Noddy, a Mouse Harboring a Missense Mutation in Protocadherin-15, Reveals the Impact of Disrupting a Critical Interaction Site between Tip-Link Cadherins in Inner Ear Hair Cells

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In hair cells of the inner ear, sound or head movement increases tension in fine filaments termed tip links, which in turn convey force to mechanosensitive ion channels to open them. Tip links are formed by a tetramer of two cadherin proteins: protocadherin 15 (PCDH15) and cadherin 23 (CDH23), which have 11 and 27 extracellular cadherin (EC) repeats, respectively. Mutations in either protein cause inner ear disorders in mice and humans. We showed recently that these two cadherins bind tip-to-tip in a “handshake” mode that involves the EC1 and EC2 repeats of both proteins. However, a paucity of appropriate animal models has slowed our understanding both of the interaction and of how mutations of residues within the predicted interface compromise tip link integrity. Here, we present *noddy*, a new mouse model for hereditary deafness. Identified in a forward genetic screen, *noddy* homozygotes lack inner ear function. Mapping and sequencing showed that *noddy* mutant mice harbor an isoleucine-to-asparagine (I108N) mutation in the EC1 repeat of PCDH15. Residue I108 interacts with CDH23 EC2 in the handshake and its mutation impairs the interaction *in vitro*. The *noddy* mutation allowed us to determine the consequences of blocking the handshake *in vivo*: tip link formation and bundle morphology are disrupted, and mechanotransduction channels fail to remain open at rest. These results offer new insights into the interaction between PCDH15 and CDH23 and help explain the etiology of human deafness linked to mutations in the tip-link interface.

Introduction

Hearing and balance depend on hair cells, the specialized mechanoreceptors of the inner ear that convert head movement or

sound into electrical stimuli (Gillespie and Muller, 2009). The mechanotransduction function of hair cells occurs within the sensory hair bundle; each bundle comprises 30–300 actin-based stereocilia coupled to one another by tip links, protein filaments that connect the tip of each stereocilium to the side of its tallest neighboring stereocilium (Pickles et al., 1984). When the hair bundle is deflected in the excitatory direction, tip links are tensioned and are thought to directly pull open mechanotransduction channels located at their lower ends (Beurg et al., 2009).

Immunocytochemical, structural, and genetic evidence indicates that each tip link is a tetramer of protocadherin 15 (PCDH15) and cadherin 23 (CDH23), two atypical cadherin superfamily members (Ahmed et al., 2006; Kazmierczak et al., 2007; Alagramam et al., 2011). Mutations in the genes encoding PCDH15 and CDH23 produce deafness and balance disorders in mice and humans (Alagramam et al., 2001a, 2001b; Ahmed et al., 2001; Di Palma et al., 2001; Bork et al., 2001). It is thought that PCDH15 and CDH23 form *cis*-homodimers, which interact in *trans* via their opposing N termini (Kazmierczak et al., 2007) to create a tip link that is ~170 nm long under endolymph-like calcium levels (Furness et al., 2008). However, the structural and

Received Sept. 21, 2012; revised Dec. 23, 2012; accepted Jan. 21, 2013.

Author contributions: R. Geng, M.S., R. Gaudet, D.F., S.D.B., D.P.C., and K.N.A. designed research; R. Geng, M.S., K.J.K., S.R.G., J.G.-S., D.H.-C.C., R.E.H.-H., G.B., A.P., R. Gaudet, D.F., S.D.B., D.P.C., and K.N.A. performed research; R. Geng, M.S., K.J.K., S.R.G., J.G.-S., D.H.-C.C., R.E.H.-H., G.B., A.P., R. Gaudet, D.F., S.D.B., D.P.C., and K.N.A. analyzed data; R. Geng and K.N.A. wrote the paper.

This work was supported by the National Institutes of Health (R01-DC010816 to K.N.A.; R01 DC02281 to D.P.C.; RC2GM093307 to National Resource for Biomedical Supercomputing/Pittsburgh Supercomputing Center), the National Science Foundation through TeraGrid/XSEDE (TRAC MCB080015), Deafness Research United Kingdom funds (D.F.), and the Medical Research Council, United Kingdom (S.D.B.). M.S. was a Howard Hughes Medical Institute Fellow of the Helen Hay Whitney Foundation and is supported by the National Institute on Deafness and Other Communication Disorders (K99DC012534). D.P.C. is an Investigator of the Howard Hughes Medical Institute. Simulations were performed at the Texas Advanced Computing Center-Ranger and Pittsburgh Supercomputing Center-Anton supercomputers. We thank Bruce Delfer for assistance with mutagenesis, Robert Powers and Monique Brewster for advice on circular dichroism and thermostability assays, Victoria D'Souza and members of her laboratory for advice on calorimetry, Sue Morse for maintenance of *noddy* colonies, and Ilya Litvak for initial work on the *noddy* project after the mice arrived at Case Western Reserve University.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.4514-12.2013

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molecular properties of the tip-link complex are just beginning to come to light (Sotomayor et al., 2010, 2012; Elledge et al., 2010).

