Molecular Pathogenesis of Genetic and Inherited Diseases

An ENU-Induced Mutation of *Cdh23* Causes Congenital Hearing Loss, but No Vestibular Dysfunction, in Mice

Shehnaaz S.M. Manji,* Kerry A. Miller,*
Louise H. Williams,* Lotte Andreassen,*
Maria Siboe,* Elizabeth Rose,[†] Melanie Bahlo,[‡]
Michael Kuiper,[§] and Hans-Henrik M. Dahl*[¶]

From the Genetic Hearing Research Laboratory,* Murdoch Childrens Research Institute, Royal Children's Hospital, Parkville; the Departments of Otolaryngology,[†] and Paediatrics,[‡] University of Melbourne, Parkville; the Bioinformatics Division,[‡] Walter & Eliza Hall Institute, Parkville; and Victorian Partnership Advanced Computing,[§] Carlton South, Victoria, Australia

Mutations in the human cadherin 23 (*CDH23*) gene cause deafness, neurosensory, autosomal recessive 12 (DFNB12) nonsyndromic hearing loss or Usher syndrome, type 1D (characterized by hearing impairment, vestibular dysfunction, and visual impairment). Reported *waltzer* mouse strains each harbor a *Cdh23*-null mutation and present with hearing loss and vestibular dysfunction. Two additional *Cdh23* mouse mutants, *salsa* and *erlong*, each carry a homozygous *Cdh23* missense mutation and have progressive hearing loss. We report the identification of a novel mouse strain, *jera*, with inherited hearing loss caused by an *N*-ethyl-*N*-nitrosourea-induced c.7079T>A mutation in the *Cdh23* gene. The mutation generates a missense change, p.V2360E, in *Cdh23*. Affected mice have profound sensorineural deafness, with no vestibular dysfunction. The p.V2360E mutation is semidominant because heterozygous mice have milder and more progressive hearing loss in advanced age. The mutation affects a highly conserved Ca²⁺-binding motif in extracellular domain 22, thought to be important for *Cdh23* structure and dimerization. Molecular modeling suggests that the *Cdh23*^{V2360E/V2360E} mutation alters the structural conformation of the protein and affects Ca²⁺-binding properties. Similar to *salsa* mice, but in contrast to *waltzer* mice, hair bundle development is normal in *jera* and hearing loss appears to be due to the loss of tip links. Thus, *jera* is a novel mouse model for DFNB12. (*Am J Pathol* 2011, 179:903–914; DOI: 10.1016/j.ajpath.2011.04.002)

Deafness is the most common sensory impairment in humans, often incurring lifelong educational, social developmental, and economic costs. Hearing loss is a multifactorial disorder, caused by environmental or genetic factors or a combination of both. It is estimated that there are 100 mapped loci that may cause monogenic nonsyndromic hearing loss and >400 syndromes that include hearing as a feature.¹ Nevertheless, when investigating a specific family affected by inherited hearing loss, the condition is usually transmitted as a monogenic disorder. It has become clear that different mutations in a gene can cause a variety of genetic and clinical features (eg, dominantly or recessively inherited hearing loss or syndromic or nonsyndromic hearing loss).

In humans, mutations in the cadherin 23 (*CDH23*) gene cause autosomal recessively inherited nonsyndromic congenital deafness (DFNB12; Online Mendelian Inheritance of Man no. 601386; <http://www.ncbi.nlm.nih.gov/omim>, last accessed March 29, 2011) or Usher syndrome, type 1D (USH1D; Online Mendelian Inheritance of Man no. 601067; <http://www.ncbi.nlm.nih.gov/omim>, last accessed March 29, 2011). USH1 is characterized by congenital hearing impairment, vestibular dysfunction, and visual impairment. DFNB12 deafness is usually caused by missense mutations in *CDH23* alleles and USH1D by null mutations.^{2–7} A synonymous single-nucleotide polymorphism, *Cdh23*^{753A}, is linked to age-related hearing loss in mice.⁸ Null mutations in mouse *Cdh23* lead to the *waltzer* phenotype, characterized by hearing loss and vestibular dysfunction.^{9–13} Two *N*-ethyl-*N*-nitrosourea (ENU)-generated mouse strains, *salsa* and *erlong*, carry a recessive *Cdh23* missense mutation.^{14,15} Both mutants have progressive hearing loss, with no vestibular abnormalities, and are, therefore, models for DFNB12 deafness. Retinal degeneration is not a clinical

Supported by grant 436944 from the National Health and Medical Research Council, the HEARing CRC, and J. & J. Calvert-Jones.

Accepted for publication April 25, 2011.

Address reprint requests to Shehnaaz S.M. Manji, Ph.D., Genetic Hearing Research, Murdoch Childrens Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Melbourne, VIC 3052, Australia. E-mail: shehnaaz.manji@mcri.edu.au.

Table 1. Allelic Variants of *Cdh23* in Mice

Mouse model	Nucleotide change	Amino acid change	Exon	EC domain	Phenotype	Predicted effect	Reference
<i>jera</i>	7486T>A	V2360E	52	EC22	DFNB12	Disruption of the Ca ²⁺ -binding motif	Present study
<i>erlong</i>	208T>C	S70P	3	EC1	DFNB12	Loss of protein function	15
<i>salsa</i>	2210A>T	E737V	22	EC7	DFNB12	Disruption of the Ca ²⁺ -binding motif	14
<i>Cdh23</i> ^V	834insG	N279EfsX39	9	EC3	USH1D	Premature stop codon	13
<i>Cdh23</i> ^{VJ}	3505delG	E1169NfsX7	29	EC11	USH1D	Premature stop codon	13
<i>Cdh23</i> ^{V-2J}	4104+1G>A		31	EC13	USH1D	Splice-site mutation	9
<i>Cdh23</i> ^{V-3J}	5291G>A	W1764X	40	EC17	USH1D	Loss of functional domains	10
<i>Cdh23</i> ^{V-4J}	8152del9	N2718del3	56	EC25	USH1D	Disruption of the Ca ²⁺ -binding motif	10
<i>Cdh23</i> ^{V-5J}	8803C>T	R2935X	60	EC27	USH1D	Loss of functional domains	10
<i>Cdh23</i> ^{V-6J}	904G>T	E302X	9	EC3	USH1D	Loss of functional domains	9
<i>Cdh23</i> ^{V-7J}	3589delT	Y1197MfsX47	30	EC11	USH1D	Premature stop codon	9
<i>Cdh23</i> ^{V-ngt}	145delG	G49VfsX3	2	EC1	USH1D	Premature stop codon	12
<i>Cdh23</i> ^{V-Alb}	1635C>Tdel119	G545G	15	EC5	USH1D	Generates ectopic splice site	9
<i>Cdh23</i> ^{Vbus}	9633+1G>A		67	CYTO	USH1D	Splice-site mutation	11
<i>Cdh23</i> ^{Ahl}	753G>A	P251P	7	EC3	AHL	In-frame skipping of exon 7	8

