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Cadherin 23 is a component of the transient lateral links in the developing hair bundles of cochlear sensory cells

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Abstract

Cadherin 23 is required for normal development of the sensory hair bundle, and recent evidence suggests it is a component of the tip links, filamentous structures thought to gate the hair cells' mechano-electrical transducer channels. Antibodies against unique peptide epitopes were used to study the properties of cadherin 23 and its spatio-temporal expression patterns in developing cochlear hair cells. In the rat, intra- and extracellular domain epitopes are readily detected in the developing hair bundle between E18 and P5, and become progressively restricted to the distal tip of the hair bundle. From P13 onwards, these epitopes are no longer detected in hair bundles, but immunoreactivity is observed in the apical, vesicle-rich, pericuticular region of the hair cell. In the P2–P3 mouse cochlea, immunogold labeling reveals cadherin 23 is associated with kinocilial links and transient lateral links located between and within stereociliary rows. At this stage, the cadherin 23 ectodomain epitope remains on the hair bundle following BAPTA or La^{3+} treatment, but is lost following exposure to the protease subtilisin. In contrast, mechano-electrical transduction is abolished by BAPTA but unaffected by subtilisin. These results suggest cadherin 23 is associated with transient lateral links that have properties distinct from those of the tip-link.

Keywords: Stereocilia; Hair bundle; Hair cell; Tip link; Lateral links; Mechano-electrical transduction; Development; Cadherin 23; Usher type 1 syndrome; Inner ear

Introduction

The sensory hair bundle is a complex array of modified microvilli (stereocilia) that is located at the apex of the hair cell. It enables the hair cell to detect mechanical stimuli and transduce these stimuli into electrical signals, receptor potentials. The hair bundle is a morphologically and functionally polarized structure, with the stereocilia arranged in rows of increasing height across the apical surface of the hair cell. The hair bundles of all hair cell types, except those in the mature auditory system, also possess a kinocilium located adjacent to the tallest row of stereocilia. Deflections of the hair bundle toward the tallest row of stereocilia increase the open probability of the hair cell's mechano-electrical transducer channel. Movements in the opposite direction decrease channel open probability. Recent evidence indicates this channel is most likely to be a member of the TRP family (Corey et al., 2004; Sidi et al., 2003), and this transducer channel is thought to be gated by the tip link, a fine filamentous link that stretches obliquely from the tip of each stereocilium (except from the tips of those in the tallest

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row) to the side of an adjacent taller stereocilium (Furness and Hackney, 1985; Kachar et al., 2000; Pickles et al., 1984).

A number of other link types are also associated with the mature sensory hair bundle. In the avian inner ear, these include the kinocilial links, horizontal top connectors, shaft connectors, and ankle links. Kinocilial links (Erneston and Smith, 1986; Goodyear and Richardson, 2003; Hillman, 1969; Hillman and Lewis, 1971) connect the kinocilium (or the kinociliary bulb) to the tallest two or three stereocilia in the hair bundle and share many similarities with tip links. They are structurally similar to tip links (Tsuprun et al., 2004), have common associated epitopes (Goodyear and Richardson, 2003; Siemens et al., 2004), and are, like tip links (Assad et al., 1991; Goodyear and Richardson, 1999; Kachar et al., 2000), insensitive to treatment with the protease subtilisin but sensitive to the trivalent cation La^{3+} and the Ca^{2+} chelators, BAPTA and EGTA (Goodyear and Richardson, 2003). The other link types observed on mature hair bundles can be distinguished from tip and kinocilial links on the basis of their relative sensitivities to BAPTA and subtilisin (Goodyear and Richardson, 1999). Shaft connectors and ankle links can also be distinguished antigenically and monoclonal antibodies are available that specifically recognize epitopes that are associated with ankle links (Goodyear and Richardson, 1999), shaft connectors (Goodyear and Richardson, 1992; Richardson et al., 1990), and both tip and kinocilial links (Goodyear and Richardson, 2003).

The surface of the developing hair bundle differs considerably from that of the mature animal (Goodyear et al., in press) and has a dense network of links connecting the stereocilia. These have yet to be characterized immunohistochemically, although high levels of antigens associated with ankle links, both tip and kinocilial links, and shaft connectors, are initially expressed over the entire surface of the developing hair bundle (Bartolami et al., 1991; Goodyear and Richardson, 1999, 2003), and it has been proposed that tip links may be derived from a subset of the rich array of links found on the hair bundle during embryogenesis (Pickles et al., 1991).