The amino acid sequence and crystal structure of extracellular cadherin repeats 1 and 2 (EC1 + EC2) of CDH23 and PCDH15 contain classic cadherin folds but also show important differences from classical cadherins. First, they lack the residues of classical cadherins, such as the conserved tryptophan at position 2 and its hydrophobic binding pocket in the partner EC1, which are involved in the β -strand swapping underlying EC1 to EC1 bonds in *trans* (Brasch et al., 2012). Second, both PCDH15 EC1 and CDH23 EC1 contain an elongated N terminus with polar residues (Sotomayor et al., 2010; Elledge et al., 2010). Therefore, cadherins that evolved to endure the strong mechanical forces produced by loud noise most likely use an interface that is different from classical cadherins. Indeed, crystal structures of EC1 and EC2 of PCDH15 and CDH23 show binding in an extended “handshake” mode that involves EC1 and EC2 repeats of both proteins (Sotomayor et al., 2012). Yet this model has not been confirmed by independent methods, and it is unclear how mutations of interface residues might affect the integrity of the tip link *in vivo*.

Here we report on a new *N*-ethyl-*N*-nitrosourea (ENU)-induced mouse mutant, *noddy*, which harbors a missense mutation (I108N) in PCDH15 EC1, the first reported missense mutation in the mouse *Pcdh15*. Circular dichroism spectra indicated that the mutation does not affect protein folding, but binding of PCDH15–CDH23 fragments *in vitro* is impaired by this mutation, as determined by isothermal titration calorimetry experiments. Thus, *noddy* mutant mice allowed us to determine the consequences of blocking the handshake interaction *in vivo*.

Materials and Methods

Experiments on mice were performed according to animal welfare guidelines at the Medical Research Council United Kingdom and Case Western Reserve University.

Chemical mutagenesis, functional studies, positional gene identification, bundle morphology evaluation, and scanning electron microscopy. Standard methods previously described include ENU recessive mutagenesis (Hardisty-Hughes et al., 2010), assessment of hearing using clickbox and ABR recording (Parker et al., 2010), and bundle morphology visualization (Alagramam et al., 2011). Sample preparation for scanning electron microscopy was performed using the osmium-thiocarbonylhydrazide impregnation method as described previously (Furness and Hackney, 1985), and the images were acquired using a Hitachi S4500 field emission scanning electron microscopy.

FM1-43 uptake assay. In brief, cochlear epithelium tissue was dissected from postnatal day 2 (P2) mice and cultured 18–20 h (P3) at 37°C with 5% CO₂ and 95% humidity in MEM (Invitrogen) supplemented with 10% (v/v) horse serum, 10 mM HEPES, and 10 μ g/ml ampicillin. Coverslips with adherent cochlear cultures were washed once with HEPES-buffered (10 mM, pH 7.2) HBSS (HBHBSS), dipped for 10 s in HBHBSS containing 3 mM FM1-43, and washed immediately three times in a large volume of HBHBSS (10 s for each wash). The coverslips were then placed on a glass slide and viewed with an upright microscope equipped with epifluorescence optics and FITC filters (488 nm excitation, 520 nm emission) using a 40 $\times$ dry objective. Images were captured with focus plane below cuticular plates at fixed time points after a dye application using a 12-bit cooled CCD camera (Retiga Exi Aqua Blue; Q-Imaging). Differential Interference Contrast (DIC) images were also taken with focus on apical surface of hair cells.

Genotyping for the *noddy* mutation. The single nucleotide change in *noddy* results in the introduction of a new restriction site, MseI. The genotyping scheme exploits this change to distinguish wild-type from *noddy* allele. Genomic DNA (~500 ng/reaction) was amplified under standard reaction conditions using Platinum TaqDNA polymerase (In-

vitrogen) for 35 cycles (94°C for 20 s, 55°C for 30 s, and 72°C for 30 s). Primer pairs are as follows: forward primer, 5'-GTGTGTTGGTGTCTA-AAACGATG-3'; and reverse primer, 5'-TCTTTTACCTCTAGG-CACATTCG-3'. PCR product was digested with restriction enzyme MseI using a reaction condition suggested by the manufacturer (New England Biolabs). The products were resolved using agarose gel (3% w/v) electrophoresis.

Genotyping for the transgene in *Pcdh15*^{Tg2742} mutation. Characterization of the transgene induced disabling mutation and phenotype in *Pcdh15*^{Tg2742} mice has been described previously (Alagramam et al., 1999 and 2001). The transgene (Tg), a linear bacterial plasmid vector containing an ampicillin resistance gene and other sequences unrelated to mouse genome, was used in a forward genetic screen to generate insertional mutants and identify genes important for development. The *Pcdh15*^{Tg2742} mouse is one of the mutants that arose from that project (Alagramam et al., 1999). The transgene was used as a unique DNA marker to clone and characterize the mutant locus. The #2742 was arbitrarily assigned and has no significance. More details about the insertional mutagenesis approach and insertional mutant *Pcdh15*^{Tg2742} were described previously (Woychik and Alagramam, 1998; Alagramam et al., 1999). The sequence of the transgene is unique and not contained in the genomic DNA of wild-type mice (Alagramam et al., 1999). Identification of the transgene was performed as follows. Genomic DNA (~500 ng/reaction) isolated from mice was amplified under standard reaction conditions using Platinum TaqDNA polymerase (Invitrogen) for 35 cycles of 94°C for 20 s, 55°C for 30 s, and 72°C for 2 min. A (forward) primer on the ampicillin gene 5'-ATAATACCGCGCCACATAGC-3' and a (reverse) primer on the human β actin promoter 5'-CCCTGGCCTTGATCTTTCA-3' were used in PCR to check for the presence of the transgene. The PCR product was resolved using agarose gel (3% w/v) electrophoresis.

