A new mouse mutant of the \textit{Cdh23} gene with early-onset hearing loss facilitates evaluation of otoprotection drugs

We report a novel mutation (erlong, \textit{erl}) of the cadherin 23 (\textit{Cdh23}) gene in a mouse model for DFNB12 characterized by progressive hearing loss beginning from postnatal day 27 (P27). Genetic and sequencing analysis revealed a 208 \textit{T} \rightarrow \textit{C} transition causing an amino-acid substitution (70S–P). Caspase expression was upregulated in mutant inner ears. Hearing was preserved (up to 35-dB improvement) in pan-caspase inhibitor Z-VAD-FMK-treated mutants compared with untreated mutants \((p < 0.05)\). Outer hair cell (OHC) loss in the cochleae of Z-VAD-FMK-treated mutants was significantly reduced compared with those of untreated mice. Thus, the \textit{erl} mutation can lead to hearing loss through apoptosis. This is the first genetic mouse model of hearing loss shown to respond to otoprotective drug therapy. The short interval from initial hearing loss to deafness (P27–P90) makes this model ideal for screening and validating otoprotective drugs.

Keywords: mouse model; \textit{Cdh23}; mutation; hearing loss; apoptosis; Z-VAD-FMK

Introduction

Cadherin 23 (CDH23) is encoded by a large \textit{Cdh23} gene containing 69 exons spanning at least 350 kb in the mouse. It is a 365 kDa transmembrane protein composed of 27 extracellular cadherin repeats, a single-pass transmembrane region and a cytoplasmic region and is an important component of the hair cell tip-link in the organ of Corti.1–3 Mutations in the \textit{CDH23} gene in humans have been linked to age-related hearing loss (AHL).4 Usher syndrome type 1 subtype D (USH1D).5,6 and a form of nonsyndromic autosomal recessive deafness designated as DFNB12.6 Different mutations in the \textit{CDH23} gene can cause either syndromic or nonsyndromic forms of deafness; only missense mutations of \textit{CDH23} have been observed in families with nonsyndromic deafness, whereas nonsense, frameshift, splice-site and missense mutations have been identified in families with Usher syndrome (syndromic).7 AHL is a characteristic of the widely used C57BL/6J mouse strain.8 AHL starts with a moderate hearing impairment in 1-year-old C57BL/6J mice, and progresses to complete hearing loss with age. Linkage studies associated AHL with a locus named \textit{ahl}.9 A single-nucleotide polymorphism in exon 7 of \textit{Cdh23} was significantly associated with AHL and the deafness modifier \textit{mdfw} (modifier of deaf waddler). The hypomorphic \textit{Cdh23}\textit{ahl} allele causes in-frame skipping of exon 7.4 Histological analysis correlated AHL with a gradual loss of hair cells, spiral
ganglion (SG) cells and degeneration of fibrocytes in the spiral ligament. Most other mouse models of point mutation in the Cdh23 gene are characterized by congenital deafness with circling behavior. In four waltzer alleles (v2f, v6b, v4d, v6f), loss of functional protein has been reported to disrupt the highly organized stereocilia bundle of hair cells in the cochlea and the vestibule during late embryonic/early postnatal development. In all previously characterized Cdh23 mouse models, the mice are either deaf at birth, show severe very early-onset hearing loss or very late-onset hearing loss with slow progression. The deaf (v-df) mutant was reported by Deol MS, that v-df mice may be deaf from the beginning or may be able to hear for a few days before weaning, but otherwise behave normally. Although the final pathological outcome is consistently observed as inner ear hair cell loss, none of these models provide an easily manageable time interval for evaluating pathophysiological changes and/or for screening and testing drug therapies. Here, we introduce a new mouse model of DFNB12, which is characterized by progressive hearing loss starting at P27 and progressing to deafness by p100. This is an ideal time window for testing otoprotective drugs. Nevertheless, we have found that apoptosis has a major role in hair cell loss in this mouse model. Most importantly, we report here that a pan-caspase inhibitor not only preserved inner ear hair cells, but also prevented hearing loss by up to 35 dB in the mutant mice.

Materials and methods

Mice, genetic linkage cross and DNA sequencing

Mice were originally housed in The Jackson Laboratory (Bar Harbor, ME, USA) research facilities and all procedures were approved by the Institutional Animal Care and Use Committee (protocols SU01NS041215 & R01DC007392). Mice were then relocated to Case Western Reserve University (CWRU, Cleveland, OH, USA). Further studies were conducted in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, and were approved by the Case Western Reserve University of the Health Sciences Institutional Animal Care and Use Committee (R01DC009246). N-ethyl-N-nitrosourea (C3H6N2O2)-induced mutagenesis was performed as part of a large-scale screening program for new mutants at the Jackson Laboratory using predominantly the C57BL/6J (B6) mouse strain (The Jackson Laboratory Neомutagenesis Facility website: http://nmf.jax.org). A detailed protocol is described at http://nmf.jax.org/protocols/genetics_scheme.html. As hearing impairment was the only measurable phenotype of erl mutant mice, we had to trace phenotype by testing hearing with auditory brainstem response (ABR) thresholds for maintaining colonies and performing all experiments. Genetic intercross generated 13 affected ((B6XC3H/He)F1–erl/+ × (B6XC3H/He)F1–erl/+ ) F2 progeny with elevated ABR thresholds as shown in Supplementary Table 1.

A DNA pooling method for gene mapping was used as previously described. Genomic DNA sequencing to identify the alteration in the erl mutant mouse was performed as follows: genomic DNA was prepared from tail tips of mice. Briefly, 2-mm mouse tail tips were digested with 0.3 ml of 50 mM NaOH in a 0.5 ml Eppendorf tube at 95 °C for 10 min. A total of 26 μl of 1 M Tris-HCl was then added to each tube. The mixtures were centrifuged at 12,000 × g for 5 min and the DNA concentration in supernatants was measured using a BioPhotometer (Eppendorf AG, Hamburg, Germany). Forty-eight pairs of PCR primers spanning exons of the Cdh23 gene were designed using the Primer3 freeware (http://frodo.wi.mit.edu/primer3/), according to the exon sequence of the Cdh23 gene in the Ensemble Mouse Genome Server (http://www.ensembl.org), and synthesized by Integrated DNA Technologies, Inc. (San Diego, CA, USA). PCR for comparative DNA analysis between Cdh23erl/erl and Cdh23ahl/ahl mice was performed according to the Tm of the primers and the expected product sizes. PCR products were purified with the QIAquick PCR Purification kit (Qiagen, Inc. Valencia, CA, USA). DNA sequencing was performed using the same primers as for DNA amplification and then run on an ABI Applied Biosystems 3730 DNA Analyzer (Life Technologies Corp., Carlsbad, CA, USA).