Recent studies have revealed that vezatin, a major component of the adherens junction, is associated with the ankle links (Küssel-Andermann et al., 2000), that the receptor-like inositol lipid phosphatase Ptprq is likely to be a major component of the shaft connectors (Goodyear et al., 2003), and that cadherin 23 is a component of tip and kinocilial links in mature hair cells (Siemens et al., 2004; Söllner et al., 2004). Cadherin 23 is the product of the USH1D gene, a gene in which mutations cause Usher syndrome type 1 (severe sensorineural deafness associated with retinitis pigmentosa), and mutations in mouse cadherin 23 cause hair bundle malformations in the waltzer mouse (Bolz et al., 2001; Bork et al., 2001; Di Palma et al., 2001a). Cadherin 23 is a large member of the cadherin superfamily of cell–cell adhesion molecules, with an ectodomain consisting of 27 cadherin repeats, a single pass transmembrane domain, and an intracellular domain that is fairly unique and is unlikely to interact with known partners for classical cadherins like beta-catenin (Di Palma et al., 2001a). It is now known (Boëda et al., 2002; Siemens et al., 2002) that the intracellular domain of cadherin 23 can interact with another hair bundle protein, the PDZ domain protein harmonin that is encoded by the Usher 1C gene (Bitner-Glindzicz et al., 2000; Verpy et al., 2000).

We previously observed the expression of cadherin 23 in the developing hair bundle, but were unable to detect it in the hair bundles of the mature inner ear (Boëda et al., 2002). More recent studies (Siemens et al., 2004; Söllner et al., 2004) have, however, indicated that cadherin 23 is a component of the tip links, structures that are a feature of mature hair cells (Furness and Hackney, 1985; Pickles et al., 1984). Also, mutations in cadherin 23 are now known to cause late onset progressive hearing loss in mice (Noben-Trauth et al., 2003), suggesting cadherin 23 is expressed in the mature cochlea. Moreover, two cadherin 23 isoforms have been predicted that differ in the cytoplasmic domain by the presence or absence of a peptide sequence encoded by exon 68 (Di Palma et al., 2001b; Siemens et al., 2002), and additional alternative transcripts may have escaped detection.

We have therefore analyzed cadherin 23 transcripts expressed in the cochlea and undertaken a detailed study of cadherin 23 expression in the developing mouse and rat cochlea using antibodies directed against unique peptide epitopes present in the intra- and extracellular domains of this protein. We have compared the sensitivities of these epitopes present in the early postnatal cochlea to BAPTA, La^{3+}, and subtilisin, and have examined whether cadherin 23 is a component of the links present on the developing hair bundle. Our results show these cadherin 23 epitopes are only detectable in developing hair bundles and that the ectodomain epitope is associated with lateral links. The ectodomain epitope remains associated with the surface of the hair bundle following BAPTA or La^{3+} treatment, but is lost following subtilisin treatment. The cadherin 23 ectodomain epitope on the developing hair bundle is therefore associated with a structure that has properties distinct from those of tip links.

Methods

RACE and RT-PCR

Rapid amplification of cDNA ends (RACE) was performed with the BD-Smart RACE cDNA Amplification kit (BD-Clontech) on P2 to P6 vestibular $polyA^+$ RNA using as reverse primer 5′-AACGGAGGCTGCCCTGGCTTGG-3′. RT-PCR was performed on P6 cochlear total RNA using as forward primers primer A: 5′-TTCTGTGGCTGGCCCA-GGGAATGG-3′ matching sequences located in the 5′ untranslated region upstream of exon 66 or primer B: 5′-

CCACAATGATACCGCCATCATC-3′ matching sequences located in exon 60.

Preparation and purification of antibodies

Rabbits were immunized with a mixture of three peptides that were based on the predicted amino acid sequence of mouse cadherin 23 and conjugated to keyhole limpet hemocyanin. Antibodies were affinity purified from the resultant immune sera on the individual peptides or with a combination of all three peptides conjugated to resin (Covalab, Lyon, France). Antibody N1 is directed against the extracellular domain peptide epitope NH2-CRGPRPLD-RERNSSH-COOH encoded by exon 29, antibody Cyto is directed against the C-terminal cytoplasmic domain peptide epitope NH2-FERNARTESAKSTPLHK-COOH encoded by exon 69, and antibody Ela3 is directed against three peptide epitopes; N1, a second extracellular domain peptide epitope, N2 (NH2-GDISVLSSLDREKKDH-COOH encoded by exon 52), and peptide epitope Cyto. Antibody Ela3N is directed against the extracellular domain peptide epitopes N1 and N2. Antibody E1 was raised against a recombinant fragment of the cytoplasmic domain of human cadherin 23 (including the peptide sequence encoded by exon 68) in a rabbit and affinity purified on the same protein fragment (Reiners et al., 2003). Fig. 1a provides a summary of the antibodies used in this study and the antigens with which they react, and displays where these antigens are