Antibody and immunofluorescence. The anti-PCDH15 antibody PB811 was kindly provided by Dr. Bechara Kachar and has been described previously (Kazmierczak et al., 2007). For immunofluorescence, cochlear epithelium tissues were dissected from P2 mice and cultured for approximately 15 h at 37°C with 5% CO₂ and 95% humidity in MEM (Invitrogen) supplemented with 10% (v/v) horse serum, 10 mM HEPES, and 10 μ g/ml ampicillin. The tissues were fixed in freshly prepared 4% (w/v) paraformaldehyde for 1 h and then washed three times for 15 min in calcium-free buffer containing the following (in mM): 140 NaCl, 0.7 NaH₂PO₄, 5.8 KCl, 2.2 MgCl₂, 5 EGTA, 5.6 D-glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH, supplemented with MEM vitamins (1:1000) and amino acids (1:500). The tissue was further incubated in calcium-free buffer at 4°C overnight and then incubated with anti-PCDH15 antibody PB811 at 2 μ g/ml in calcium-free buffer at room temperature for 1 h. After three 15-min washes with calcium-free buffer, the tissues were incubated with Alexa Fluor-488 conjugated anti-rabbit antibody (1:500 dilution, Invitrogen) and Alexa Fluor-546 conjugated phalloidin (1:100 dilution, Invitrogen) in calcium-free buffer supplemented with 0.1% Triton X-100 at room temperature for 1 h. After three 15 min washes, tissues were mounted on slides and imaged with a Leica confocal microscope.

Structural and biochemical analysis. Cloning, expression, and purification of mouse CDH23 and PCDH15 EC1 + EC2 were previously described (Sotomayor et al., 2010, 2012). The *noddy* I108N mutation in PCDH15 EC1 + EC2 was generated using the QuikChange Lightning mutagenesis kit (Stratagene) and verified by DNA sequencing. Size exclusion chromatography (SEC) experiments were done on a Superdex75 column (GE Healthcare). Circular dichroism (CD) spectra were recorded at 10°C using a 1 mm-path-length cuvette on a Jasco J-815 spectrometer. Samples for CD were purified in 20 mM Tris HCl, pH 8.0, 100 mM KCl, and 1 mM CaCl₂, and concentrated to 3.46 (WT) and 3.13 μ M (I108N). Thermostability assays (Niesen et al., 2007) were performed in triplicate with protein samples at 0.329 (WT) and 0.292 (I108N) mg/ml in a white thin-walled PCR plate (50 μ l per sample) mixed with the SYPRO Orange dye (Invitrogen). Measurements used a Bio-Rad Opticon 2 Real-Time PCR detector (sample plate heated using 1°C steps and a holding time of 1 min). Melting temperatures were estimated from the peak of the first derivative of the fluorescence. Isothermal titration calorimetry (ITC) experiments were performed using a MicroCal iTC₂₀₀

Figure 1. Characterization of the *noddy* mice and localization of *noddy* mutation in PCDH15. **A**, Hearing loss in control and *noddy* mutant mice. Pure-tone-evoked ABR shows auditory thresholds for control (+/n) ($n = 3$) and *noddy* (n/n) ($n = 5$) at P18. **B**, Sequence chromatogram from wild-type (top) and *noddy* mutant (bottom) mice reveals a T-to-A mutation in the exon coding for EC1 of PCDH15, resulting in an Ile-108-Asn substitution. **C**, The I108N substitution affects an amino acid that is conserved across species. **D**, Domain structure of PCDH15, indicating the 11 EC repeats (purple squares). Missense mutations in the *noddy* mutant mice and human PCDH15 EC1 and EC2 are indicated. Other mutations in mouse PCDH15 referred to in this report (av3J and av6J) are also indicated in this diagram.

calorimeter with buffer-matched samples at 10°C (20 mM HEPES, pH 7.5, 150 mM KCl, 1 mM CaCl_2). In all ITC experiments, a 0.5 μl injection (disregarded in analysis) was followed by 14 2.48 μl injections of CDH23 EC1 + EC2 (933 μM ; $\Delta t = 3$ min). PCDH15 EC1 + EC2 or PCDH15 EC1 + EC2 I108N (107 μM) was placed in the sample cell. Protein concentra-

tions were measured using absorbance at 280 nm and theoretical extinction coefficients.

Systems for molecular dynamics simulations were prepared with the Visual Molecular Dynamics software (Humphrey et al., 1996) as previously described (Sotomayor et al., 2012); the PCDH15 I108N mutation was generated *in silico* using the Visual Molecular Dynamics Mutator plugin. The simulation box comprised the complex of PCDH15 EC1 + EC2 I108N and CDH23 EC1 + EC2, water molecules, and ions (108,687 atoms total). A 1.1 ns equilibration of the mutant complex was performed with Nanoscale Molecular Dynamics (Phillips et al., 2005) and followed by 0.727 μs of dynamics using Anton (Shaw et al., 2010), with parameters as described previously (Sotomayor et al., 2012). The number of water molecules within 5 Å of I108 and I108N Ca atoms was monitored throughout trajectories of a 1- μs -long simulation of the wild-type complex (Sotomayor et al., 2012) and the 0.727- μs -long simulation reported here. Molecular images were created with Visual Molecular Dynamics.