To confirm the mutation and to identify potential aberrant exon splicing, Cdh23erl/erl and Cdh23ahl/ahl mice at 2 weeks of age were used for RNA extraction and reverse transcription PCR. After mice were killed under anesthesia (avertin 5 mg per 10 g), the inner ears were quickly removed. Total RNA (DNA-free) was prepared using the pure-LinkTM Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) synthesis was carried out using the SuperScript™ First-Strand Synthesis System (catalog no. 11904–018). PCR primers (CdhmF and CdhmR in Table 2) were designed in exon 2 and exon 4 yielding a 228-bp PCR product containing exon 3 and its flanking regions from exons 2 and 4.

Auditory-evoked brainstem response

ABR was measured at various intervals for Cdh23erl/erl and Cdh23ahl/ahl mice (at ages over P14). A computer-aided evoked potential system (Intelligent Hearing Systems, Miami, FL, USA) was used to test mice for ABR thresholds as previously described. Briefly, mice were anesthetized and body temperature maintained at 37–38 °C by placing them on a heating pad in a sound-attenuating chamber. Subdermal needle electrodes were inserted at the vertex of (active) and ventrolaterally to (reference) the right ear and to the left ear (ground). Clicks and 8-, 16- and 32-kHz tone-bursts were channeled through plastic tubes into the animal’s ear canals. The amplified brainstem responses were averaged by a computer and displayed on a computer screen. Auditory thresholds were obtained for each stimulus by reducing the sound pressure level (SPL) at 10-dB steps and finally at 5-dB steps up and down to identify the lowest level at which an ABR pattern could be recognized. ABR threshold values above 55 (for click stimulus), 40 (for 8 kHz), 35 (for
16 kHz) or 60 (for 32 kHz) dB SPL were considered to be hearing impaired.8

**Distortion product oto-acoustic emission (DPOAE)**
To test the function of outer hair cells (OHCs) of different mice at different time points, we used the IHS Smart EP 3.30. USBez Software (Intelligent Hearing Systems) for DPOAE measurement, which was conducted for pure tones from 2 to 36 kHz.17 An Etymotic 10B + (Etymotic Research, Inc., Elk Grove Village, IL, USA) probe was inserted into the external ear canal and used with two different types of transducers depending on the range of the stimulation frequency. For frequencies ranging from 2 to 16 kHz, an Etymotic ER2 stimulator was used and for frequencies ranging from 16 to 30 kHz, an IHS high-frequency transducer was used. Stimulus response signals were sampled at a rate of 128 kHz using a 16-bit D/A converter; L1 and L2 amplitudes were set to the same level. Frequencies were acquired with an F2:F1 ratio of 1.22. The stimuli were presented starting from the lowest frequencies tested and increasing to the highest frequencies tested. Five stimulation levels ranging from 65 to 25 dB SPL in 10-dB steps were used.

**Histological analyses of inner ears**
Histological analyses of inner ears were performed following the methods described previously.20 Briefly, anesthetized mice were perfused through the left ventricle of the heart with phosphate-buffered saline (PBS) followed by Bouin’s (for hematoxylin/eosin staining) or 4% paraformaldehyde (for all others) fixative. For microscopic analysis of cross-sections, inner ears from Cdh23ertl/ertl and Cdh23ahl/ahl mice were dissected, perfused with fixative, immersed in same for 48 h, decalcified with Cal-EX solution for 6 h, and embedded in paraffin. Sections (5 μm) were cut, mounted on glass slides and counterstained in hematoxylin/eosin.

**Cytocochleograms**
Cytocochleograms were obtained by a modified method as described previously.20 Briefly, the organ of Corti was carefully microdissected out and mounted in glycerin on glass slides. The surface preparations were stained for F-actin with Alexa Fluor 568 conjugated to phalloidin to show hair bundles and examined with a fluorescence microscope (Leica DM4000 B, Leica Microsystems, Wetzlar, Germany). Hair cells were counted as present if V-shapes of hair bundles were intact. Inner and outer hair cell counts were made by subdividing the cochlea into 10 regions at 10% distance intervals, beginning at the apex and continuing toward the base. Individual cochleograms were constructed to show the percentage of hair cells missing as a function of distance from the apex.

**Semi-quantitative reverse transcription PCR for measuring mRNA accumulation levels of apoptosis-related genes**
Cdh23ertl/ertl and Cdh23ahl/ahl mice were killed under avertin anesthesia at 2 weeks or 2 months of age. The inner ears and left temporal brain lobes (50 mg) were quickly isolated for total RNA and cDNA preparation as described in the previous section. One μg of total RNA from each sample was used as template for cDNA synthesis. The 20 μl reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 9.0 (at 25 °C), 0.01% Triton X-100, 2 mM MgCl₂, 250 nM of each primer (forward and reverse), 200 μM dNTPs, 1 μl of cDNA and 0.5 U of Taq DNA polymerase (New England BioLabs, Inc., Ipswich, MA, USA). PCR primers are listed in Table 2. PCR was performed in a Bio-Rad PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc. Hercules, CA, USA). Amplification conditions were 94 °C for 2 min; followed by 28 cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 50 s; followed by 5 min at 72 °C. Ten μl of the PCR products were subject to agarose gel electrophoresis and the gray intensity of each band was digitized using ImageJ software (http://rsb.info.nih.gov/ij/ NIH, Bethesda, MD, USA) and corrected by the glyceraldehyde 3-phosphate dehydrogenase mRNA accumulation level of the same sample.

**Caspase-3/7 activity measurement**
Caspase-3/7 activity was detected in inner ears of 9 Cdh23ertl/ertl and 7 Cdh23ahl/ahl mice by the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. In brief, the inner ears were isolated and immersed in 300 μl of 0.1 M PBS and homogenized on ice with a Tissue Mincer (Fisher Scientific, Pittsburgh, PA, USA). After centrifugation at 12,000 × g for 15 min at 4 °C, the supernatant was retained and the protein concentration determined by the Lowry method. For the caspase assay, the nonfluorescent caspase substrate (Z-DEVD-R110, diluted 100-fold in the provided buffer) was mixed with an equal volume (20 μl) of solution containing the same amount of total inner ear protein (20 μg) for each sample. The protein/substrate mixture was incubated at room temperature for 4 h and diluted 300-fold afterward. The fluorescent product was detected using a spectrofluorometer configured to an excitation wavelength of 499 nm and an emission wavelength of 521 nm. The relative fluorescence unit as a measure of caspase activity was calculated as the fluorescence counts divided by the total amount of protein.