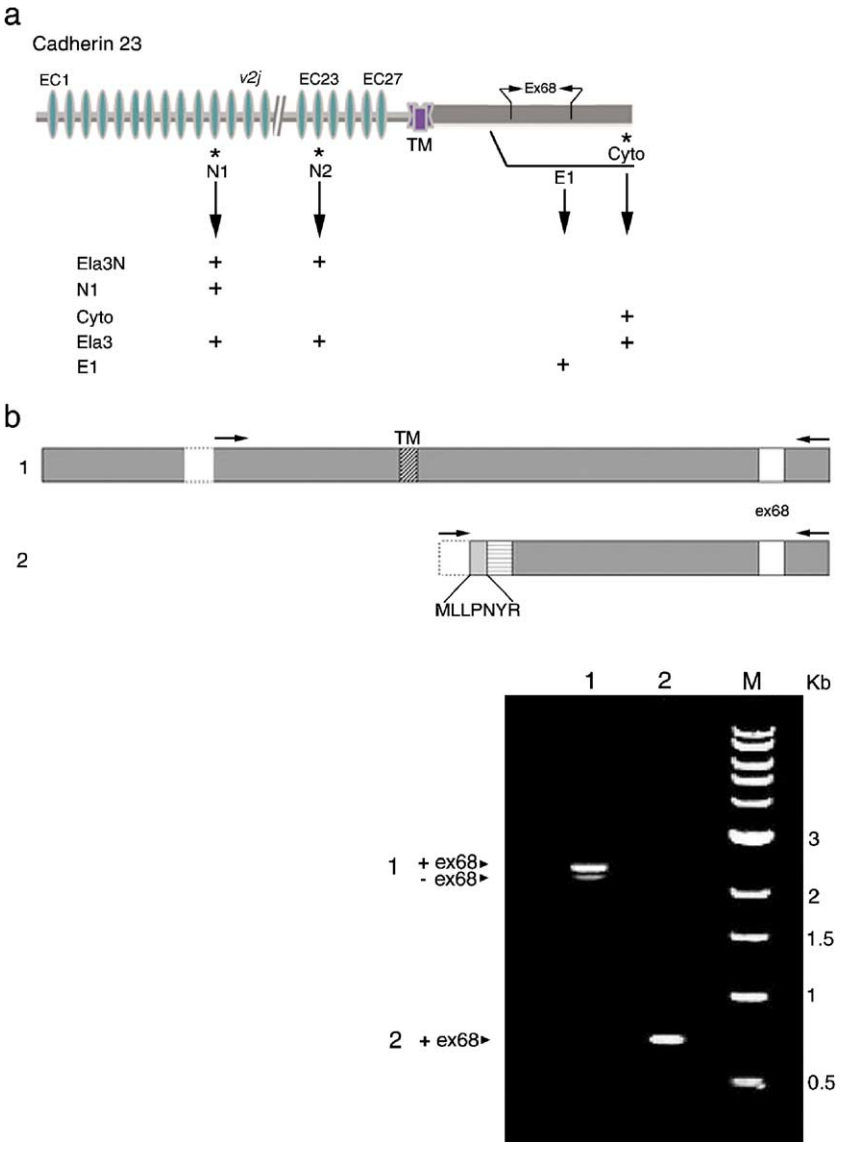

Fig. 1. Summary of antibodies used in the study and RT-PCR analysis of cadherin 23 expression in the cochlea. (a) Diagram showing the predicted structure of cadherin 23 and the location of the antigens used to generate the different antibodies used in this study. (b, upper) Location of primers used and schematic representation of the different isoforms predicted from RT-PCR. (1) Transmembrane form, (2) cytoplasmic form. TM, transmembrane domain; ex 68, exon 68. (Lower) RT-PCR analysis of cadherin 23 expression in the cochleae at P6 reveals (lane1) transmembrane forms with and without exon 68, and (lane 2) cytosolic form with exon 68. Lane M is a 1-kb marker.

located within the predicted structure of the cadherin 23 molecule.

Preparation of mouse cochlear cultures

Cochlear cultures were prepared from CD1 or waltzer v^{2J} mice from P0 to P2 using methods described previously (Russell and Richardson, 1987). The cochlear coils were dissected in HEPES-buffered (10 mM, pH 7.2) Hanks' balanced salt solution (HBHBSS), plated onto hydrated collagen gels prepared on glass coverslips, fed with one drop (approximately 50 μl) of medium (93% DMEM/F12, 7% fetal calf serum, 10 mM HEPES, pH 7.2, 10 μg/ml ampicillin), sealed into Maximow slide assemblies, and grown for 1–2 days at 37°C.

Treatment of cochlear cultures with BAPTA, La^{3+} and subtilisin

Coverslips with adherent cultures were removed from the Maximow slide assemblies, placed in 35-mm diameter plastic Petri dishes, washed twice with HBHBSS or, if to be treated with La^{3+}, twice with phosphate/Mg^{2+}-free control saline, and incubated for 15 min at room temperature with either HBHBSS, Ca^{2+} free-HBHBSS containing 5 mM BAPTA, HBHBSS containing 50 μg/ml subtilisin, phosphate/Mg^{2+}-free control saline, or phosphate/Mg^{2+}-free saline containing 5 mM $LaCl_3$. Following treatment with HBHBSS, BAPTA, and subtilisin, the solutions were removed and fixative (3.7% formaldehyde in 0.1 M sodium phosphate, pH 7.4) was added. Following treatment with phosphate/Mg^{2+}-free saline or La^{3+}, cultures were briefly washed once with phosphate/Mg^{2+}-free saline prior to fixation to prevent the La^{3+} from forming a precipitate with the phosphate buffer. Following fixation for 1 h at room temperature, the collagen films with adherent cultures were removed from the glass coverslips, placed in preblock solution for 1 h, and immunolabeled as described below. Each experiment was repeated 3 or more times with a minimum of four explants (two apical and two basal-coil cultures) being exposed to the different conditions in each experiment. Data shown are from apical coil cultures.