feature in any of the mice with *Cdh23* mutations. Previously reported allelic variants of *Cdh23* in mice are detailed in Table 1.

Auditory hair cells, located within the organ of Corti, have a bundle of stereocilia with a central kinocilium, located at their apical surface. These structures act as mechanoelectrical transduction sensors and are, therefore, essential for normal hearing. Stereocilia and kinocilium are connected via transient lateral links, kinociliary links, and tip links in immature cochlear hair cells (Figure 1). *Cdh23*, together with protocadherin 15 (*Pcdh15*), is a major component of these links.^{16–18} The tip links are connected to transduction channels on the stereocilia, in which the movement of stereocilia causes the rigid tip links to pull open the transduction channels and thereby initiate mechanotransduction.^{19,20} Therefore, *Cdh23* is thought to be directly involved in this process. Also,

Figure 1. Schematic diagram of stereocilia and kinocilium location in an immature (<P8) cochlear hair cell and the position of associated tip links formed by the interaction of *Cdh23* (red) and *Pcdh15* (green).

Cdh23 may be involved in hair bundle development, as this process is defective in *waltzer* mice with *Cdh23*-null mutations.^{9,12,13,21–27} *salsa* mice confirmed the role of *Cdh23* in mechanotransduction, as hair cells and hair bundles develop normally, but the function of these cells was affected.¹⁴ A recent report by Lelli et al²⁸ provided physiological evidence to support the involvement of CDH23 and PCDH15 *N*-terminal regions in tip link formation. The *Cdh23* gene codes for several different splice isoforms.¹⁰ The longest isoform of *Cdh23* in mouse comprises 71 exons and codes for a 3354–amino acid protein. It is expressed in the mouse inner ear but not in the mouse retina.²⁹ Cadherins are adhesion molecules that mediate Ca²⁺-dependent cell-cell adhesion via extracellular (EC) domains. *Cdh23* contains 27 EC domains; and each EC domain consists of three Ca²⁺-binding motifs (consensus sequences DXD, LDRE, and DXNDN) that are essential for dimerization, linearization, and rigidification.¹⁰ Different EC domains are affected in *waltzer*, *salsa*, and *erlong* mice. We have screened ENU mouse mutant libraries for recessively inherited hearing loss; in this study, we describe a novel mouse strain, *jera*, with a missense mutation in *Cdh23*. The hearing loss in *jera* is more severe than that in *salsa* and *erlong*. Similar to *salsa* and *erlong*, the development of hair bundles appears normal in *jera*; therefore, it is a new model for DFNB12.

Materials and Methods

jera Mouse

This project was approved by the Royal Children's Hospital Animal Ethics Committee (application numbers A488 and 585). The *jera* mouse was generated by ENU mutagenesis at the Australian Phenomics Facility, Can-

berra, in a program aimed at identifying recessive conditions. Male C57BL/6 mice were treated weekly for 3 weeks with 100 mg/kg ENU. To identify recessive phenotypes, G_2 siblings were mated to generate G_3 offspring homozygous for ENU mutations. We screened 5750 ENU C57BL/6 mutant G_3 mice for hearing loss. The initial click box hearing test elicits a Preyer reflex or a startle response in hearing mice by producing an 18.9-kHz burst of 106-dB sound pressure level (SPL) at a distance of 10 cm (Institute of Hearing Research, Nottingham, UK). Mice that failed the initial click box hearing test were subsequently tested by an evoked auditory brainstem response (ABR) (Bio-logic Systems Corp, Chicago, IL). Subdermal active, reference, and ground electrodes were placed at the vertex, ventrolateral to the left ear, and the abdomen, respectively, of the anesthetized mouse. A specific auditory stimulus in the form of broadband clicks was delivered in a range of decibel SPLs and the ABR was recorded. Mice were also screened for the presence of vestibular dysfunction that was identified by the display of hyperactivity that manifests as circling, head tossing/tilting, and/or star-gazing behavior.

Heritability and Genetic Mapping

Mice that failed both the click box and ABR hearing test were intercrossed to the congenic strain C57BL/10 for heritability testing. We expected 25% of the G_2 offspring to be deaf if the mutation was recessively inherited and fully penetrant. Deaf mice were then outcrossed to the CBA/H strain, and the F_2 progeny were generated for homozygosity mapping and subsequent identification of candidate regions. Genomic DNA samples from 10 affected mice were analyzed by genomewide scans using 120 microsatellite markers (AGRF, Melbourne, Australia). Fine mapping was performed using Amplifluor-based single-nucleotide polymorphism assays (Australian Phenomics Facility) on 53 additional affected mice. For our statistical analysis, we used the normal approximation to the binomial test for proportions of homozygous C57BL/6 genotypes (hearing loss mutants) to map the deafness loci. In chromosome regions of linkage, the proportion of homozygous C57BL/6 genotypes would approach 1.0; at unlinked loci, this proportion would be 0.25. Linkage intervals were examined for candidate genes using the UCSC genome bioinformatics (<http://genome.ucsc.edu>).