**Immunostaining for active caspases**
A time course immunocytochemistry study of caspase expression was carried out for Cdh23ertl/ertl and Cdh23ahl/ahl. The following antibodies from Cell Signaling Technology, Inc. (Danvers, MA, USA) were used. Cleaved caspase-3 (A1P75) antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175 (This antibody does not recognize full-length caspase-3 or other cleaved caspases.); caspase-8 antibody (mouse-specific) detects endogenous levels of the large 18 kDa subunit of active caspase-8; cleaved caspase-9 (A13P53) antibody (mouse-specific) detects endogenous levels of the 37 kDa subunit of mouse caspase-9 only after cleavage at aspartic acid 353. It does not cross-react with full-length caspase-9 or with other caspases at endogenous levels. Mice were subjected to ABR testing (age ≥ 14 days) under anesthetizing conditions and then
killed. The inner ears were removed and cryosections were made and fixed in 4% PFA (diluted in 1 × PBS) for 2 h. The sections were washed in PBS at room temperature twice for 5 min and permeabilized in 0.5% Triton X-100 for 30 min. After being washed twice in 1 × PBS for 5 min and blocked in 5% BSA for 1 h, the samples were immersed in anti-active caspase-3, caspase-8 or caspase-9 (1:200 dilution) and incubated at 4 °C overnight. After being washed twice in 1 × PBS for 5 min, the samples were immersed in anti-rabbit secondary antibody Alexa 488 (1:500 dilution) for 1 h. The samples were also stained with propidium iodide (10 mg ml⁻¹ in PBS) for 30 min at room temperature. The sample mounts were observed under immunofluorescent microscopy.

**Intraperitoneal injection and treatment regime optimization**

Cdh23<sup>erl/erl</sup> was used as a model for testing anti-apoptotic drug therapy. In all, 60 Cdh23<sup>erl/erl</sup> mice at the age of 7 days (a starting point based on the aim of preventing caspase increases, detected as early as P14 in untreated Cdh23<sup>erl/erl</sup> mice) were divided into 3 groups: a test group, a dimethyl sulfoxide (DMSO) group and an untreated group. The following treatment regime was selected as the best treatment from three sets of preliminary regimes (data not shown). In the test group, 22 mice were injected intraperitoneally under sterile conditions with Z-VAD-FMK (1 mg ml⁻¹, Z-Val-Ala-Asp(OMe)-Fluoromethylketone, Alexis Biochemicals, Farmingdale, NY, USA) in PBS-diluted DMSO (1:1) at the dosage of 1.5 mg g⁻¹ mouse weight: first, starting at P7, eight injections, once every other day; second, four injections, once every 3 days; then, one injection every 4 days until the time of euthanization for experimental procedures. In all, 18 mice in the DMSO group received 1.5 µl of PBS-diluted DMSO (1:1) per gram of mouse weight at the same time points as the test group. ABR and DPOAE were tested at 4, 6, 8 and 12 weeks for the mice in each group and 4–5 mice from each group were killed at each time point for histological investigation.

**Statistical methods**

The analysis of variance was used for all data analysis except that hair cell loss data were analyzed by the χ²-test. A value of P < 0.05 was considered significant.

**Results**

**Linkage analysis and genetic complementation tests showed that erl is a new allele of the Cdh23 gene**

A new hearing-impaired mouse mutant was discovered at the Jackson Laboratory on the C57BL/6J (B6) background initially on the basis of its lack of a Preyer reflex when presented with a calibrated 20 kHz 90 dB SPL tone burst from a click box at two months of age. This mutant was originally generated from the Neuroscience Mutagenesis Facility (http://nmf.jax.org/), was designated #NMF308), but here is named erl (symbol: erl), meaning hearing impaired in the Chinese language. Subsequently, hearing loss (starting at P27, Table 1) was shown to progress to deafness (at P90) by ABR threshold testing in the homozygous colony (data not shown). No balance defect was observed throughout the lifespan of these mice. Initially, the mutant was mated with an unrelated B6 mouse to determine the heritability pattern of the mutation. ABR testing revealed no hearing loss in (erl × B6) F1 progeny up to 3 months of age, thus indicating that erl is a recessive hearing loss mutation. B6-erl/erl mice were outcrossed with C3H/HeJ mice to generate the (B6XC3H/HeJ)F1-erl/+ mice. These F1 mice were intercrossed (F1 × F1) and yielded a Mendelian proportion of a single-gene recessive inheritance pattern, generating roughly 1/4 (13/51) erl/erl F2 progeny with ABR thresholds above 80 dB SPL and 1/4 (38/51) ?/ erl/erl F2 progeny with ABR thresholds below 50 dB SPL at 4 months of age (see Supplementary Table 1).

Our previous publications showed that, for AHL, many strains have a chromosome 10 (Cdh23) defect<sup>4,8,20,22–25</sup> that causes hearing loss; therefore, we first tested this possibility

| Parental mating | ID | Age (days) | ABR (dB SPL) | Genotypes |
|-----------------|----|-----------|--------------|-----------|
| ahl/v2J × erl/erl F1 | 1 | 26 | 50 | 50 | 70 | 80 | Normal | erl/v2J (codominant) |
| ahl/v2J × erl/erl F1 | 2 | 26 | 45 | 40 | 60 | 90 | Normal | erl/v2J (codominant) |
| ahl/v2J × erl/erl F1 | 3 | 26 | 40 | 40 | 40 | 70 | Normal | erl/v2J (codominant) |
| ahl/v2J × erl/erl F1 | 4 | 26 | 40 | 40 | 40 | 70 | Normal | erl/v2J (codominant) |
| ahl/v2J × erl/erl F1 | 5 | 26 | 40 | 40 | 40 | 70 | Normal | erl/v2J (codominant) |
| ahl/v2J × erl/erl F1 | 6 | 26 | 40 | 40 | 40 | 70 | Normal | erl/v2J (codominant) |
| ahl/v2J × erl/erl F1 | 7 | 26 | 40 | 40 | 40 | 70 | Normal | erl/v2J (codominant) |
| ahl/v2J × erl/erl F1 | 8 | 26 | 40 | 40 | 40 | 70 | Normal | erl/v2J (codominant) |
| C57BL6J/xv2J/ahl/xv2J | 9 | 26 | 100 | 100 | 100 | 100 | Normal | erl/V2j |
| C57BL6J/xv2J/ahl/erl | 10 | 26 | 40 | 50 | 20 | 50 | Normal | erl/erl |
| C57BL6J/xv2J/ahl/erl | 11 | 40 | 40 | 40 | 70 | 90 | Normal | erl/erl |
| v2J/xv2J × C57BL6J/ahl/ahl | 12 | 90 | 40 | 40 | 20 | 50 | Normal | ahl/v2J |