Results

Generation and phenotype of *noddy* mutant mice

From a recessive ENU mutagenesis screen undertaken at MRC Harwell, United Kingdom, we sought mice with hearing impairment and abnormal balance. One such line was identified by the lack of an auditory startle (Preyer) reflex to a “clickbox” (~ 20 kHz at 90 dB sound pressure level) at P30. It also showed head-bobbing, and so was named *noddy*. Auditory brainstem recordings (ABRs) confirmed deafness in *noddy* homozygotes as early as P18 (Fig. 1A), which fail to show a response to any of the stimulus frequencies at the highest stimulus level (100 dB SPL).

Wild-type siblings displayed controlled head and body movements indicative of normal balance function. In contrast, *noddy* homozygotes displayed head-bobbing and circling behavior, as early as 2–3 weeks of age, indicative of early-onset vestibular dysfunction. *Noddy* homozygotes were further evaluated for balance by a swim test. When placed in water, wild-type mice surfaced immediately and maintained a horizontal bodyline; they used their tail effectively to remain prone. In contrast, *noddy* homozygotes mice remained submerged, completely disoriented, turning and tumbling beneath the surface. The erratic head movements and disoriented swimming behavior indicate a lack of balance function in *noddy* homozygotes. Overall, *noddy* mutant mice lack inner ear function.

First reported missense mutation in mouse *Pcdh15*

The affected genomic locus was initially mapped using a panel of SNP markers selected from the Mouse Phenome Database (MPD)

to a 45 Mb interval on chromosome 10. Fine mapping using additional SNP markers and larger numbers of affected *noddy* mutant mice reduced this to an 11.54 Mb interval between SNPs rs13480628 (chromosome 10: 67238174) and rs13459122 (chromosome 10: 80795365) that contained ~40 genes. Among these genes was *Pcdh15*, which when mutated in mice causes early onset deaf/circling behavior (Alagramam et al., 2001a; Hampton et al., 2003; Washington et al., 2005), making it the best candidate to harbor the *noddy* mutation. Analysis of the entire coding sequence of *Pcdh15* in *noddy* mutant mice compared with the coding sequence in the database (GenBank accession NM_0231153) revealed a point mutation, T822A, in exon 6, which produces an Ile-to-Asn substitution at position 134 within the PCDH15 protein sequence (Fig. 1B). Counting from the first residue after the cleaved 26 amino acid signal sequence, we refer to this mutation as “I108N.” Isoleucine at position 108 is highly conserved across species and is located in the PCDH15 EC1 domain (Fig. 1C,D).

To confirm that the newly defined mutation in *noddy* mutant mice is indeed associated with *Pcdh15*, we performed a classical complementation analysis. Mice homozygous for the *noddy* allele were crossed to mice heterozygous for the *Pcdh15*^{Tg2742} allele, a previously reported allele of *Pcdh15* (Alagramam et al., 2001a). Offspring with either normal or deaf-circling behavior were obtained from these crosses. Whereas offspring with normal behavior carried one wild-type *Pcdh15* allele and one *noddy* mutant allele, mice showing deaf-circling behavior showed the compound heterozygous genotype of *noddy*/*Tg2742* (Fig. 2). Lacking a wild-type allele at the *Pcdh15* locus, mice with both the *noddy* mutant allele and the *Tg2742* allele express the phenotype, unequivocally demonstrating by noncomplementation that the hair cell phenotype in the *noddy* mutant mice is caused by a mutation in *Pcdh15* rather than in some other locus. Thus, this new mutation was designated *Pcdh15*^{*noddy*}. Although other mutations in the mouse PCDH15 sequence have been described (Alagramam et al., 2001a; Hampton et al., 2003; Washington et al., 2005; Zheng et al., 2006), this is the first report of a missense mutation in mouse PCDH15. Because the EC1 domain is involved in the PCDH15–CDH23 interaction according to current models of the tip link (Kazmierczak et al., 2007; Sotomayor et al., 2012), we decided to test the functional consequences of the *noddy* mutation *in vitro* and *in vivo*.

PCDH15-I108N mutation impairs *in vitro* binding between PCDH15 and CDH23

To understand the mechanism of the *Pcdh15*^{*noddy*} mutation, we performed a series of structural and biochemical analyses *in vitro*. The I108N mutation replaces a large hydrophobic isoleucine side

Figure 2. Confirmation of allelism to *Pcdh15*. **A**, Schematic diagram showing point mutation in exon 6 of *Pcdh15* in *noddy* mutant mice. The *Noddy* mutation introduces a *MseI* restriction endonuclease site, as shown in the expanded view of the *noddy* mutation site below. Positions of forward primer (FP) and reverse primer (RP) used for genotyping are indicated. **B**, *MseI* restriction enzymes recognition site. **C**, PCR products digested with *MseI*. DNA band pattern for wild-type (+/+), heterozygote (+/n), and homozygote (n/n) for the mutation. **D**, Physical map of the mutant locus in the *Pcdh15*^{Tg2742} allele. The integration of the transgene (Tg) disrupts genomic DNA at the site of insertion and serves as a unique sequence (or “tag”) to identify the mouse carrying the mutant allele. Forward and reverse arrows indicate primers to the transgene used in PCR to identify the *Pcdh15*^{Tg2742} allele of *Pcdh15*. **E**, Detection of the 2000 bp PCR product confirms presence of the transgene. M, DNA marker ladder; Tg, Tg2742-positive genomic DNA was used as the template; WT, wild-type genomic DNA was used as the template. **F**, Classical genetic complementation analysis. Mice heterozygous for the *Pcdh15*^{Tg2742} allele were mated with mice homozygous for the *noddy* mutation. A litter of 8 from one of those crosses is shown here. Mice with either normal or *noddy* behavior were identified in this litter. Genotyping confirmed that all mice with the phenotype carried both alleles, namely, the compound heterozygous genotype (*noddy*/*Pcdh15*^{Tg2742}). Mice 5–8 are positive for the transgene (left panel) and the *noddy* allele (right panel). In contrast, mice 1–4 are negative for the transgene (left panel) and positive for the *noddy* allele (right panel). Both the wild-type and transgenic (nonfunctional) allele of *Pcdh15* from the female carry wild-type sequence with respect to exon 6, hence the appearance of a wild-type band (289 bp) in all offspring (right panel); offspring also inherits one *noddy* allele from the male, hence the appearance of *noddy* mutant bands (189 and 104 bp) in all lanes as well (right panel). Mice showing phenotype carry two nonfunctional copies of *Pcdh15*, resulting in noncomplementation, whereas mice showing normal behavior carry at least one functional/wild-type allele of *Pcdh15*.