Mutation Analysis

To scan for mutations in the *Cdh23* gene (transcript reference, ENSMUST00000105464; protein reference, ENSMUSP00000101104), 1 μ g of total RNA from mouse brain was reverse transcribed using random hexamers (Roche, Penzberg, Germany). cDNA products were PCR amplified using HotStar Taq polymerase (Qiagen, Hilden, Germany) with 16 primer sets, generating overlapping amplicons that covered the 10,572-bp *Cdh23* RNA coding region transcript and most of the 5' and 3' untranslated regions. The PCR products were sequenced in both directions using a Big Dye Sequencing kit, version 3.1 (ABI, Carlsbad, CA) and analyzed

on an Applied Biosystems DNA Analyzer (model 3730xl; ABI). DNA sequences from deaf and hearing littermates were compared using Mutation Surveyor version 2.60 (SoftGenetics, State College, PA).

Tissue Collection

Mice [postnatal day (P) 14 and older] were anesthetized with isoflurane and euthanized by cervical dislocation, whereas mice younger than P14 were euthanized by tribromoethanol (Avertin) overdose, according to the National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes (Royal Children's Hospital Animal Ethics Committee approval A488 and 585). Intact cochleae were surgically removed using a posterolateral approach, and the brain was dissected from mice of the same strain, age, and sex. Brains were stored in RNAlater buffer (Qiagen GmbH, Hilden, Germany) at -70°C for total RNA isolation. Adult mouse cochleae were prepared as described by Whitlon et al³⁰ and processed for H&E staining. P5 mouse half heads were fixed in 4% paraformaldehyde at 4°C for 2 hours, followed by PBS washes three times for 20 minutes, before isolation of the cochlear sensory epithelium (SE) for whole-mount immunofluorescence analysis. The P14 to P30 half heads were fixed in 4% paraformaldehyde at room temperature for 2 hours before PBS washes and fine dissection.

H&E Staining

Processed cochleae were embedded in OCT (Sakura Finetek USA, Inc., Torrance, CA) and sectioned (10 μ m) using a cryostat (Leica Microsystems, Wetzlar, Germany) onto SuperfrostPlus microscope slides (Gerhard Menzel GmbH, Braunschweig, Germany) and stored at -20°C until use. For H&E staining, cochlear sections were processed through H&E staining, coverslipped with Pertex mounting media, and visualized using a Leica DMI3000 microscope (Leica Microsystems).

Immunohistochemical Staining for Cdh23 Protein

Cochlear SE isolated from P5 to P30 mice were processed for immunohistochemical staining using either a cadherin 23 antibody (provided by Prof. Thomas Friedman and Dr. Ayala Lagziel, National Institute on Deafness and Other Communication Disorders, Bethesda, MD)²⁹ or a polyclonal rabbit anti-human Cdh23 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Briefly, SEs were rinsed three times in PBS and then permeabilized for 30 minutes with 0.3% to 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS. SEs were then blocked in 5% to 10% goat serum (with or without 2% bovine serum albumin) in PBS for 1 to 2 hours. Isolated SEs were incubated with an anti-Cdh23 antibody [1:250 (antibody from National Institute on Deafness and Other Communication Disorders) or 1:500 (antibody from Santa Cruz Biotechnology, Inc.), respectively] overnight at 4°C . SEs

were rinsed three times in PBS with 10- to 20-minute intervals. This was followed by incubation with an Alexa Fluor 488- or an Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (Invitrogen-Molecular Probes, Eugene, OR) in 1% to 10% goat serum in PBS (1:2500) at room temperature for 2 hours and washes three times with PBS. SEs were counterstained with Alexa Fluor 594- or Alexa Fluor 488-phalloidin (1:250; Molecular Probes) in PBS for 20 minutes, then washed a further three times with PBS for 10 minutes. SEs were incubated with a rabbit IgG antibody (Invitrogen Corp., Carlsbad, CA) as a control. SEs were positioned on slides, mounted with Prolong Gold Antifade reagent with DAPI (Molecular Probes), and viewed using a Carl Zeiss Imager.M1 (MTB2004 AXIO; Carl Zeiss MicroImaging GmbH, Gottingen, Germany) or a Leica TCS SP2 laser scanning confocal microscope. Images were generated using AxioVision Rel. 4.7 or Leica Confocal Software (Leica Microsystems).

Scanning Electron Microscopy

Inner ears from P14, P30, and 13-week-old mice were isolated; the round and oval windows were cleared; and the cochlear apex was pierced to allow thorough perfusion of the fixative. Inner ears were fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) with 3 mmol/L CaCl_2 for 3 hours at room temperature. Samples were then washed in PBS and further dissected by removing the bony shell, stria vascularis, and Reissner's and tectorial membranes to reveal the cochlear SE. Cochlear specimens were incubated in osmium tetroxide for 2.5 hours, washed, dehydrated through an ethanol gradient, and then critical point dried (CPD030; Bal-Tec GmbH, Schalksmuhle, Germany). Specimens were mounted on stubs using a conductive silver paint, gold sputter coated (Edwards S150B sputter coater, approximately 10 nm; Edwards High Vacuum Inc., Crawley, West Sussex, UK), and viewed using a Philips XL30 FE scanning electron microscope (Philips Electron Optics, Eindhoven, the Netherlands).

Molecular Modeling of Cdh23 Mutations

The mouse E-cadherin 1EDH crystal structure³¹ was used to model the effect of the Cdh23 p.V2360E mutation on the structure and function of the protein. Both wild-type and mutant models were simulated for 4 nanoseconds of fully solvated molecular dynamics at 310 K (36.85°C) using the NAMD software package (<http://www.ks.uiuc.edu/Research/namd>).³² Simulations were run with the calcium ions in the crystallographically identified binding sites and with the calcium ions moved away from the binding sites. Molecular modeling images were generated using the VMD software package (<http://www.ks.uiuc.edu/Research/vmd>).³³

Figure 2. **A:** Chromatograms of wild-type (top) and *jera* (bottom) mice. A T>A transversion of nucleotide 7079, localized in the EC22 domain, is shown in the *Cdh23*^{V2360E/V2360E} mutant, resulting in amino acid 2360 changing from a valine (V) to a glutamic acid (E). **B:** The mutation is localized in a highly conserved LDRE Ca^{2+} -binding domain (red).