Abbreviations: ABR, auditory brainstem response; SPL, sound pressure level.
by analyzing the genotype of a marker (D10Mit194, Chr location: 46 MB) close to the Cdh23 gene (Chr location: 59 MB). A PCR assay on pooled genomic DNA from the 13 hearing-impaired F2 progeny showed a distinctive single PCR band for the B6 allele of D10Mit194, suggesting that erl might be another AHL-related allele of the Cdh23 gene on Chr 10 on which we previously worked 4,8,9,22,24,25 or another locus in the same region. Then individual DNA samples of the S1 F2 progeny were genotyped with respect to D10Mit194, D10Mit166 (Chr location: 6 MB) and D10Mit42 (Chr location: 82 MB). The results showed that the candidate interval was between D10Mit194 and D10Mit42 at 29 cM on chromosome 10, whereas the Cdh23 gene is located at 30.3 cM as shown in the Supplementary Table 1. These results prompted us to do a genetic complementation test with mice carrying the most severe Cdh23 allele, waltzer 2 Jackson (abbreviated v-2j). The results showed that Cdh23v designation is C57BL/6J-Cdh23/v-2J allele, waltzer 2 Jackson (abbreviated v-2J allele, waltzer 2 Jackson (abbreviated v-2J allele, waltzer 2 Jackson (abbreviated v-2J allele, waltzer 2 Jackson (abbreviated v-2J). The three mice gave a clear single band (of the predicted size and identical to a normal control, data not shown) on agarose gel electrophoresis suggesting that no aberrant splicing is caused by this mutation. The erl mutation did not quantitatively alter the expression level of Cdh23 mRNA (Supplementary Figure 1a). The mutation results in an amino-acid substitution (aa 70S–P) in the middle of a regular secondary structure element—proline backbone dihedral angle at ~75°, giving proline an exceptional conformational rigidity compared with other amino acids. Proline functions as a structural disruptor in the middle of a regular secondary structure element— α-helix (aa 73–76)—and leads to changes in the surrounding extended strands (specifically, an extended strand formed by...

Identification of a novel mutation in the Cdh23 gene of the hearing-impaired mutant mouse

Genomic DNA screening to identify the alteration in the Cdh23 gene was performed using PCR and sequencing of all exons of the Cdh23 gene with detailed comparisons between mutants and controls using Sequencher 4.0 (http://genecodes.com; Gene Codes Corporation, Ann Arbor, MI, USA). A point mutation (208T>C) in the middle region of exon 3 (genbank accession number: NM_023370) was identified by sequencing the PCR products of primer pair ex3-F1 and ex3-R1 (for three mutant and two control genomics DNA samples, see Table 2 for primer sequences) and confirmed by sequencing the cDNA of this gene (Figure 1a). No other mutations were found in any other exons of this entire gene. The reverse transcription PCR products with primers flanking exon 3 and adjacent exons gave a clear single band (of the predicted size and identical to a normal control, data not shown) on agarose gel electrophoresis suggesting that no aberrant splicing is caused by this mutation. The erl mutation did not quantitatively alter the expression level of Cdh23 mRNA (Supplementary Figure 1a). The mutation results in an amino-acid substitution (aa 70S–P) in the middle of a regular secondary structure element—proline backbone dihedral angle at ~75°, giving proline an exceptional conformational rigidity compared with other amino acids. Proline functions as a structural disruptor in the middle of a regular secondary structure element— α-helix (aa 73–76)—and leads to changes in the surrounding extended strands (specifically, an extended strand formed by...
aa 65–68 in the wild-type mice shifts to that formed by aa 65–67 in the mutant, and another one formed by aa 77–80 in the wild-type mice to that formed by aa 76–80 in the mutant mice) affecting the secondary structure of the protein when analyzed by the GOR IV secondary structure prediction method. The substituted amino acid is not within, but nearby a conserved calcium-binding site (aa 59–61, sequence DMD, highlighted in Figure 1c). Loss of the α-helix and changes in the extended strands will probably affect protein function, such as formation of tip-links in the stereocilia or binding efficiency to calcium ions. On the basis of the mutation, a genotyping method was established to distinguish mice carrying the Cdh23erl allele from mice carrying the Cdh23ahl (C57BL/6J) allele by size differences on an agarose gel of PCR products digested with BstN I (Figure 1b). This confirms in another way that the erl mutation was accurately identified. By sequencing, we confirmed that all of the erl mice carry the ahl allele, thus this allele causes in-frame skipping of exon 7 and may contribute to conformational changes of CDH23.4

DPOAE and ABR testing reveal early onset, progressive hearing loss in Cdh23erl/erl mice
DPOAEs were measured to determine the mutant effect on OHC function over time in Cdh23erl/erl mice as compared with Cdh23ahl/ahl controls (Figure 2a). Three-week-old Cdh23erl/erl mice had normal DPOAE amplitudes that were indistinguishable from those of Cdh23ahl/ahl mice, which have normal hearing as determined by ABR thresholds at 3 weeks. At 7 weeks of age, Cdh23erl/erl mice had DPOAE amplitudes 10–20 dB lower than those of Cdh23ahl/ahl at frequencies from 10 to 18 kHz. By 22 weeks of age, Cdh23erl/erl mice had no detectable DPOAE at any frequency indicating complete deafness.

ABR recordings yielded normal wave patterns (data not shown), but elevated thresholds at every frequency tested (click, 8, 16, 32 kHz from 30–99 dB, Figure 2b and Table 1) with high frequencies affected first, as early as P40, progressing to deafness at P90 and thereafter remaining deaf to at least 9 months of age. The Cdh23erl/erl mice show hearing loss much earlier (P40) than do mice expressing other Cdh23 alleles such as Cdh23ahl/ahl mice (12 months onset, Figure 2b and Table 1). The vestibular system in these mutant mice seems to be unaffected by the erl mutation as Cdh23erl/erl mice showed normal swimming ability (3 Cdh23erl/erl mice at 4 months of age had similar normal swimming as B6 mice) and gait pattern as observed for all time points examined up to 9 months.

Histological examination reveals progressive OHC loss that follows hearing loss in Cdh23erl/erl mice
To investigate cochlear pathology in Cdh23erl/erl versus Cdh23ahl/ahl control mice, cochleae were stained for F-actin with Alexa Fluor 568 conjugated to phalloidin to examine all turns and reveal the presence and morphology of OHCs (Figure 2c). The OHCs of Cdh23ahl/ahl mice at P100 (Figure 2c, upper panel) showed normal V-shaped arrangement of stereocilia. No OHC loss was observed in any of the
cochlear turns of Cdh23ahl/ahl mice, as correlates with normal ABR thresholds up to 9 months (Figure 2b). In contrast, large segments of OHC loss were evident in the middle cochlear turns of Cdh23erl/erl mice at P100 (Figure 2c, lower panel). This OHC loss at P100 follows ABR threshold shifts observed at P27–P90 (Figure 2b).