chain with a polar asparagine. The mutation might generally impair proper folding, making PCDH15 unavailable for tip-link formation, or could impair protein function more specifically by preventing its binding to CDH23.

To test folding, we produced a recombinant PCDH15 EC1 + EC2 fragment bearing the I108N mutation. PCDH15 EC1 + EC2 I108N behaved just like the wild-type protein during the refolding and purification step, and the refolded mutant protein had an elution profile equivalent to that observed for the wild-type fragment in SEC experiments (data not shown). In addition, the CD spectrum of the I108N mutant fragment (Fig. 3A) is very similar to that of the wild-type protein, and both correspond well to that of a properly folded cadherin with antiparallel β strands (Prasad and Pedigo, 2005). Thermostability assays using both CD (data not shown) and the thermofluor method (Fig. 3A, inset) (Niesen et al., 2007) yielded well-defined melting temperatures (WT:

Figure 3. Effect of the I108N mutation on the structure of PCDH15 and its interaction with CDH23. **A**, Circular dichroism spectra of the refolded wild-type (gray) and I108N (blue) PCDH15 EC1 + EC2 fragments. Inset, Thermostability assay for the same fragments (gray, wild-type; blue, I108N; green, buffer control), with data shown as average of triplicates $\pm$ SD. Red bar represents melting temperature. Spectra and melting data indicate proper folding of both fragments. **B**, Schematic of cadherin tip link (left) and ribbon diagram (right) of PCDH15 EC1 + EC2 (purple) and CDH23 EC1 + EC2 (blue). Red box indicates location of the cadherin handshake shown in ribbon. Residue I108 of PCDH15 is shown in stick representation, and Ca^{2+} ions are shown as green spheres. **C**, Detail of PCDH15's I108 and neighboring residues, from black box in **B**. (See structure file pdb 4APX; Sotomayor et al., 2012). **D**, Raw power versus time in ITC experiments for wild-type (gray) and I108N (blue) PCDH15 EC1 + EC2, titrated with CDH23 EC1 + EC2 at 10°C . Inset, Change in molar enthalpy. The decreased enthalpic change and the lack of saturation indicate impaired interaction between PCDH15 EC1 + EC2 I108N and CDH23 EC1 + EC2. **E**, Normalized histograms of the number of water molecules within 5 Å of I108 (gray) and I108N (blue) C α atoms, from microsecond-long molecular dynamics simulations of the complex. More water is within the interface with the mutation.

$40.3 \pm 0.5^{\circ}\text{C}$; I108N: $38.3 \pm 0.5^{\circ}\text{C}$). Thus, the mutation does not seem to significantly affect the protein fold.

We then considered the structure of wild-type PCDH15 EC1 + EC2 bound to CDH23 EC1 + EC2 (Sotomayor et al., 2012). Residue I108 is at the surface of PCDH15 and is part of the interface between PCDH15 and CDH23 (Fig. 3B, C). The large hydrophobic isoleucine side chain flanks a disulfide bond at the tip of PCDH15 EC1. It is part of the hydrophobic core of the interface, surrounded by residues Y8, K12, T106 (not in view), and H110 of PCDH15, and by residues G139, A140, S143, Q187, and K189 of CDH23 (Fig. 3C). The structure therefore suggests that the I108N mutation could affect PCDH15's ability to bind to CDH23 and thereby destabilize tip-link connections between stereocilia.

To test this hypothesis, we used SEC and ITC to compare the interaction of both wild-type and mutant PCDH15 EC1 + EC2 fragments with CDH23 EC1 + EC2. We observed lack of binding in SEC experiments with the mutant fragment (data not shown). In addition, ITC experiments (Fig. 3D) showed decreased enthalpic heats and lack of saturation with the PCDH15 fragment carrying the I108N mutation, which suggests impaired binding. The enthalpic heats observed here are significantly smaller than those observed under similar conditions for the human PCDH15 R113G deafness mutation (Sotomayor et al., 2012), suggesting that the I108N mutation has a more dramatic effect on the interaction between PCDH15 and CDH23.