Solutions for experiments were prepared as follows: HBHBSS was prepared from 10× Ca^{2+}/Mg^{2+}-free HBSS without $NaHCO_3$ and contained a final concentration of 1× HBSS, 1.3 mM $CaCl_2$, 0.9 mM Mg^{2+}, and 10 mM HEPES, pH 7.2. BAPTA was prepared from 10× Ca^{2+}/Mg^{2+}-free HBSS without $NaHCO_3$ and contained a final concentration of 1× HBSS, 5 mM BAPTA, 0.9 mM Mg^{2+}, and 10 mM HEPES, pH 7.2. Subtilisin (Sigma Protease type XIV) was prepared as a 5 mg/ml stock solution in HBHBSS and diluted into HBHBSS to a final concentration of 50 μg/ml. Phosphate/Mg^{2+}-free La^{3+} control saline was prepared from 10× stock solutions and contained 155 mM NaCl, 6 mM KCl, 3 mM glucose, 4 mM $CaCl_2$, 10 mM HEPES, pH 7.2. La^{3+} solution was of the same ionic composition with the addition of 5 mM $LaCl_3$, as described by Kachar et al. (2000).

Procedures for immunofluorescence microscopy

For immunofluorescence microscopy, mouse cochlear cultures were washed twice with HBHBSS, fixed for 1 h at room temperature with 3.7% formaldehyde in 0.1 M sodium phosphate buffer pH 7.4, washed three times in PBS (150 mM NaCl, 10 mM sodium phosphate pH 7.4), and preblocked for 1 h in TBS (150 mM NaCl, 10 mM Tris–HCl pH 7.4) containing 10% heat-inactivated horse serum (Life Technologies, Paisley, UK). When using the Cyto antibody directed against the cadherin 23 intracellular domain, preblock contained 0.1% Triton X-100 (TX-100) and the entire staining procedure was done in the presence of 0.1% TX-100. When using the Ela3N or N1 antibodies directed against extracellular domain peptides, TX-100 was usually omitted until the final double labeling step with fluorochrome-conjugated antibodies and phalloidin. The omission or inclusion of TX-100 did not affect the staining patterns observed with these extracellular domain antibodies. Following the preblock, samples were incubated overnight in preblock solution containing affinity purified antibodies Ela3N, N1, or Cyto at a final concentration of 1–3 μg/ml with gentle agitation, washed 5 times in TBS/0.1% horse serum, and incubated for 1–3 h in FITC-conjugated swine anti-rabbit Ig (Dako, High Wycombe, UK) either with or without 10 ng/ml rhodamine-conjugated phalloidin. Following 3 washes in TBS/0.1% horse serum and 2 washes in TBS, cultures were mounted in Vectashield (Vector Laboratories, Peterborough, UK). Specimens were viewed with a Zeiss Axioplan microscope equipped with a 100-W Attoarc mercury lamp using ×40 Plan Neofluar NA 0.75, ×63 Plan Apochromat NA 1.4 oil immersion, or ×100 Plan Apochromat NA 1.4 oil immersion lenses, and digital images were captured with a Spot RT slider camera at a resolution of 1600 × 1200 pixels, and with a Zeiss LSM 510 confocal microscope using a ×63 NA C-Apo 1.2 NA water immersion lens.

Wholemount preparations of rat and mouse cochleae at different stages of development were prepared as follows. The animals were killed by exposure to CO_2 followed by decapitation, and the inner ears were removed and placed in PBS. The organ of Corti was exposed by removing the stria vascularis, and the tissues were fixed in microwells containing 60 μl 4% paraformaldehyde in PBS for 1 h at room temperature. The tissues were washed three times in PBS (10 min for each wash), incubated for 1 h at room temperature in PBS containing 20% normal goat serum and 0.3% TX-100, washed twice with PBS, and stained overnight with the affinity purified anti-cadherin 23 peptide antibodies diluted 1:100 (1–3 μg/ml) in PBS containing 1% bovine serum albumin (PBS/1% BSA). As a control, primary antibodies were omitted and the samples were incubated overnight in PBS/1% BSA. Following three 10-min washes with PBS, the

tissues were incubated in Alexa 488-conjugated goat anti-rabbit Fab́2 antibodies diluted 1:500 in PBS/1% BSA containing TRITC-conjugated phalloidin (1:2000 dilution) for 1 h at room temperature. After three washes with PBS, the tectorial membrane was carefully dissected away and the pieces were mounted under glass coverslips with the hair bundles directed toward the coverslip in Fluorosave (Calbiochem, USA). Samples were viewed with a ×63 Plan Apochromat oil immersion lens (NA 1.2) using a Zeiss LSM-510 confocal microscope.