To quantify OHC loss and to determine the developmental time course of OHC loss, we generated cytocochleograms from 25 Cdh23erl/erl mice ranging from 1 to 9 months of age. For each cytocochleogram, we calculated the percentage of missing OHC and inner hair cell (IHC) by subdividing the cochlea into 10 regions at 10% distance intervals from the apex of the cochlea. Scatter plots were constructed showing the mean percentage of OHC loss as a function of age for Cdh23erl/erl mice (Figure 2d). Cdh23erl/erl mice showed no evidence of OHC loss from P0 to P28. However, OHC loss was observable at 3 months, became progressively worse over time, and moved from the cochlear base to the apex in Cdh23erl/erl mice. OHC loss in the 75–100% region increased to a nearly complete loss after 3 months of age, corresponding to a high frequency hearing loss (16 and 32 kHz, top two lines in Figure 2b) earlier than that of low frequency hearing loss (8 kHz, bottom line, Figure 2b) as measured by ABR. Because IHC loss was not significant until after 5 months of age, IHCs were excluded from this analysis.

mRNA accumulation and activity of apoptosis-related genes was upregulated in the inner ears of Cdh23erl/erl mice

To determine whether OHC loss might be the result of OHC apoptosis, we examined expression of apoptosis-related genes in Cdh23erl/erl mice. Gene transcription levels of apoptosis-related genes were measured in the inner ears of Cdh23erl/erl and control Cdh23ahl/ahl mice at 2 weeks and 2 months of age (Figure 3). The extrinsic caspase-8 and intrinsic caspase-9 initiators, and activator caspase-3 were significantly upregulated (P < 0.05 for each) in Cdh23erl/erl samples relative to the levels measured in control
Cdh23ahl/ahl samples (Figures 3a and b) though the intensity for caspase-8 was higher and earlier than that of caspase-9, suggesting that the extrinsic pathway was initiated earlier than the intrinsic. At the 2-month time point, the expression level of caspase-9 was also upregulated \((P < 0.05)\) in Cdh23erl/erl samples. However, there was no significant difference in mRNA accumulation levels of caspase-3, caspase-8 and caspase-9 genes in brain tissue between Cdh23erl/erl and Cdh23ahl/ahl mice at the ages of 2 weeks or 2 months \((P > 0.05, \text{data not shown})\), indicating that the apoptotic gene expression measured in ear tissue was not a generalized phenomenon.

To confirm whether increased mRNA levels were correlated with changes at the protein level, the Apo-ONE homogenous caspase-3/7 assay (Promega) was used to measure caspase-3 and -7 activities in inner ears of erl mutants and control mice. The relative fluorescence unit is a measure of caspase activity in the homogenized inner ear tissue from the mice in each group, on addition of a caspase substrate to the homogenate. At P14 (2 weeks) and P60 (2 months), the relative caspase-3/7 activity was significantly higher in Cdh23erl/erl mice than in Cdh23ahl/ahl mice (Figures 3c and d).

Immunostaining reveals signs of apoptosis early, increasing toward the 3-month time point in the organ of Corti of Cdh23erl/erl mice. To investigate inner ear apoptosis in situ in the Cdh23erl/erl mouse model and determine whether the observed OHC loss can be explained by apoptosis of the OHCs, we stained tissue sections with fluorescein-labeled antibodies to detect cells that express caspases. The antibodies used specifically detect only the active forms of caspase-3, caspase-8 and caspase-9. Cdh23ahl/ahl mice were used as controls. We investigated three time points leading up to the time of complete hearing loss at 3 months, and beginning at the P14 time point (Figure 4). At P14, caspase-8 and caspase-9 were expressed in the middle turns of cochleae (mainly in the OHCs and SG cells for caspase-8 and caspase-9) of the Cdh23erl/erl mice (Figures 4d and e) compared with the same areas of the Cdh23ahl/ahl mice (Figures 4a and b). Primary antibody-omitted sham sections of the cochleae of both mouse groups showed that this staining was specific and not an artifact of nonspecific secondary antibody staining (Figures 4c and f). Notably, caspase-8 stained OHC stronger than SG (Figure 4d), whereas caspase-9 stained OHC weaker than SG (Figure 4e).

A time course observation of caspase-3 staining in the middle turns of cochleae of Cdh23erl/erl mice is shown in Figure 5. Anti-caspase-3 (the activated, downstream and effector caspase) staining was not visible in any areas of the cochleae at P14 (Figure 5a); however, it was evident in the cytoplasm of OHCs of Cdh23erl/erl mice by P23 (Figure 5b) and became stronger by P57 (Figure 5c). Active caspase-3 was also positive in the spiral ligament at both time points at P57. An enlarged image of OHCs from Figure 5c showed the overlapping staining (Figure 5f) of PI-stained nuclei (Figure 5e) and anti-caspase-3-stained cytoplasm (Figure 5d). Anti-caspase-3 signals were not detected in any cochlear turns from Cdh23ahl/ahl mice.
Upregulation of gene expression in the extrinsic (transforming growth factor (TNF)-α) and intrinsic (calpain) apoptosis pathways in Cdh23erl/erl mice

The relative mRNA levels of TNF-α, caspase-12, m-calpain and μ-calpain were significantly upregulated in the Cdh23erl/erl mice at both time points of 2 weeks (Supplementary Figure 1a, c) and 2 months (Supplementary Figure 1b, d), indicating multiple apoptosis pathways are involved in the hearing loss of these mutant mice. But TNF-α (Supplementary Figure 1a) was upregulated at the younger...
age (2 weeks) more significantly than other genes (Supplementary Figure 1c), suggesting the extrinsic pathway may have an important role for initiation of apoptosis. The fact that caspase-8 was upregulated at the younger age (2 weeks; Figure 3a) more significantly ($P < 0.01$) than other genes in the Cdh23erl/erl mice also supports this point, as TNF-$\alpha$ and caspase-8 are both important indicators of the extrinsic apoptotic pathway. Calpains, caspase-12 (Supplementary Figure 1b, d, $P < 0.05$) and caspase-9 (Figure 3b, $P < 0.05$) were also upregulated slightly, but significantly, as indicators of intrinsic apoptosis.

OHC protection in Cdh23erl/erl mice
Cdh23erl/erl was used as a model for testing anti-apoptotic drug therapy. In all, 60 Cdh23erl/erl mice were divided into a test group, a diluent (DMSO) control group and an untreated control group. Each mouse in the test group received Z-VAD-FMK (in DMSO, 1:1 diluted with PBS) intraperitoneally at a dosage of 1.5 $\mu$g g$^{-1}$ body weight at different time points: first, eight injections, on a schedule of one injection every other day, beginning at age P7; then four injections, on a schedule of one injection every 3 days; and finally, one injection every 4 days until the time of euthanization for analysis at various time points. Each mouse in the DMSO group received an equivalent volume of DMSO following an identical injection schedule to its cohort test group. ABR and DPOAE were tested at 4, 6, 8 and 12 weeks of age. ABR thresholds were measured at each time point (indicating mouse age) on the $x$ axis (Figure 6). ABR thresholds were measured at the frequencies indicated on the $y$ axis of each plot. Each point represents the mean ABR threshold for a group, with error bars indicating s.e. from the mean. The results show that ABR thresholds were significantly lower in the DMSO + Z-VAD-FMK-treated mice than those of the DMSO-treated or the untreated mice at stimulus frequencies of click, 8, 16 and 32 kHz at all time points ($*P < 0.05$ at 4 weeks; $**P < 0.01$, respectively for 6, 8 and 12 weeks). There were also significant differences for the ABR thresholds between the untreated and DMSO-treated mice at 12 weeks at the stimulus frequency of 16 kHz ($P < 0.05$).