Replacement of isoleucine's bulky hydrophobic side chain by the slightly smaller and hydrophilic asparagine side chain could

break specific van der Waals contacts, increase hydration at the interface, or both. To elucidate the underlying molecular mechanisms of impaired binding, we performed molecular dynamics simulations of a model of the complex with the mutation (Sotomayor et al., 2010, 2012). During the simulations, more water molecules were observed within the interface than in wild-type (Fig. 3E). The hydrophilic character of the asparagine favors increased hydration of the interface, which may either prevent formation of the complex or promote rupture once formed. Together, the experimental and computational results strongly suggest that the I108N mutation severely impairs binding between PCDH15 and CDH23.

Loss of bundle integrity in *Pcdh15*^{noddy} homozygotes

To test the consequences of blocking the handshake interaction *in vivo*, we examined hair bundles by light and electron microscopy in *Pcdh15*^{noddy} homozygotes. Scanning electron microscopy of heterozygote and homozygote neonatal hair cells (Fig. 4A–E) revealed disorganized hair bundles in both inner and outer hair cells of *Pcdh15*^{noddy} homozygotes (Fig. 4A, B), which was observed also by light microscopy (Fig. 5A, B). At postnatal day 3 (P3), bundles were misoriented (Fig. 4B), were fragmented into smaller clumps of stereocilia (Fig. 4D), showed abnormal row structure (Fig. 4E), and had abnormally positioned kinocilia compared with heterozygote controls (Fig. 4C, D, letter “K”). These defects are very similar to those reported for the presumptive null allele *Pcdh15*^{av3J} (Alagramam et al., 2001a, 2001b; Paw-

lowski et al., 2006; Kikkawa et al., 2008), suggesting that the *Pcdh15^{noddy}* mutation is in some respects as severe as a null mutation. The defects become more severe within a few days of development; at P10, the heterozygote showed virtually normal ranking of stereocilia (Fig. 4*F, G*), whereas the mutant showed disorder in both position and height of the different rows (Fig. 4*H–J*).

Perhaps this missense mutation affects protein stability or normal localization of PCDH15, rather than creating a specific functional deficit in an otherwise well-folded and stable protein. Thus, we used immunocytochemistry to determine whether PCDH15-I108N is degraded or mistargeted in hair cells. Organs of Corti harvested from wild-type and *Pcdh15^{noddy}* homozygotes at P3 were stained with PB811, an antibody specific for the amino acid sequence LSLKDNVDYWLLDPVK (Kazmierczak et al., 2007), which is 37 amino acids upstream of I108. In both wild-type and *Pcdh15^{noddy}* homozygote bundles, PB811 labeled the tips of stereocilia of both inner and outer hair cells (Fig. 5*C, E*). Lack of bundle labeling in the presumptive null allele *Pcdh15^{av3f}* confirmed the specificity of PB811 (Fig. 5*D*). Close examination of hair bundles in *Pcdh15^{noddy}* homozygotes shows that PCDH15 is concentrated at the tips of stereocilia (Fig. 5*F, G*), even though the bundles were fragmented, suggesting that the observed phenotype is not the result of loss of PCDH15 expression or localization. Instead, the I108N mutation apparently produces a specific deficit in the function of EC1.

Pcdh15^{noddy} homozygotes fail to develop normal tip links

Early in hair-bundle development, numerous links connect stereocilia to each other (Furness et al., 1989; Michel et al., 2005). As bundles mature, links segregate into horizontal top connectors, which run horizontally between all adjacent stereocilia, and tip links, which run nearly vertically from shorter to taller stereocilia along the excitatory axis. At P3, control mice formed normal tip links, whereas *Pcdh15^{noddy}* homozygotes, in contrast, had links between stereocilia but rarely any that could be described as bona fide tip links (data not shown). To make sure that no true tip links form in these mice, we examined older animals (P10) for tip links, where the heterozygote showed clearly identifiable tip links (Fig. 4*G*). These were completely absent in the homozygote (Fig. 4*J*). Thus, tip links in *Pcdh15^{noddy}* homozygote mice were absent.

Pcdh15^{noddy} homozygotes lack FM1-43 uptake

In the absence of tip links, *Pcdh15^{noddy}* homozygotes should show loss of mechanotransduction. The amphipathic styryl dye, FM1-

Figure 4. Scanning electron microscopy of hair cells from heterozygous (control) and *noddy* mice at P3 (*A–E*) and P10 (*F–J*). *A, B*, Low-power examination of P3 controls showed normally oriented hair bundles in all rows of outer hair cells (OHC) and in inner hair cells (IHC), whereas in *noddy*, bundles were both fragmented and misoriented. *C*, Higher-power examination of single P3 OHC bundles from controls revealed the typical V-shaped bundle with rows of stereocilia organized in graded heights and a kinocilium at the vertex (letter “K”). *D*, In the P3 *noddy* cochleae, the V shape was disrupted and the bundle fragmented into clumps associated with abnormally positioned kinocilia (letter “K”). *E*, The stereociliary row structure was abnormal in *noddy* OHCs. *F, G*, at P10, the control specimens showed well-ordered hair bundles with few defects in row structure and well-defined tip links (arrows). *H–J*, Low-power examination of P10 *noddy* specimen showed that bundles were both fragmented and misoriented (*H*), the row structure in OHC bundles was disordered and tip links could not be identified (*I, J*). Scale bars: *A, B*, 10 μ m; *C–F, I, J*, 1 μ m; *G, H*, 4 μ m; *G, J*, 400 nm.