Immunogold labeling for electron microscopy

Cochlear cultures were washed twice with HBHBSS to remove medium and fixed in 3.7% formaldehyde in 0.1 M sodium phosphate buffer pH 7.4 for 1 h at room temperature. The collagen films with adherent cultures were then removed from the glass coverslips and placed into preblock (see above) for 1 h. Cultures were then incubated with gentle agitation overnight at 4°C in preblock containing affinity purified Ela3N or N1 antibodies at a concentration of 1 μg/ml, or non-immune rabbit IgG (Sigma, Poole, UK) at the same concentration. After multiple washes in TBS containing 0.05% Tween, cultures were incubated for 24–48 h at 4°C with goat anti-rabbit Ig conjugated to 5-nm gold particles (British BioCell, Cardiff, UK) diluted 1:10 in TBS/0.05%Tween/1 mM EDTA/1 mM sodium azide. Following extensive washing, the cultures were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h, washed three times with cacodylate buffer, and osmicated (1% OsO_4 in 0.1 M sodium cacodylate buffer) for 1 h. Following a brief wash with H_2O, cultures were dehydrated with ethanol and embedded in TAAB 812 resin. Sections were cut at a thickness of 90 nm, mounted on copper grids, stained with uranyl acetate followed by lead citrate, and viewed with a Hitachi 7100 transmission electron microscope operating at 80 kV.

Measurements of mechano-electrical transducer currents in mouse outer hair cells

Apical coil OHCs (n = 13) of CD-1 mice were studied either in organotypic cochlear cultures (ages P2–P3: P1 plus 1 or 2 days in vitro) or following acute dissection of the organ of Corti (P6–P7). Organotypic cultures were prepared as described above. After acute dissection, apical coil organs of Corti were transferred to a recording chamber in which they were immobilized under a nylon mesh fixed to a stainless steel ring. Extracellular solution was continuously bath-applied at a rate of 6 ml/h and contained (in mM): 135 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 0.7 NaH_2PO_2, 2 Na-pyruvate, 5.6 D-glucose, 10 HEPES. Amino acids and vitamins for Eagle's MEM were added from concentrates (Life Technologies, UK). The pH was adjusted to 7.5 with NaOH.

Mechano-electrical transducer currents were elicited in OHCs using fluid jet stimulation (45 Hz sine waves filtered at 1 kHz, 8-pole Bessel) and recorded under whole-cell voltage clamp (EPC8 HEKA, Germany) as described previously (Kros et al., 1992). Patch pipettes (resistance in the bath 2–3 MΩ) were pulled from soda glass capillaries and coated with wax. Intracellular solutions contained (in mM): 147 CsCl, 2.5 $MgCl_2$, 1 EGTA, 2.5 Na_2ATP, 5 HEPES; pH adjusted to 7.3 with 1 M CsOH. Data were acquired using Asyst software (Keithley Instruments, Rochester, NY, USA), filtered at 2.5 kHz, sampled at 5 kHz, and stored on computer for off-line analysis. Membrane capacitance (C_m) was 6.5 ± 0.1 pF and series resistance after electronic compensation of up to 70% (R_s) was 2.8 ± 0.3 MΩ, resulting in voltage clamp time constants of 18 ± 2 μs (n = 14). Membrane potentials were corrected for a −4 mV liquid junction potential between pipette and bath solutions, but not for any voltage drop (usually less than 3 mV) across the residual series resistance. All experiments were conducted at room temperature (22–25°C). All means given in the text are expressed ± SEM. The criterion for statistical significance was set at $P < 0.05$.

For examining the effects of subtilisin on transduction, cochleae were treated for 15 min with subtilisin (50 μg/ml, Sigma, Gillingham, UK) before transferring them into the recording chamber containing normal extracellular solution. Transducer currents were usually recorded between 10 and 50 min after the application of subtilisin. To study the effects of calcium chelation, hair cells were locally superfused with 5 mM BAPTA (Molecular Probes, Leiden, the Netherlands), added to the extracellular solution, through a multibarreled pipette positioned close to the patched cells. In some of these experiments, Ca^{2+} and/or Mg^{2+} were omitted from the superfused solution. In all BAPTA solutions used, the concentration of NaCl was adjusted to keep the osmolality constant. For every solution change, the fluid jet used for stimulating the hair bundles was filled with the new solution by suction through its tip to prevent dilution of the BAPTA in the superfused solution around the hair bundle during stimulation.