![Figure 6](image)

**Figure 6** ABR thresholds to evaluate hearing in Cdh23erl/erl mice with no treatment or after treatment with diluents of DMSO or DMSO + Z-VAD-FMK, over a period of 11 weeks. Treatments were initiated at mouse age P7 (1 week). The number of mice tested was 14, 14 and 19 at 4 weeks; 10, 11 and 14 at 6 weeks; 10, 7 and 9 at 8 weeks; and 6, 4 and 6 at 12 weeks, for no treatment, DMSO and DMSO + Z-VAD-FMK groups, respectively. ABR thresholds were measured at each time point (indicating mouse age) on the $x$ axis. (a–d) ABR thresholds were measured at the frequencies indicated on the $y$ axis of each plot. Each point represents the mean ABR threshold for a group, with error bars indicating s.e. from the mean. The results show that ABR thresholds were significantly lower in the DMSO + Z-VAD-FMK-treated mice than those of the DMSO-treated or the untreated mice at stimulus frequencies of click, 8, 16 and 32 kHz at all time points ($*P < 0.05$ at 4 weeks; $**P < 0.01$, respectively for 6, 8 and 12 weeks). There were also significant differences for the ABR thresholds between the untreated and DMSO-treated mice at 12 weeks at the stimulus frequency of 16 kHz ($P < 0.05$).
in the DMSO-treated mice than in the untreated mice at the basal turn \((P < 0.05)\). IHC loss was not substantially altered by the treatment. In addition, we have not observed any adverse effects of Z-VAD-FMK treatment (such as tumor development in the intestine, colon, liver or lungs or at the intraperitoneal injection site) on the treated mice during the experimental period up to 3–4 months of ages (data not shown).

**Z-VAD-FMK treatment prevents OHC loss in Cdh23\(^{v/v}^{2J}\) mice**

As represented in Supplementary Figure 2, serial sections showed that Z-VAD-FMK treatment could protect OHCs from loss in Cdh23\(^{v/v}^{2J}\) mice, but failed to improve the hearing of the mice at any level. The survival of inner ear hair cells may still be important to release neurotrophic factors for keeping SG neurons viable and for responding to electrical stimuli in cochlear-implanted Usher syndrome type 1 subtype D patients, despite the inability of rescued hair cells to function in hearing. Thus, anti-apoptosis treatment in Usher syndrome type 1 subtype D patients might be useful in the future, but will require further studies.

**Discussion**

The Cdh23\(^{erl/erl}\) mice introduced in this study are of the C57BL/6J background and proved to be carrying a new allele of Cdh23. Cdh23\(^{erl/erl}\) is characterized by progressive hearing loss starting about 1 month after birth and becoming deaf at 3 months of age. Hair cell loss began near the base of the cochlea and spread toward the apical turn, and lesions of the SG were also involved at a later stage (P149, data not shown). The Cdh23\(^{erl/erl}\) mouse, therefore, provides a promising model for investigating the pathogenesis of DNF12. It is also an ideal mouse model for otoprotection drug therapy and discovery.

**Figure 7** A time course observation in the three treatment-based Cdh23\(^{erl/erl}\) mouse groups of DPOAEs to stimulus frequency of 16 kHz (f2) at 4 weeks (weeks), 6, 8 and 12 weeks. DPOAE amplitudes (dB SPL) gradually decreased with time in the untreated group (\(n = 8, 10, 10\) and 8, respectively, at each time point), the DMSO-treated group (\(n = 11, 7, 7, 7\) and 6) and DMSO + Z-VAD-FMK-treated group (\(n = 14, 9, 9\) and 6). The mean amplitude was significantly higher in the DMSO + Z-VAD-FMK-treated mice than in the untreated (\(P = 0.03294\)) or DMSO-treated (\(P = 0.03955\)) group at the age of 8 weeks. There were no significant differences for the DPOAE amplitudes between the untreated group and DMSO-treated group at any time points. *\(P < 0.05\). Error bars indicate s.e. from the mean.

**Figure 9** Hair cell losses in the cochlea of the Z-VAD-FMK-treated Cdh23\(^{erl/erl}\) mice at the age of 2 months. Surface preparations of four cochleae from four mice in each mouse group were examined. The mean percentages of outer hair cell (OHC) loss in the apex, middle and basal regions of the cochleae of the Z-VAD-FMK-treated mice (black bars) were significantly lower than that of the untreated (white bars) and DMSO-treated (gray bars) mice. There were also significant differences in OHC loss between the untreated and DMSO-treated mice at the basal regions (\(P < 0.05\)). No significant difference in inner hair cell loss occurred between the three mouse groups. *\(P < 0.05\); **\(P < 0.01\). Error bars represent s.e. from the mean.
Given that the hearing loss in Cdh23<sup>erl<sup>erl</sup> mice happens before hair cell loss (P60 see Figure 2d), but after caspase signals increase (P14), we hypothesized that apoptosis might be the mechanism for causing a physiological deficit to occur before hair cell loss. There are mainly two pathways of cellular apoptosis as shown in Figure 10. In response to extra-cellular stimuli, the initiator of the extrinsic pathway (caspase-8) undergoes self-processing, releasing active enzyme molecules into the cytosol and activating caspase-3, caspase-6 and caspase-7. These activated caspases proteolytically cleave and activate other caspases, as well as relevant targets in cells. Alternatively, the intrinsic pathway involves release from the mitochondrion of proapoptotic proteins that activate caspase enzymes, mainly caspase-9, which ultimately trigger apoptosis by activating caspase-3, caspase-6, and caspase-7. Therefore, we attempted to determine the role of apoptosis in the pathogenesis of the Cdh23<sup>erl<sup>erl</sup> mouse model by examining expression of apoptosis-related proteins in the inner ears of these mice. Our results showed significantly upregulated transcriptional levels of caspase-3, caspase-8 and caspase-9 in the inner ears of Cdh23<sup>erl<sup>erl</sup> mice at 2 weeks and/or 2 months of age, indicating both extrinsic and intrinsic pathway activation. Though the extrinsic pathway was activated slightly earlier and more strongly than the intrinsic pathway, this presumably was the result of increased TNF-α signalling triggered by debris from broken stereocilia composed of misfolded CDH23 erl<sup>erl</sup> protein, because of the ϕ backbone dihedral angle (−75°) caused by the proline from the amino-acid substitution (70S–P). A detailed mechanistic explanation of why mutated CDH23 increases TNF-α signalling is yet to be determined. Another possibility is that the 70S–P introduced conformational changes in the first ectodomain, ultimately leading to intracellular domain and catenin complex signal changes that modulate apoptosis.