43, is known to enter hair cells via mechanotransduction channels that are partially open at rest. Because resting tip-link tension is required to keep the channels partially open, lack or loss of tip links prevents rapid internalization of FM1-43 (Gale et al., 2001; Meyers et al., 2003). We examined cochlear cultures from wild-type and *Pcdh15^{noddy}* homozygotes for their ability to load with FM1-43 in response to a brief (10 s) exposure to the dye. Hair cells from control mice showed dye loading in the apical and basal turn as expected (Fig. 6*A, E*). In contrast, hair cells from the *Pcdh15^{noddy}* homozygotes did not load with FM1-43 dye in any turn of the cochlea (Fig. 6*B, F*) indicating that mechanotransduction channels are closed at rest. The same was reported for the

Figure 5. Hair bundle morphology and PCDH15 expression in *noddy* homozygote hair cells at P3. **A, B**, Outer and inner cochlear hair cells in *noddy* heterozygotes (control) (**A**) and *noddy* homozygotes (*noddy*) mice (**B**), stained with phalloidin (green). Both inner and outer hair cell bundles in the homozygotes were severely disrupted in P3 hair cells. **C–G**, Cochlear hair cells stained with the PCDH15 EC1-specific polyclonal antibody PB811 (green) and phalloidin (red). PCDH15 protein is near the tips of stereocilia in control (**C**) and *noddy* homozygotes (**E–G, G'**), but not in the bundle of the *av3J* presumptive null allele of PCDH15 (**D**). Close-up view of a single outer hair cell bundle from a *Pcdh15* stained with PCDH15 antibody shows labeling at the tips of the stereocilia (**G, G'**). To detect residual staining for PCDH15 in the *av3J* bundle, a higher gain setting was used for the green channel, but no staining was found in the bundle (**D**), validating the specificity of PB811 for PCDH15. The few green dots observed outside of the bundle and on supporting cells represent nonspecific background.

presumptive null allele of *Pcdh15* (*Pcdh15^{av3J}*), which also lacked clearly identifiable tip links and transducer currents (Alagramam et al., 2011). Therefore, the I108N mutation apparently affects the ability of PCDH15 to form links with CDH23 *in vivo* as well as *in vitro*.

Discussion

We first reported that PCDH15 is essential for hearing and balance in the mouse and specifically for normal development of hair bundles (Alagramam et al., 2001a, 2001b; Pawlowski et al., 2006; Kikkawa et al., 2008). More recently, we showed that an in-frame deletion of 47 amino acids in EC9 of PCDH15 (associated with the *av6J* allele of *Pcdh15* in the mouse) does not prevent formation of tip links, although the *av6J* links are apparently not stable over time (Alagramam et al., 2011). Similarly, a missense mutation in EC7 of mouse CDH23 does not affect the formation

of tip links, although they are progressively lost (Schwander et al., 2009). In contrast, *Pcdh15^{noddy}* homozygotes show that a point mutation in EC1 of PCDH15 disrupts bundle integrity, much like the null allele *Pcdh15^{av3J}*. The hair bundle arrangement at P3 in *Pcdh15^{noddy}* homozygotes was severely disrupted and misoriented; the kinocilia were often abnormally positioned as well. PCDH15 I108N was expressed in the hair bundle in *Pcdh15^{noddy}* homozygotes, but tip links did not form; thus, the observed phenotype is likely the result of impaired interaction with its partner, CDH23, rather than degradation or mislocalization of the mutant protein.

Data presented here and the phenotypic similarity to the *Pcdh15^{av-3J}* mice (Alagramam et al., 2011) suggest that *noddy* homozygotes lack mechanotransduction. Both *noddy* and *Pcdh15^{av-3J}* homozygotes failed to (1) develop normal tip links, (2) develop normal bundle morphology, and (3) load FM1-43. In contrast to controls, hair cells of *Pcdh15^{av3J}* homozygotes have links between stereocilia but rarely with the stereotypical alignment along the excitatory axis from short to tall. Hair cells from *Pcdh15^{av-3J}* mice fail to produce normal mechanotransduction currents in response to fluid-jet stimulation, indicating that the observed links are not bona fide tip links. Because the hair cell phenotype in the *noddy* homozygote is a phenocopy of the defects observed in *Pcdh15^{av-3J}* mutant (points 1–3 above), it is likely that mechanotransduction is impaired in *noddy* mutant hair cells as well.

The hair bundle phenotypes in *Pcdh15^{av3J}* and *Pcdh15^{noddy}* indicate that loss of PCDH15 function affects normal hair bundle development concomitant with lack of tip link formation in mouse cochlear hair cells. We hypothesize that, in addition to its role in the tip links, PCDH15 and CDH23 in mouse cochlear

hair cells also serve as key components of intercilary links, including kinociliary links. These intercilary links might be essential for bundle cohesion and for the polarized, V-shaped arrangement of the bundle with the kinocilia at the vertex. There is some support for this idea. Using immunogold labeling in vestibular hair cells of chicken, Goodyear et al. (2010) showed that the kinociliary links are made of PCDH15 and CDH23 proteins, that CDH23 localizes to the stereocilium, and that PCDH15 localizes to the kinocilium. Therefore, it is conceivable that loss of the PCDH15–CDH23 interaction in the mouse cochlear hair bundle causes the loss of bundle cohesion and orientation.