Results

Cadherin 23 transcripts expressed in the inner ear were analysed by RT-PCR. 5' RACE PCR was performed on poly A^+ mRNA extracted from vestibular neuroepithelium microdissected from mice at P2 to P6 using a primer located in the last coding exon, exon 69. Two types of transcripts were identified. One is predicted to encode a transmembrane form of cadherin 23. The other is shorter and contains an open reading frame of 234 or 199 amino acids, depending on the presence or absence of exon 68, respectively, and begins with an ATG initiation codon within the context of a Kozak consensus sequence (CCTATGC). The 5' untranslated region of this shorter type of transcript is 320 bp long and has an in-frame stop codon located 24 bp upstream of the alternative translation start site. These shorter transcripts are

predicted to encode forms of cadherin 23 that lack a transmembrane domain and do not have a signal peptide, and are therefore expected to be cytosolic. These cytosolic forms begin with 7 amino acids, (M)LLPNYR, derived from sequences located immediately upstream from the previously defined 5' boundary of exon 66. Comparative genome analysis reveals the putative initiator ATG is preceded by an upstream stop codon in the rat, although two of the predicted 7 amino acids are different. In the human genome, such a sequence upstream of exon 66 is not detected. Primers were designed to explore the expression of mRNAs encoding the short and transmembrane isoforms in the cochlea. Splice variants encoding transmembrane and cytosolic forms of cadherin 23 were also found in the cochlea at P6; a single short splice variant was detected which contains exon 68 sequences and two variants encoding transmembrane forms, a highly prevalent one containing the sequence from exon 68, and a minor one in which this exon is spliced out (Fig. 1b). Cytosolic forms expressed in transfected COS cells, either with or without the sequence encoded by exon 68, were recognized by the Cyto antibody in immunolabeling experiments (data not shown).

All of the affinity-purified antibodies used in this study, Ela3N directed against two peptides in the cadherin 23 ectodomain, N1 directed against the distal, N-terminal ectopeptide, Cyto directed against a C-terminal peptide, Ela3 directed against all three peptides (N1, N2 and Cyto), and E1 directed against the recombinant cytoplasmic domain of cadherin23 (see Fig. 1a), were tested for specificity by staining cultures prepared from the cochleae of heterozygous $+/v^{2J}$ and homozygous v^{2J}/v^{2J} waltzer mice. In v^{2J}/v^{2J} waltzer mice, the first nucleotide of intron 32 alters the wild type donor splice site. A number of aberrant transcripts are produced from this allele, and almost all analysed were found to introduce a premature stop codon (Di Palma et al., 2001a). All the antibodies provided bright staining of the distal ends of the hair bundles in the heterozygous controls (Figs. 2a, e, i, m, and q), whereas staining could not be detected in the hair bundles of the homozygous mutants (Figs. 2c, g, k, o, and s). Phalloidin labeling clearly revealed the presence of hair bundles in the homozygous v^{2J}/v^{2J} cultures, and these exhibited varying degrees of disorganization (Figs. 2d, h, i, p, and t).

The extracellular domain peptide epitopes recognized by the antibody N1 can only be detected in the hair bundles of the cochlea during their development (Figs. 3a–h). In the rat cochlea, the entire surface of the emerging hair bundles of the inner hair cells (IHCs) is labeled at E18 (Fig. 3a). The hair bundles of the outer hair cells (OHCs) are very faintly labeled by the N1 antibody at this stage. At P1, the hair