Results

jera Mice Harbor a Cdh23 Point Mutation

Genomewide homozygosity mapping, followed by fine mapping, positioned the deafness locus in the *jera* mutant to mouse chromosome 10, between nucleotides 58,149,600 and 66,453,600. Because this region contained the known "deafness" gene, *Cdh23*, we sequenced the *Cdh23* cDNA. A novel mutation was detected: a T-to-A transversion at nucleotide position 7079 (c.7079T>A) in exon 52, resulting in amino acid 2360 changing from valine to glutamic acid (p.V2360E) (Figure 2A). This amino acid is within a highly conserved Ca^{2+} -binding motif (Figure 2B) in EC domain 22. All reported *Cdh23* mutations in mouse, including *waltzer* (except one *Cdh23*^{b^{us}}), *salsa*, *erlong*, and *jera*, are located in the EC domains (Figure 3A). Many missense mutations that cause DFNB12, including those in *salsa* and *jera*, affect the Ca^{2+} -binding domains (ie, DXD, LDRE, and DXNDN). Nonsense and splice-site mutations that lead to *CDH23*-null alleles cause USHD1 and the *waltzer* phenotype in mice (Figure 3B).

Protein Conformation Is Disrupted in Cdh23^{V2360E/V2360E} Mice

To assess the structural consequences of the Cdh23 p.V2360E mutation, we examined computer-generated molecular models of the EC Cdh23 calcium-binding domains based on the mouse E-cadherin 1EDH crystal structure.³¹ The amino acid that corresponds to Cdh23 Val2360 in the mouse E-cadherin 1EDH fragment is Leu66. In the mutant modeling simulations, Leu66 is, therefore, replaced with Glu66. The modeling studies revealed that EC domains of E-cadherins form parallel symmetric dimers, held together primarily by a network of hydrogen bonding at an interface region coordinated by calcium ions (Figure 4A). Removal of the calcium ions allows greater flexibility in the calcium-binding regions and, consequently, appears to destabilize the dimer complex.³⁵ The Leu66 residue stabilizes the EC domains by participating in hydrophobic interactions, with adjacent residues directing the side chain portion internally.

Figure 3. **A:** Schematic diagram of Cdh23 protein structure showing the location of the *jera* mutation (red) relative to *waltzer*, *salsa*, and *erlong* mutations. USH1D human mutations within close proximity to a reported *waltzer* mutation are shown in parentheses.^{5,34} Details of these mouse mutations are included in Table 1. **B:** The 27 EC domain sequences of mouse Cdh23. Yellow boxes depict the calcium-binding motifs DXD, LDRE, and DXNDN within each EC domain. The positions of reported *waltzer* (blue); *salsa*, *erlong*, and *jera* (green); and known human DFNB12 (red) mutations are shown.

Simulations in which Leu66 was replaced with Glu66 consistently showed that Glu66 is repelled from the hydrophobic pocket and is instead directing itself externally to the solvent, causing a local conformational change. The simulations also suggest that the mutated residue forms a new

calcium-binding arrangement with residue Asp67, thereby severely affecting calcium-binding properties. Finally, the mutant form appears to lead to other structural changes in adjacent β sheets, altering the positioning of Lys14, which is likely to affect its ability to form intermolecular salt bridging with Glu138. These studies suggest that the Cdh23

Figure 4. **A:** Schematic diagram of the wild-type dimeric E-cadherin 2.0A crystal structure (1EDH) highlighting the amino acids that are involved in stabilizing the Ca²⁺ ions in the E-cadherin domains. **B:** Protein remodeling of a Cdh23 p.V2360E mutation showing the Ca²⁺ ions and amino acids for the wild-type (pink) and mutant (green) molecules. The mutation alters the structural conformation, interfering with the Ca²⁺ interaction that results in destabilization of the region.

Figure 5. Hearing profile of the *Cdh23*^{V2360E/V2360E} mutant, compared with littermates. The *Cdh23*^{V2360E/V2360E} homozygous mutant (MM) displays profound hearing loss at the age of 4 weeks. A two-way analysis of variance revealed that the *Cdh23*^{V2360E/+} heterozygotes (NM) exhibit an elevated hearing threshold at the age of 24 weeks compared with *Cdh23*^{+/+} wild-type (NN) littermates (**P* = 0.0018).

Figure 6. H&E staining of *Cdhl23*^{V2360E/V2360E} and *Cdhl23*^{+/+} cochleae. **A** and **D**: Normal cochlear morphological features (*Cdhl23*^{+/+} at 4 weeks). **B** and **E**: Mutant cochleae at 4 weeks showed modest signs of degeneration with a slight collapse of the OC in the basal region. **C** and **F**: At the age of 12 weeks, cochleae from *Cdhl23*^{V2360E/V2360E} mice show degeneration of the basal through the midcochlear turns, as seen by collapse of the OC (arrowhead) and loss of SG neurons (arrow). **D**, **E**, and **F**: The basal cochlear turn. OC indicates organ of Corti; SG, spiral ganglion. Scale bar = 200 μ m.

p.V2360E mutation alters the structural conformation of the protein and affects Ca^{2+} binding (Figure 4B).

Loss of Auditory Function in *Cdhl23*^{V2360E/V2360E} Mice

The effect of the *Cdhl23* p.V2360E mutation on auditory function was assessed by ABR (Figure 5). The homozygous mutant, *Cdhl23*^{V2360E/V2360E}, displays profound hearing loss at 4 weeks (>120 dB SPL), whereas ABR thresholds were within the normal range in 4-week-old *Cdhl23*^{V2360E/+} and *Cdhl23*^{+/+} mice. *Cdhl23*^{+/+} mice show a moderate increase in hearing thresholds with increasing age, which we attribute to the presence of the age-related hearing loss *Cdhl23*^{753A} allele in C57BL6/J mice.⁸ However, *Cdhl23*^{V2360E/+} heterozygous mice exhibit a statistically significant ($P = 0.0018$) increase in hearing thresholds at the age of 24 weeks when compared with *Cdhl23*^{+/+} wild-type littermates using two-way analysis of variance (Figure 5). There was no sign of vestibular dysfunction in *jera* because behavioral test results, including movement, circling, head tossing/tilting, star-gazing behavior, and righting response (curl trunk test), were normal in all three genotypes.