Immunostaining showed that caspase-8 was activated in the organ of Corti at P14, whereas caspase-3 was activated at P23 and that the staining for both became more intense at
P57. In addition, there were signs of apoptosis in the SG and the stria vascularis. In contrast to a previous report in rat of a strong active caspase-3 signal in the lateral wall of the cochlear basal turn with drug-induced damage,21 our mouse experiments showed that caspase immunoreactivities were first present and strong in the basal turn and middle turns of the organ of Corti. On the basis of these results, we conclude that apoptosis has a major role in Cdh23erl/erl mice in the death of hair cells, SG cells, cells of the spiral ligament and stria vascularis in the inner ear, and that the cell death mechanism of drug-induced damage in rat may differ from the mechanism in a genetic mutant such as the Cdh23erl/erl mouse. The upregulated apoptosis is probably localized to the inner ear in Cdh23erl/erl mice, as there was no significant difference in mRNA accumulation levels for caspase-3, caspase-8 or caspase-9 genes in brain tissue between Cdh23erl/erl and Cdh23blablhd mice. We acknowledge that other mechanisms may apply for possible causes of hearing loss in other mutations.

Cadherins are glycoproteins involved in Ca\(^{2+}\)-mediated cell–cell adhesion, migration and compaction.2 CDH23 is an important component of the Ush1 interactome and of the tip-links in the mechanotransduction (MET) system of the organ of Corti.15,31–33 It is involved in regulating the activity of mechanically gated ion channels in hair cells.3,34,35 It is reported that, of the 21 missense mutations in CDH23 (Figure 10). (1) The intricate relationship between CDH23 and other proteins of the Usher syndrome type complex (Figure 10). (2) We investigated Z-VAD-FMK as a candidate drug for maintenance of hearing and hair cell viability in Cdh23erl/erl mice, though the mechanism of cell death in Cdh23erl/erl mice may differ from that in drug-induced damage. After Z-VAD-FMK treatment of the Cdh23erl/erl mice, there was a significantly reduced amount of OHC loss compared with that of the untreated or DMSO-treated mice in all regions of the cochlea. The protective effect of Z-VAD-FMK on OHC was further confirmed by significantly higher DPOAE amplitudes in the DMSO + Z-VAD-FMK-treated mice than in the untreated or DMSO-treated mice at the age of 8 weeks. Furthermore, ABR thresholds were preserved at levels ~15–40 dB better in the Z-VAD-FMK-treated group than in the untreated or DMSO-treated groups of litter mate Cdh23erl/erl mice from 6 weeks to 12 weeks of age. The involvement of apoptosis in the pathology of Cdh23erl/erl inner ears was thus confirmed by anti-apoptotic drug therapy. Z-VAD-FMK can preserve cells (hair cells, for example) in the inner ears by stopping apoptosis. These results offer the potential that DNNB12 could be preventable in the future by anti-apoptotic intervention. However, the hearing loss of the Cdh23erl/erl mice could not be completely prevented by Z-VAD-FMK treatment in the time course observation, especially at 12 weeks of age, indicating that other factors related to alteration in CDH23 structure may also be involved in the pathology of these mouse inner ears. In addition, we noticed that DMSO has a minor, but slightly significant, rescue effect on hearing (Figures 6c and 9). This is consistent with another recent report.40

A mouse model of DFNB12 was reported recently, in which the mutation salsa was predicted to affect Ca\(^{2+}\) binding by altering the extracellular CDH23 domain, resulting in severe hearing loss at P21.15 The early onset and rapid progression of hearing loss in salsa mice may make it difficult to find a time period for drug intervention. Therefore, the Cdh23erl mouse model may provide a better
platform for further anti-apoptotic drug screening and testing.

In summary, a point mutation in the Cdh23 gene (208T>C) of C57BL/6J mice results in hearing loss around 1 month after birth. Given the strain background on which the erl mutation arose, the Cdh23<sup>erm</sup> mice also carries the ah1 mutation. Thus, the combination of mutations on the C57BL/6J background produces an accelerated hearing loss phenotype that is well suited for an experimental model. The erl mutation influences cell fate by increasing cellular apoptosis, which may be attenuated by anti-apoptotic drug therapy. These results may provide avenues for improving hearing in patients with DFNB12 by an anti-apoptosis approach in the near future. Anti-apoptosis drugs could be adapted for preventive pre-clinical application after detailed evaluation of the primary effects and the side effects under various treatment regimens in this mouse model. Because this is a preventive drug, we suggest that only after a patient is clearly genetically diagnosed as DFNB12 younger than a determined age of hearing loss should a patient be included for trial of this drug.

Conflict of interest

QYZ, FH and HY have filed a patent covering the general approach of Z-VAD-FMK as a therapeutic approach for genetic hearing loss and using the erl mouse model for evaluating otoprotective drugs.