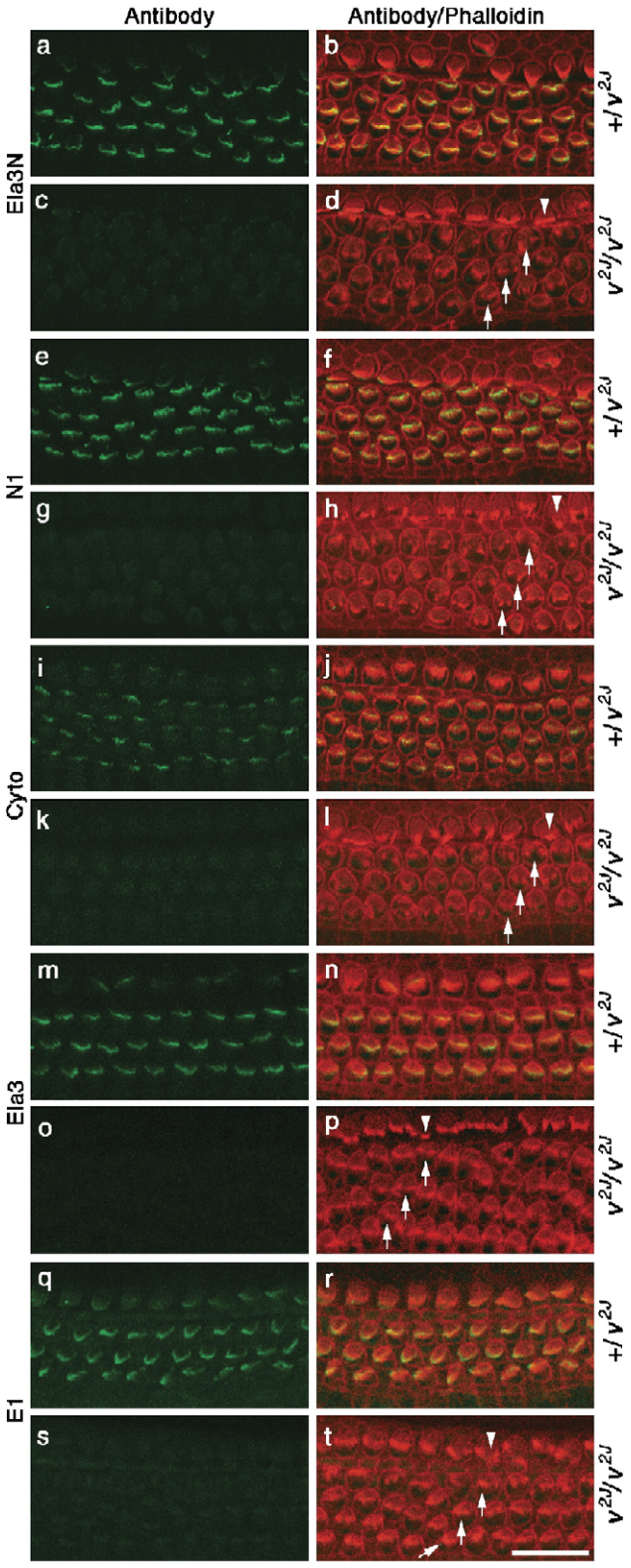

Fig. 2. Specificity of anti-cadherin 23 peptide antibodies. Apical coil cochlear cultures from $+/v^{2J}$ (a, b, e, f, i, j, m, n, q, r) and v^{2J}/v^{2J} (c, d, g, h, k, l, o, p, s, t) mice double labeled with rhodamine phalloidin and affinity purified antibodies Ela3N (a–d), N1 (e–h), Cyto (i–l), Ela3 (m–p), and E1 (q–t). Images on the left are the signal from the antibody label, images on the right are the overlays of the same images with the corresponding signal from phalloidin. Each antibody specifically stains the hair bundles in the heterozygotes, but does not stain the hair bundles in the homozygotes. Arrowheads in d, h, l, p, and t indicate hair bundles of IHCs, arrows indicate hair bundles of OHCs. Images are compressed confocal *z* stacks. Scale bar = 20 μm.

