrated according to IEC 61672-2 standards (2004) using a frequency analyzer (Bruel & Kjaer, type 2260) and corrected for the sound field. The EEG recording system was externally triggered by a stimulus generator implemented in a PC using a National Instruments sound interface card using a digital-to-analog converter (type 6062E) to trigger the tone bursts. Click stimuli were internally generated and triggered by the EEG system. Before ABR measurements, mice were anesthesized by i.p. injections of ketamine (200 mg/kg) and xylazine (14 mg/kg). ABRs were obtained using an EEG recording system (Modeled Synergy, Oxford Instruments, UK) with bandpass filter settings set between 300 Hz (HP) and 5000 Hz (LP), auto reject mode (set at 50 $\mu$V) and a 60 Hz notch filter. A minimum of 500 averages were recorded suprathreshold; at threshold levels the minimum of averages was set at 1500 averages. For identification of possible electrical stimulus artifacts, the recording time window was set at 1.5 millisecond prestimulus time, and lasted 13.5 milliseconds, comprising all brainstem potentials. Recording needle electrodes were placed at Cz and M1 (non-inverting and inverting electrode respectively), a ground electrode was placed in the tail. Electrode impedances were measured before ABR recording and revealed inter-electrode impedances of less than 5 kOhm. The auditory threshold was defined at the level where the brainstem responses show just noticeable reproducible peaks. Brainstem peaks were visually determined and the interwave interval (IWI) JI-IV were evaluated for any prolonged interpeak latencies to exclude retrocochlear pathology.

**Immunohistochemistry.** Immersion-fixed (5 hours; methanol:acetone:water [2:2:1]) paraffin-embedded sections of 4-day-old wild-type ($n$ = 6) inner ears were used for immunohistochemistry. Paraffin-embedded sections were dewaxed, rehydrated, rinsed in phosphate-buffered saline (PBS) containing 0.1% Triton-X100 and boiled for 10 minutes in sodium citrate buffer (pH 6.0). The sections were blocked in 3.0% blocking reagent (supplied in TSA fluorescein system: Perkin-ElmerLife Science, Boston, MA) in PBS for 1 hour at room temperature and incubated overnight with either 1:200 dilution of rabbit anti-ATP8B1-C6 (15, 16) or 1:200 diluted preimmune serum. After washing, endogenous peroxidase activity was blocked with 3% $H_2O_2$ in PBS. As secondary antibody horseradish peroxidase (HRP)–conjugated goat anti-Rabbit IgG was used. HRP-based signal amplification was applied by using the Alexa Fluor 488 tyramide fluorescein system procedure according to the manufacturer's description. Sections were counterstained with propidium iodide. Immunofluorescent labeling was visualized by confocal laser scan microscopy using a Nikon Eclips E600 microscope. As

a positive control sections were stained with rabbit anti-calbindin-$D_{28K}$ (1:500; Sigma Chemicals).

**Murine Cochlear Whole Mounts.** Perfusion-fixed (4% paraformaldehyde in PBS) inner ears of 3-month-old wild-type mice ($n = 8$) were dissected to expose the cochlear organ of Corti containing the sensory hair cells with stereociliar bundles, by removing the cartilaginous capsule, the lateral part of the cochlear duct, and the tectorial membrane. The organ of Corti together with the medial part was then detached as a single spiral coil from the central modiolus of the cochlea. These cochlear whole mounts were permeabilized with 0.5% Tween-20 in PBS and incubated overnight with either rabbit anti-ATP8B1-C6 (15, 16) (1:200) or preimmune serum (1:200) diluted in a blocking solution of 0.1% ovalbumin with 0.5% fishgelatin in PBS. As secondary antibody goat anti-rabbit conjugated to Alexa 488 (Molecular Probes) was used. After washing, the specimens were mounted in Vectashield and examined with a Leica FCS sp2 AOBS confocal microscope using 63× oil immersion lens (numerical aperture 1.4).

**Light Microscopy.** Perfusion-fixed (2.5% glutaraldehyde in 0.1 mol/l phosphate buffer) inner ears of 4 days, 16 days, 1 month, and 6-month-old wild-type ($n =$ 3/age group) and *Atp8b1*$^{G308V/G308V}$ mutant ($n =$ 3/age group) mice were isolated, decalcified for 2 (16-day-old mice), 5 (1-month-old mice), or 7 days (6-month-old mice) in 10% ethylenediaminetetraacetate (EDTA) and embedded in the plastic glycol methacrylate (JB4). Sections (2 $\mu$m) were stained with 2.5% toluidine blue and viewed with a standard Zeiss Axioskop microscope.

**Scanning Electron Microscopy.** For exposing the cochlear hair cells with their stereocila, perfusion-fixed (2.5% glutaraldehyde in 0.1 mol/l phosphate buffer) inner ears of wild-type ($n = 2$) and *Atp8b1*$^{G308V/G308V}$ mutant ($n = 2$) mice 4 days, 16 days, 1 month, and 6 months of age were dissected by removing the surrounding bone, stria vascularis, and tectorial membrane. Tissues were processed in 1% osmium tetroxide, dehydrated, and critical point dried. Hereafter the specimens are sputter-coated with gold and examined at 15 kV with a Jeol 6310 scanning electron microscope.

**Transmission Electron Microscopy.** Dissected cochleae from perfusion-fixed (2.5% glutaraldehyde in 0.1 mol/l phosphate buffer) inner ears of 1-month-old wild-type ($n = 3$) and *Atp8b1*$^{G308V/G308V}$ mutant ($n = 3$) mice were decalcified for 5 days in 10% EDTA containing 1.25% glutaraldehyde. The specimens were embedded in epon, sectioned, contrasted via standard procedures and examined with a Jeol 1010 transmission electron microscope.

**Statistical Analysis.** Individual psycho-acoustical auditory threshold data of BRIC type 1 and PSC patients as well as all electrophysiological data of mice were analyzed and compared between groups using two-tailed *t*-tests for independent samples after groups were tested for equal variances (Levene statistic). All statistical analyses were performed using SPSS statistical package (V14.0).

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