Abnormal Cochlear Morphological Features in *Cdhl23*^{V2360E/V2360E} Mice

A comparison of H&E-stained cochlear sections from 4-week-old *Cdhl23*^{V2360E/V2360E}, *Cdhl23*^{V2360E/+}, and *Cdhl23*^{+/+} mice revealed only minor differences in the mutant mice. There was some indication of hair cell degeneration in the basal cochlear region occurring in the mutant mouse because the tunnel of Corti was less well formed than in *Cdhl23*^{+/+} mice at this age (Figure 6, A, B,

D, and E). However, at the ages of 8 weeks (data not shown) and 12 weeks, signs of hair cell degeneration, leading to collapse of the organ of Corti and loss of spiral ganglion neurons, were clearly apparent in the basal and midcochlear turns (Figure 6, C and F) of *Cdhl23*^{V2360E/V2360E} mice. Cochlear morphological features in *Cdhl23*^{V2360E/+} mice resembled those of wild-type mice.

Cdhl23 Expression Is Lost in *Cdhl23*^{V2360E/V2360E} Mice

Immunofluorescence analysis was performed using an anti-*Cdhl23* antibody on cochlear SE from *Cdhl23*^{V2360E/V2360E}, *Cdhl23*^{V2360E/+}, and *Cdhl23*^{+/+} mice. Confocal microscopy confirmed that expression of *Cdhl23* protein was localized at the stereocilia of both the outer hair cells (OHCs) and inner hair cells (IHCs) of hearing mice up to P14, when similar levels were observed in the SE from *Cdhl23*^{V2360E/V2360E} and *Cdhl23*^{+/+} mice. A comparison of *Cdhl23*^{+/+}, *Cdhl23*^{V2360E/+}, and *Cdhl23*^{V2360E/V2360E} mice at P30 shows that *Cdhl23* expression is lost in both OHCs and IHCs of *Cdhl23*^{V2360E/V2360E} mice by this age (Figure 7). There also appeared to be reduced levels of *Cdhl23* mRNA expression in the hair cells of both P2 *Cdhl23*^{V2360E/V2360E} mutant compared with wild-type *Cdhl23*^{+/+} mice (Figure 8).

Cdhl23^{V2360E/V2360E} Mice Exhibit Hair Cell Degeneration and Loss of Stereocilia Tenting

An examination of cochlear SEs using scanning electron microscopy (SEM) revealed that, although hair cells appear normal at P14 (data not shown), by P30, there was significant loss of hair cells (primarily OHCs) in the basal through the midcochlear regions (Figure 9, A and B). P30

Figure 7. Immunofluorescence of whole mount cochlear SE. Stereocilia, labeled with phalloidin (red in **A** and **C** and green in **B** and **D–H**), are arranged normally in wild-type, heterozygote, and mutant mice. Levels of Cdh23 protein (green in **A** and **C** and red in **B** and **D–H**) localizing to the stereocilia appear normal at P5 (**A** and **C**) and P14 (**E** and **G**) in *Cdh23*^{+/+} (**A** and **E**) and *Cdh23*^{V2360E/V2360E} (**C** and **G**) mice. A comparison of *Cdh23*^{+/+} (**B**), *Cdh23*^{V2360E/+} (**D**), and *Cdh23*^{V2360E/V2360E} (**F**) mice at P30 shows Cdh23 expression is lost in both the OHCs and IHCs of *Cdh23*^{V2360E/V2360E} mice by this age. Isotype control showing no nonspecific staining (**H**). Scale bar = 8 μm. RIgG indicates rabbit.

Cdh23^{V2360E/V2360E}-mutant cochleae also showed a lack of stereociliary tip tenting of IHC second tallest row stereocilia compared with *Cdh23*^{V2360E/+} and *Cdh23*^{+/+} mice, indicative of disrupted tip links (Figure 9, G–I). As seen in the IHCs of *Cdh23*^{+/+} mice (Figure 9, J–L), this row of stereocilia commonly has pointed tips (examples are indicated by the arrowheads in Figure 9K), attributable to membrane tenting that is thought to occur because of the pulling force of the tip link that connects the top of these stereocilia to midway up the tallest row of

stereocilia. OHC hair bundles appeared normal in *Cdh23*^{V2360E/V2360E} mice, with lateral links seen between stereocilia (Figure 9, M–O).

Hair Cell Degeneration Is Apparent in Aging *Cdh23*^{V2360E/+} Mice

Because of the increased hearing thresholds observed in *Cdh23*^{V2360E/+} mice at 24 weeks, we used SEM to exam-

Figure 8. *In situ* hybridization of *Cdh23* in developing mouse cochlea. *Cdh23* expression was observed in both the IHCs and OHCs of *Cdh23*^{+/+} (A) and *Cdh23*^{V2360E/V2360E} (B) P2 mouse cochleae. Control *Cdh23* sense probe (C).

ine cochleae from 13-week-old *Cdh23*^{V2360E/+} and *Cdh23*^{+/+} mice to determine whether there were any observable differences between hair cells from these mice. *Cdh23*^{V2360E/+} mice exhibited normal tip tenting of IHC stereocilia (Figure 10, G–I); however, there was evidence of hair cell degeneration in the basal cochlear turn, with approximately 50% of OHCs missing in this region (Figure 10, A–C).

Discussion

In this study, we identified and characterized a novel ENU-generated mouse model, *jera*, with inherited hearing loss because of a c.7079T>A mutation in *Cdh23* (*Cdh23* p.V2360E). The *Cdh23*^{V2360E/V2360E} mice show profound hearing loss at the age of 4 weeks. Many mice (>80%) had a hearing threshold of ≥ 120 dB SPL when the ABR was tested at 4 weeks, with the remaining mutants at 110 to 115 dB SPL.

Heterozygous *Cdh23*^{V2360E/+} mice had a later onset and milder hearing loss. Therefore, the *Cdh23* p.V2360E allele is semidominant because *Cdh23*^{V2360E/+} heterozygous mice show significantly elevated hearing threshold levels at 24 weeks when compared with wild-type littermates. This finding is in agreement with previous studies³⁶ that show mice heterozygous for the *Cdh23*^V *waltzer* allele have hearing loss that deteriorates with age and have an increased susceptibility to noise-induced hearing loss. SEM analysis of 13-week-old *Cdh23*^{V2360E/+} mice revealed degeneration of hair cells in the basal cochlear region, which is likely to extend throughout the cochlea as the mice age and attribute to the elevated hearing thresholds we observed in these mice at the age of 24 weeks.