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References

1 Di Palma F, Holme RH, Bryda EC, Belyantseva IA, Pellegrino R, Kachar B et al. Mutations in Cdh23, encoding a new type of cadherin, cause stereocilia disorganization in waltzer, the mouse model for Usher syndrome type 1D. Nat Genet 2001; 27: 103–107.
2 Di Palma F, Pellegrino R, Noben-Trauth K. Genetic structure, alternative splice forms and normal and mutant alleles of cadherin 23 (Cdh23). Gene 2001; 281: 31–41.
3 Siemens J, Lillo C, Dumont RA, Reynolds A, Williams DS, Gillespie PG et al. Cadherin 23 is a component of the tip link in hair-cell stereocilia. Nature 2004; 428: 950–955.
4 Noben-Trauth K, Zheng QY, Johnson KR. Association of cadherin 23 with polygenic inheritance and genetic modification of sensorineural hearing loss. Nat Genet 2003; 35: 21–23.
5 Bolz H, von Brederlow B, Ramirez A, Bryda EC, Kutsche K, Nothwang HG et al. Mutation of CDH23, encoding a new member of the cadherin gene family, causes Usher syndrome type 1D. Nat Genet 2001; 27: 108–112.
6 Bork JM, Peters LM, Riazuddin S, Bernstein SL, Ahmed ZM, Ness SL et al. Usher syndrome 1D and nonsyndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene CDH23. Am J Hum Genet 2001; 68: 26–37.
7 Astuto LM, Bork JM, Weston MD, Askew JW, Fields RR, Orten DJ et al. CDH23 mutation and phenotype heterogeneity: a profile of 107 diverse families with Usher syndrome and nonsyndromic deafness. Am J Hum Genet 2002; 71: 262–275.
8 Zheng QY, Johnson KR, Erway LC. Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. Hear Res 1999; 130: 94–107.
9 Johnson KR, Erway LC, Cook SA, Willott JF, Zheng QY. A major gene affecting age-related hearing loss in C57BL/6J mice. Hear Res 1997; 114: 83–92.
10 Shinerson A, Devigne C, Pujol R. Age-related changes in the C57BL/6J mouse cochlea. II. Ultrastructural findings. Brain Research 1981; 254: 77–88.
11 Hequembourg S, Liberman MC. Spiral ligament pathology: a major modifier of age-related cochlear degeneration in C57BL/6J mice. J Assoc Otolaryngol 2001; 2: 118–129.
12 Bryda EC, Ling H, Flaterty L. A high-resolution genetic map around waltzer on mouse chromosome 10 and identification of a new allele of waltzer. Mamm Genome 1997; 8: 1–4.
13 Wada T, Wakabayashi Y, Takahashi S, Ushiki T, Kikkawa Y, Yonekawa H et al. A point mutation in a cadherin gene, Cdh23, causes deafness in a novel mutant, Waltzer mouse niigata. Biochem Biophys Res Commun 2001; 283: 113–117.
14 Lefere G, Michel V, Weil D, Lepelletier L, Bizard E, Wolfrum U et al. A core cochlear phenotype in Ush1 mouse mutants implicates fibrous links of the hair bundle in its cohesion, orientation and differential growth. Development 2008; 135: 1427–1437.
15 Swander M, Xiong W, Tokita J, Lelli A, Elledge HM, Kazmierczak P et al. A mouse model for nonsyndromic deafness (DFNB12) links hearing loss to defects in tip links of mechanosensory hair cells. Proc Natl Acad Sci USA 2009; 106: 5252–5257.
16 Deol M. A gene for uncomplicated deafness in the mouse. J Embryol Exp Morphol 1956; 4: 190–195.
17 Johnson KR, Cagnon LH, Webb LS, Peters LL, Hawes NL, Chang B et al. Mouse models of Ush1C and DFNB18: phenotypic and molecular analyses of two new spontaneous mutations of the Ush1c gene. Hum Mol Genet 2003; 12: 3075–3086.
18 Taylor BA, Navin A, Phillips SJ. PCR-amplification of simple sequence repeat variants from pooled DNA samples for rapidly mapping new mutations of the mouse. Genomics 1994; 21: 626–632.
19 Polak M, Eshraghi AA, Nehme O, Ahsan S, Guzman J, Delgado RE et al. Evaluation of hearing and auditory nerve function by combining ABR, DPOAE and eABR tests into a single recording session. J Neurosci Methods 2004; 134: 141–149.
20 Zheng QY, Ding D, Yu H, Salvi RJ, Johnson KR. A locus on distal chromosome 10 (ah4) affecting age-related hearing loss in A/J mice. Neurobiol Aging 2009; 30: 1693–1705.
21 Mizutari K, Matsunaga T, Kamiya K, Fujinami Y, Fujii M, Ogawa K. Caspase inhibitor facilitates recovery of hearing by protecting the cochlear lateral wall from acute cochlear mitochondrial dysfunction. J Neurosci Res 2008; 86: 215–222.
22 Johnson KR, Zheng QY, Erway LC. A major gene affecting age-related hearing loss is common to at least ten inbred strains of mice. Genomics 2000; 70: 171–180.
23 Johnson KR, Zheng QY, Noben-Trauth K. Strain background effects and genetic modifiers of hearing in mice. Brain Res 2006; 1091: 79–88.
24 Zheng QY, Johnson KR. Hearing loss associated with the modifier of deaf waddler (mdfw) locus corresponds with age-related hearing loss in 12 inbred strains of mice. Hear Res 2001; 154: 45–53.
25 Zheng QY, Yan D, Ouyang XM, Du LL, Yu H, Chang B et al. Digenic inheritance of deafness caused by mutations in genes encoding cadherin 23 and protocadherin 15 in mice and humans. Hum Mol Genet 2005; 14: 103–111.
26 Garnier J, Gibb JF, Robson B. GOR method for predicting protein secondary structure from amino acid sequence. Methods Enzymol 1996; 266: 540–553.
27 Yin XM. Signal transduction mediated by Bid, a pro-death Bcl-2 family protein, connects the death receptor and mitochondria apoptosis pathways. Cell Res 2000; 10: 161–167.
28 Creagh EM, Conroy H, Martin SJ. Caspase-activation pathways in apoptosis and immunity. Immunological Reviews 2003; 193: 10–21.
29 George SJ, Beeching CA. Cadherin:catenin complex: a novel regulator of vascular smooth muscle cell behaviour. *Atherosclerosis* 2006; 188: 1–11.
30 Zhu W, Leber B, Andrews DW. Cytoplasmic O-glycosylation prevents cell surface transport of E-cadherin during apoptosis. *EMBO J* 2001; 20: 5999–6007.
31 Adato A, Michel V, Kikkawa Y, Reiners J, Alagramam KN, Weil D et al. Interactions in the network of Usher syndrome type 1 proteins. *Hum Mol Genet* 2005; 14: 347–356.
32 Kazmierczak P, Sakaguchi H, Tokita J, Wilson-Kubalek EM, Milligan RA, Muller U et al. Cadherin 23 and protocadherin 15 interact to form tip-link filaments in sensory hair cells. *Nature* 2007; 449: 87–91.
33 Yap AS, Briehler WM, Gumbiner BM. Molecular and functional analysis of cadherin-based adherens junctions. *Annu Rev Cell Dev Biol* 1997; 13: 119–146.
34 Howard J, Hudspeth AJ. Compliance of the hair bundle associated with gating of mechanoelectrical transduction channels in the bullfrog's saccular hair cell. *Neuron* 1988; 1: 189–199.
35 Jia S, Dallos P, He DZ. Mechanoelectric transduction of adult inner hair cells. *J Neurosci* 2007; 27: 1006–1014.
36 Chan DK, Lieberman DM, Musatov S, Goldfein JA, Selensnick SH, Kaplitt MG. Protection against cisplatin-induced ototoxicity by adeno-associated virus-mediated delivery of the X-linked inhibitor of apoptosis protein is not dependent on caspase inhibition. *Otol Neurotol* 2007; 28: 417–425.
37 de Brouwer AP, Pennings RJ, Roeters M, Van Hauwe P, Astuto LM, Hoefsloot LH et al. Mutations in the calcium-binding motifs of CDH23 and the 35delG mutation in GJB2 cause hearing loss in one family. *Hum Genet* 2003; 112: 156–163.
38 Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A, Chishti AH. Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol Cell Biol* 2001; 21: 2213–2220.
39 Erez N, Zamir E, Gour BJ, Blaschuk OW, Geiger B. Induction of apoptosis in cultured endothelial cells by a cadherin antagonist peptide: involvement of fibroblast growth factor receptor-mediated signalling. *Exp Cell Res* 2004; 294: 366–378.
40 Melki SJ, Heddon CM, Frankel JK, Levitt AH, Momin SR, O'Brien RG et al. Pharmacological protection of hearing loss in the mouse model of endolymphatic hydrops. *The Laryngoscope* 2010 (in press).
41 Chen M, Ona VO, Li M, Ferrante RJ, Fink KB, Zhu S et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat Med* 2000; 6: 797–801.
42 Zender L, Hutker S, Liedtke C, Tillmann HL, Zender S, Mundt B et al. Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc Natl Acad Sci USA* 2003; 100: 7797–7802.
43 Ehret G. Masked auditory thresholds, critical ratios, and scales of the basilar membrane of the housemouse (Mus musculus). *J Comp Physiol* 1975; 130: 329–341.

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