In previous studies based on the crystal structure of CDH23 EC1 + EC2, it was proposed that CDH23 interacts with PCDH15 via a calcium-binding site created by polar residues at the tips of

EC1 of both proteins (Sotomayor et al., 2010; Elledge et al., 2010). Through cocrystallization of the proteins, we instead discovered an extended “handshake” interface linking EC1 + EC2 of CDH23 and PCDH15 (Fig. 3*B*) (Sotomayor et al., 2012). Structural analyses reported here provide further support for this interface and demonstrate that I108 of PCDH15 is in a critical part of the binding interface between the EC1 domain of PCDH15 and the EC2 domain of CDH23. The PCDH15-I108N mutation in *Pcdh15^{noddy}* mutant mice shows that residues more distal from the extreme tip of EC1 are important for binding. The *in vivo* and complementary *in vitro* data presented here further support an extended binding interface between PCDH15–CDH23 EC1 and EC2.

The extended binding interface model is consistent with the unique requirements for cadherin binding in the inner ear. It is likely that tip links endure greater mechanical stress resulting from loud sound stimuli than do classic cadherins at cell–cell junctions. The extended interface might create a tip-link bond that is mechanically stronger than that proposed for a single Ca^{2+} binding site at the tip of EC1. Indeed, steered molecular dynamics simulations forcing unbinding of the PCDH15 and CDH23 complex suggest that the heterophilic bond is mechanically stronger than that of a typical homophilic C-cadherin bond (Sotomayor et al., 2012).

The data presented here also highlight the variety of phenotypes and molecular mechanisms associated with deleterious mutations in PCDH15 and CDH23. Although many mutations in human CDH23 and PCDH15 replace canonical calcium-binding residues (de Brouwer et al., 2003), the *Pcdh15^{noddy}* mutation impairs binding but does not modify a residue in or near any of the calcium-binding domains (Fig. 1*C*). Thus, the interaction between PCDH15 and CDH23 can be affected by mutations in amino acids not involved in calcium binding. *Pcdh15^{noddy}* mice may serve as a good model for the human PCDH15-R113G mutation (reported as R139G, including the signal sequence) (Ahmed et al., 2003), which is similarly located in EC1 but not at calcium-binding sites. The PCDH15-R113G mutation impairs binding *in vitro* of full-length PCDH15 to CDH23 (Kazmierczak et al., 2007), and it inhibits binding of PCDH15 protein fragments to tip links in cochlear cultures (Lelli et al., 2010). The lack of a vestibular phenotype for PCDH15-R113G is consistent with a more dramatic effect of the *Pcdh15^{noddy}* mutation on the interaction between PCDH15 and CDH23 in *in vitro* ITC experiments (Sotomayor et al., 2012).

In contrast, the zebrafish PCDH15-V116Q mutation in EC1 (reported as V142Q, including signal sequence) causes severe auditory and vestibular phenotypes by modifying a residue close to the calcium-binding domain of PCDH15 EC1 + EC2 (Seiler et al., 2005). This mutation might impair folding or decrease calcium-binding affinity. Similarly, the *salsa* mu-

Figure 6. Function of hair cells from *noddy* mice. Basal activity of the mechanotransduction channel was tested with FM1-43 uptake in *noddy* heterozygous (control) and homozygous (*noddy*) cochlear hair cells in culture at P3. **A–D**, Apical turn. **E–H**, Midbasal turn. After washout of dye, fluorescence remained in intracellular organelles, a hallmark of FM1-43 entry through transduction channels (**A**, **E**). **A**, **E**, Arrow points to the location of inner hair cells. Hair cells from *noddy* mice completely failed to load (**B**, **F**). **C**, **D**, **G**, **H**, Differential interference contrast microscopy images of **A**, **B**, **E** and **F**, respectively, show the location of the hair cells.

tation E737V directly modifies a calcium-binding residue in EC7 of CDH23 and shows only progressive hearing loss and gradual loss of tip links over time (Schwander et al., 2009). Other mutations in human CDH23 affecting calcium-binding sites produce a similar phenotype (Bork et al., 2001; Pennings et al., 2004; Wagatsuma et al., 2007; de Brouwer et al., 2003).

Recently, a mouse carrying a mutation in the EC1 domain of CDH23 was reported (Han et al., 2012). This CDH23-S47P mutation results in hearing loss, consistent with the idea that, like the EC1 domain of PCDH15, the EC1 domain of CDH23 is important for the tip-link interaction. However, the CDH23-S47P mutation is not located at the “handshake” interface, it does not impair PCDH15–CDH23-S47P binding *in vitro* (Sotomayor et al., 2012), and it causes a much milder phenotype than the I108N mutation in PCDH15 EC1. Specifically, the CDH23-S47P mutation causes late-onset hearing loss (starting at P27) without obvious vestibular dysfunction. The more dramatic effect of the PCDH15-I108N mutation supports the idea that the I108 residue of PCDH15 is in a critical part of the binding interface. This rich repertoire of molecular mechanisms and phenotypes suggests that many specific animal models are required to understand diseases associated with missense mutations in PCDH15 and CDH23. *Noddy* mutant mice provide a unique *in vivo* model for understanding the consequences of mutations to the tip-link binding interface.

In conclusion, data presented here reveal the pathogenic mechanism underlying the *Pcdh15^{noddy}* mutation in PCDH15 and important properties of the interaction between PCDH15 and CDH23, critical for hearing and balance. The *noddy* mouse is also the first mouse model for missense mutations that directly affect PCDH15 and CDH23 binding, and in this context may emerge as a tool to explore molecular therapies.

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