Cdh23 forms part of the cochlea hair cell tip link filaments where the protein interacts with PCDH15.¹⁸ The *Cdh23* p.V2360E mutation affects the highly conserved Ca²⁺-binding VDYE motif located in EC domain 22 (Figure 2B). Amino acid changes in the calcium-binding motifs are likely to cause structural changes and to alter calcium-binding properties.³⁷ Our protein modeling supports the hypothesis that the *Cdh23* p.V2360E mutation leads to a conformational change that severely interferes with local Ca²⁺-binding properties, thereby compromising the structural integrity and dimerization capability of the mutant protein.

We detected similar levels of *Cdh23* protein localizing to the stereocilia of *Cdh23*^{V2360E/V2360E} mutants when compared with wild-type mice at P5 and P14, but *Cdh23* was undetectable by P30 in the *Cdh23*^{V2360E/V2360E} mutants. Our SEM analysis of P14 mice (data not shown) revealed no obvious differences between mutant and wild-type mice at this age; stereociliary tenting was observed in IHCs, and hair bundle formation was normal for IHCs and OHCs. This suggests that, despite being composed of mutant *Cdh23* protein, tip links are initially able to form in the *Cdh23*^{V2360E/V2360E} mice and are intact up until approximately the time of onset of hearing (at P12 to P14). However, we propose that the tip links would be unable to function correctly when hearing would normally be established and would break readily when under tension caused by the deflection of the stereocilia bundle in response to sound. This is supported by reduced tenting of stereocilia tips observed in IHCs at P30 in *Cdh23*^{V2360E/V2360E} mice, indicating disruption of stereociliary tip links. This also supports the hypothesis that tip links regenerate from the reserve pool of transduction molecules, as suggested by Lelli et al.²⁸ However, the rate of regeneration in the *Cdh23*^{V2360E/V2360E} mutant would be insufficient to fully reestablish tip links; and the reserve pool would eventually be depleted, rendering the hair cell insensitive to mechanical stimulation. Interestingly, we were still able to detect what appear to be interstereociliary links connecting stereocilia in OHC hair bundles. Rzadzinska and Steel³⁸ reported a similar finding for the *Cdh23*^{V2J} *waltzer* mouse; reduced tenting was observed in the IHCs of the mutant mouse, despite the presence of “tip link–like structures” in hair bundles of both IHCs and OHCs. We hypothesize that the lateral links, which we are able to detect by SEM in P30 mutant mice, may not be under the same degree of tension as the tip links and are, therefore, more likely to remain intact despite incorporating mutant *Cdh23* protein. Alternatively, there may be other molecular components that compose the various stereociliary links that might compensate for the presence of mutant *Cdh23*.

The degeneration of hair cell bundles, most notable in the basal cochlear region, in *Cdh23*^{V2360E/V2360E} mice at P30 (and also in the *Cdh23*^{V2360E/+} mice at 13 weeks) is likely to be a secondary consequence of the hair cells being nonfunctional.

In humans, mutations in *CDH23* can cause deafness-blindness (USH1D) or nonsyndromic deafness (DFNB12). *CDH23*-null mutations appear to cause USH1D, whereas missense mutations cause nonsyndromic deafness.² Most mice with *Cdh23* mutations present with the *waltzer* phenotype: hearing loss and vestibular dysfunction.^{9–13} However, in mice, mutations in *Cdh23* have not been associated with retinal degeneration. Gene expression studies²⁹ have shown that the longest *Cdh23* isoform (*CDH23_V1*) is expressed in the primate retina but not in the murine retina. This *Cdh23* isoform was the only isoform affected by the *Cdh23*^{V6J} nonsense mutation located in EC domain 3. Tissue-specific differences in the expression of the long isoform of *Cdh23* in human and mouse eyes could explain why no retinal degeneration is seen in *waltzer* mice.²⁹ *Waltzer* mutations are predomi-

Figure 9. Disruption of tip links and hair cell degeneration in P30 *Cdh23*^{V2360E/V2360E} mice. **A** through **F**: SEM images of the cochlear SEM reveal loss of OHCs in the basal through the midcochlear region in *Cdh23*^{V2360E/V2360E} mice. **G** through **L**: An examination of IHCs indicates a defect in stereociliary tip tenting (shown by peaked stereocilia tips: **arrowheads** in **K**) in *Cdh23*^{V2360E/V2360E} mice, most obvious in the mid and apical regions. **M** through **R**: OHCs appeared normal in the mid to apical region of *Cdh23*^{V2360E/V2360E} mice. Lateral links could be observed between stereocilia (**arrows** in **N**). Basal OHCs (**M**), when still present, showed signs of degeneration, such as fused stereocilia (**asterisk**). Scale bars: 10 μ m (**A–F**); 2 μ m (**G–R**).

Figure 10. A through F: *Cdh23*^{V2360E/+} mice show degeneration of basal hair cells. SEM analysis of cochlear SE revealed substantial OHC loss in the basal turn of *Cdh23*^{V2360E/+} mice compared with *Cdh23*^{+/+} mice. G through I: The results of an examination of IHC stereocilia indicated normal tip tenting in *Cdh23*^{V2360E/+} mice. Scale bars: 10 μm (A–F); 2 μm (G–I).

nantly located in EC domains and appear to be functional null alleles.^{9–11} The null mutations in *waltzer* mice result in lack of organization of cochlear hair cell stereocilia and misplaced kinocilia.^{39,40} Recently, two ENU-generated *Cdh23* mouse mutants, *salsa* and *erlong*, were reported; recessively inherited progressive hearing loss commenced at 3 to 4 weeks without any vestibular dysfunction, unlike the *waltzer* mutants.^{14,15} The *salsa* mutant harbors a point mutation, *Cdh23* p.E737V, that is located in the more distal EC7 Ca^{2+} -binding domain and leads to loss of tip links and tenting of stereociliary tips because of its effect on interaction/binding with *Pcdh15*.¹⁴ The *erlong* missense mutation *Cdh23* p.S70P resides in the EC1 domain but not within a Ca^{2+} -binding motif and has a less severe hearing loss phenotype than *salsa*.¹⁵ Similar to *salsa*, the *jera* *Cdh23* p.V2360E missense mutation is located within a Ca^{2+} -binding domain in EC22. A similar process of tip link loss occurs in the *jera* mutant, as

reported in *salsa*. Our results also support the hypothesis that missense mutations in a Ca^{2+} -binding domain affect mechanotransduction, but not hair bundle development, because hair bundles appear normal at P14. The deafness observed in these mutants suggests a genotype-phenotype correlation in which missense mutations residing in Ca^{2+} -binding domains lead to severe hearing loss and increased severity influenced by the location of the mutation. Because the mutant protein is present in the stereocilia links of affected mice, hair bundle development could be regulated by another *Cdh23* domain and perhaps its interaction with other proteins. The data we present herein provide supportive evidence that the *Cdh23* protein is directly involved in establishing a stereociliary tip link and tenting, which are crucial for the mechanotransduction process.

Furthermore, polymorphisms in *CDH23* have been associated with noise-induced hearing loss in humans.

Mice carriers for the *Cdh23* waltzer-null allele show increased susceptibility to noise trauma. The compilation of well-characterized allelic variants in the *Cdh23* gene (or any other deafness gene) provides a great opportunity to generate a “carrier susceptibility matrix” to environmental insults, including predisposition to noise trauma or ototoxic drugs. This information would be extremely useful in the detection, counseling, and management of patients and their families (who are carriers) with DFNB12 hearing loss.

In conclusion, *jera* is a novel mouse model for deciphering the molecular pathways that lead to the different forms of DFNB12 hearing loss that will enable more detailed studies on the role of *Cdh23* in the development and function of cochlear hair cells, its role in stereocilia tip link mechanotransduction, and its role for screening otoprotective drugs under the various DFNB12 conditions.

Acknowledgments

We thank the Australian Phenomics Facility for its contribution, Jessica Cardwell and Melissa Arnold for their technical help, and Prof. Thomas Friedman and Dr. Ayala Lagziel (National Institute on Deafness and Other Communication Disorders) for providing the TF8-*Cdh23* antibody.

References

- Petit C: Genes responsible for human hereditary deafness: symphony of a thousand. *Nat Genet* 1996, 14:385–391
- Bork JM, Peters LM, Riazuddin S, Bernstein SL, Ahmed ZM, Ness SL, Polomeno R, Ramesh A, Schloss M, Srisailpathy CR, Wayne S, Bellman S, Desmukh D, Ahmed Z, Khan SN, Kaloustian VM, Li XC, Lalwani A, Riazuddin S, Bitner-Glindzicz M, Nance WE, Liu XZ, Wistow G, Smith RJ, Griffith AJ, Wilcox ER, Friedman TB, Morell RJ: 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
- Bolz H, von Bredelow B, Ramirez A, Bryda EC, Kutsche K, Nothwang HG, Seeliger M, del C-Salcedo Cabrera M, Vila MC, Molina OP, Gal A, Kubisch C: Mutation of *CDH23*, encoding a new member of the cadherin gene family, causes Usher syndrome type 1D. *Nat Genet* 2001, 27:108–112
- Friedman TB, Griffith AJ: Human nonsyndromic sensorineural deafness. *Annu Rev Genomics Hum Genet* 2003, 4:341–402
- Astuto LM, Bork JM, Weston MD, Askew JW, Fields RR, Orten DJ, Ohliger SJ, Riazuddin S, Morell RJ, Khan S, Riazuddin S, Kremer H, van Hauwe P, Moller CG, Cremers CW, Ayuso C, Heckenlively JR, Rohrschneider K, Spandau U, Greenberg J, Ramesar R, Reardon W, Bitoun P, Millan J, Legge R, Friedman TB, Kimberling WJ: *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
- Oshima A, Jaijo T, Aller E, Millan JM, Carney C, Usami S, Moller C, Kimberling WJ: Mutation profile of the *CDH23* gene in 56 probands with Usher syndrome type I. *Hum Mutat* 2008, 29:E37–E46
- Becirovic E, Ebermann I, Nagy D, Zrenner E, Seeliger MW, Bolz HJ: Usher syndrome type 1 due to missense mutations on both *CDH23* alleles: investigation of mRNA splicing. *Hum Mutat* 2008, 29:452
- 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
- Di Palma F, Holme RH, Bryda EC, Belyantseva IA, Pellegrino R, Kachar B, Steel KP, Noben-Trauth K: 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
- Di Palma F, Pellegrino R, Noben-Trauth K: Genomic structure, alternative splice forms and normal and mutant alleles of cadherin 23 (*Cdh23*). *Gene* 2001, 281:31–41
- Yonezawa S, Yoshizaki N, Kageyama T, Takahashi T, Sano M, Tokita Y, Masaki S, Inaguma Y, Hanai A, Sakurai N, Yoshiki A, Kusakabe M, Moriama A, Nakayama A: Fates of *Cdh23/CDH23* with mutations affecting the cytoplasmic region. *Hum Mutat* 2006, 27:88–97
- Wada T, Wakabayashi Y, Takahashi S, Ushiki T, Kikkawa Y, Yonekawa H, Kominami R: 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
- Wilson SM, Householder DB, Coppola V, Tessarollo L, Fritzsche B, Lee EC, Goss D, Carlson GA, Copeland NG, Jenkins NA: Mutations in *Cdh23* cause nonsyndromic hearing loss in waltzer mice. *Genomics* 2001, 74:228–233
- Schwander M, Xiong W, Tokita J, Lelli A, Elledge HM, Kazmierczak P, Sczaniecka A, Kolatkar A, Wiltshire T, Kuhn P, Holt JR, Kachar B, Tarantino L, Muller U: A mouse model for nonsyndromic deafness (DFNB12) links hearing loss to defects in tip links of mechanosensory hair cells. *Proc Natl Acad Sci U S A* 2009, 106:5252–5257
- Han F, Yu H, Tian C, Chen HE, Benedict-Alderfer C, Zheng Y, Wang Q, Han X, Zheng QY: A new mouse mutant of the *Cdh23* gene with early-onset hearing loss facilitates evaluation of otoprotection drugs. *Pharmacogenomics J* 2010, [Epub ahead of press] doi:10.1038/tj.2010.60
- Siemens J, Lillo C, Dumont RA, Reynolds A, Williams DS, Gillespie PG, Muller U: Cadherin 23 is a component of the tip link in hair-cell stereocilia. *Nature* 2004, 428:950–955
- Sollner C, Rauch GJ, Siemens J, Geisler R, Schuster SC, Muller U, Nicolson T: Mutations in cadherin 23 affect tip links in zebrafish sensory hair cells. *Nature* 2004, 428:955–959
- Kazmierczak P, Sakaguchi H, Tokita J, Wilson-Kubalek EM, Milligan RA, Muller U, Kachar B: Cadherin 23 and protocadherin 15 interact to form tip-link filaments in sensory hair cells. *Nature* 2007, 449:87–91
- Beurg M, Fettiplace R, Nam JH, Ricci AJ: Localization of inner hair cell mechanotransducer channels using high-speed calcium imaging. *Nat Neurosci* 2009, 12:553–558
- Sotomayor M, Weihofen WA, Gaudet R, Corey DP: Structural determinants of cadherin-23 function in hearing and deafness. *Neuron* 2010, 66:85–100
- Alagramam KN, Murcia CL, Kwon HY, Pawlowski KS, Wright CG, Woychik RP: The mouse Ames waltzer hearing-loss mutant is caused by mutation of *Pcdh15*, a novel protocadherin gene. *Nat Genet* 2001, 27:99–102
- Gibson F, Walsh J, Mburu P, Varela A, Brown KA, Antonio M, Beisel KW, Steel KP, Brown SD: A type VII myosin encoded by the mouse deafness gene *shaker-1*. *Nature* 1995, 374:62–64
- Johnson KR, Gagnon LH, Webb LS, Peters LL, Hawes NL, Chang B, Zheng QY: 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
- Kikkawa Y, Shitara H, Wakana S, Kohara Y, Takada T, Okamoto M, Taya C, Kamiya K, Yoshikawa Y, Tokano H, Kitamura K, Shimizu K, Wakabayashi Y, Shiroishi T, Kominami R, Yonekawa H: Mutations in a new scaffold protein *Sans* cause deafness in Jackson shaker mice. *Hum Mol Genet* 2003, 12:453–461
- Lefevre G, Michel V, Weil D, Lepelletier L, Bizard E, Wolfrum U, Hardelin JP, Petit C: 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
- Pawlowski KS, Kikkawa YS, Wright CG, Alagramam KN: Progression of inner ear pathology in Ames waltzer mice and the role of protocadherin 15 in hair cell development. *J Assoc Res Otolaryngol* 2006, 7:83–94
- Senften M, Schwander M, Kazmierczak P, Lillo C, Shin JB, Hasson T, Geleoc GS, Gillespie PG, Williams D, Holt JR, Muller U: Physical and functional interaction between protocadherin 15 and myosin VIIa in mechanosensory hair cells. *J Neurosci* 2006, 26:2060–2071
- Lelli A, Kazmierczak P, Kawashima Y, Muller U, Holt JR: Development and regeneration of sensory transduction in auditory hair

- cells requires functional interaction between cadherin-23 and protocadherin-15. *J Neurosci* 2010, 30:11259–11269
29. Lagziel A, Overlack N, Bernstein SL, Morell RJ, Wolfrum U, Friedman TB: Expression of cadherin 23 isoforms is not conserved: implications for a mouse model of Usher syndrome type 1D. *Mol Vis* 2009, 15: 1843–1857
30. Whitton DS, Szakaly R, Greiner MA: Cryoembedding and sectioning of cochleas for immunocytochemistry and in situ hybridization. *Brain Res Brain Res Protoc* 2001, 6:159–166
31. Nagar B, Overduin M, Ikura M, Rini JM: Structural basis of calcium-induced E-cadherin rigidification and dimerization. *Nature* 1996, 380: 360–364
32. Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, Chipot C, Skeel RD, Kale L, Schulten K: Scalable molecular dynamics with NAMD. *J Comput Chem* 2005, 26:1781–1802
33. Humphrey W, Dalke A, Schulten K: VMD: visual molecular dynamics. *J Mol Graph* 1996, 14:33–38, 27–28
34. Roux AF, Faugere V, Le Guedard S, Pallares-Ruiz N, Vielle A, Chamber S, Marlin S, Hamel C, Gilbert B, Malcolm S, Claustres M: Survey of the frequency of USH1 gene mutations in a cohort of Usher patients shows the importance of cadherin 23 and protocadherin 15 genes and establishes a detection rate of above 90%. *J Med Genet* 2006, 43:763–768
35. Sotomayor M, Corey DP, Schulten K: In search of the hair-cell gating spring elastic properties of ankyrin and cadherin repeats. *Structure* 2005, 13:669–682
36. Holme RH, Steel KP: Progressive hearing loss and increased susceptibility to noise-induced hearing loss in mice carrying a Cdh23 but not a Myo7a mutation. *J Assoc Res Otolaryngol* 2004, 5:66–79
37. de Brouwer AP, Pennings RJ, Roeters M, Van Hauwe P, Astuto LM, Hoefsloot LH, Huygen PL, van den Helm B, Deutman AF, Bork JM, Kimberling WJ, Cremers FP, Cremers CW, Kremer H: 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. Rzadzinska AK, Steel KP: Presence of interstereocilia links in waltzer mutants suggests Cdh23 is not essential for tip link formation. *Neuroscience* 2009, 158:365–368
39. Holme RH, Steel KP: Stereocilia defects in waltzer (Cdh23), shaker1 (Myo7a) and double waltzer/shaker1 mutant mice. *Hear Res* 2002, 169:13–23
40. Lagziel A, Ahmed ZM, Schultz JM, Morell RJ, Belyantseva IA, Friedman TB: Spatiotemporal pattern and isoforms of cadherin 23 in wild type and waltzer mice during inner ear hair cell development. *Dev Biol* 2005, 280:295–306