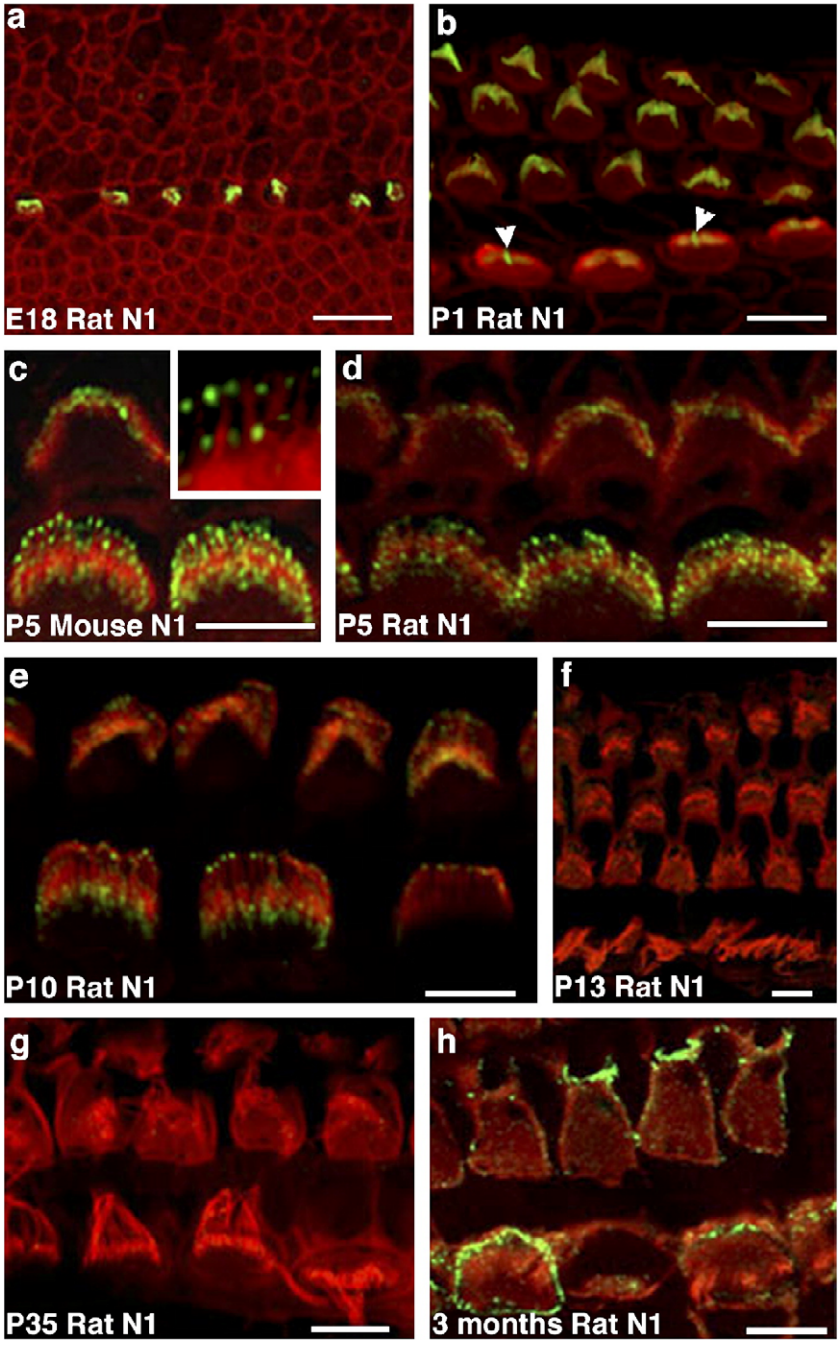

Fig. 3. Expression of N1 epitopes during cochlear development. Wholemount preparations of the rat (a–b and d–h) and mouse (c) cochlea double labeled with TRITC phalloidin (red channel) and affinity purified N1 antibody (green channel). N1 labels IHC hair bundles at E18 (a) and those of OHCs at P1 (b). By P5, N1 labeling of hair bundles in mouse (c) and rat (d) is restricted to the distal ends of the stereocilia. N1 labeling diminishes by P10 (e) and cannot be detected at either P13 (f) or P35 (g). N1 labeling can be detected at 3 months of age in the apical, pericuticular region of both inner and outer hair cells (h). Arrowheads in b indicate the kinocilium. Inset in c reveals staining between the stereocilia. Images are single confocal sections. Scale bars = 10 μm.

bundles of IHCs are labeled only at their extreme distal tips, while those of the OHC are labeled over the distal-most two-thirds (Fig. 3b). The kinocilium is also clearly labeled by N1 at this stage of development (Fig. 3b). By P5, these epitopes become progressively restricted to the distal regions of the stereocilia in all rows, including those in the tallest row (Figs. 3c and d). In IHCs at this stage, staining is observed between the distal ends of adjacent stereocilia in the tallest and second highest rows (Fig. 3c, inset). Punctate staining is also observed around the base of the IHC hair bundle. Staining becomes further reduced by P10, and only a few spots that are brightly stained by the N1 antibody can be observed in the hair bundles of IHCs and OHCs at this stage (Fig. 3e). The N1 epitopes cannot be detected on the hair bundles of IHCs or OHCs at either P13, P35, or 3 months of age (Figs. 3f–h). Increasing the concentration at which the

N1 antibody was used for immunolabeling 10-fold failed to reveal the presence of hair bundle staining at P13. At 3 months of age, however, epitopes recognized by the N1 antibody re-appear within the hair cell cytoplasm of inner and outer hair cells in the rat cochlea, in the vesicle-rich zone surrounding the cuticular plate that is known as the pericuticular necklace (Fig. 3h). In the fully developed mouse cochlea (at P21), hair bundle staining could not be detected with antibodies to the ectodomain of cadherin 23 in either wholemount preparations or cryosections of tissues that had been fixed and decalcified with EDTA (not shown). The decalcification procedure did not prevent the ectodomain epitopes from being detected in cryosections of the early postnatal inner ear.

The intracellular domain epitopes recognized by the antibody Cyto can be detected in the hair bundles of the IHCs at E18 (Fig. 4a), and in those of the OHCs by P1 (data not shown), at the same time that the ectodomain epitopes can be recognized. As observed for the ectodomain epitopes, the intracellular domain epitopes can no longer be detected in the hair bundles of either IHCs or OHCs by P13 (Fig. 4b), but they re-appear somewhat earlier than the ectodomain epitopes, by P21, in the apical pericuticular region, in the region of the basal body, and in deeper regions of the cytoplasm in IHCs and OHCs (Fig. 4c). In the OHCs at P35, Cyto staining in the apical, pericuticular region appears to be closely associated with the plasma membrane (Fig. 4d).

Results similar to those obtained with antibodies directed against the individual peptides N1 and Cyto were obtained with the Ela3 antibodies directed against all three peptides (Fig. 5). The hair bundles of IHCs were stained intensely at E18 (Fig. 5a), and staining of both the IHC and OHC hair bundles was visible by P1 (Fig. 5b). Prominent staining of the kinocilium was observed at this stage, especially on the OHCs (Fig. 5b). By P5, hair bundle staining was less intense (Fig. 5c). By P13, hair bundle staining was no longer visible, however, labeling was visible in the pericuticular necklace region of the IHCs (Fig. 5d). At P21 and P35, bright, clearly defined labeling of the pericuticular necklace was observed, especially in IHCs (Figs. 5e–g).

Immunogold labeling of mouse cochlear cultures prepared from early postnatal mice with Ela3N and N1 antibodies confirmed that cadherin 23 is concentrated in the distal end of the hair bundles at these stages (Fig. 6a). Labeling is associated with the filamentous links that are present between the different rows of stereocilia (Figs. 6a and b), and with those that run between the stereocilia within a row (Figs. 6c and d). Labeling is also associated with the kinocilial links that are found at this stage of development located between the top of the kinocilium and the immediately adjacent stereocilia (Figs. 6e and f). Gold labeling was not observed in cultures that had been stained with rabbit non-immune IgG (data not shown).

Mouse cochlear cultures prepared from early postnatal mice were treated with either BAPTA, La^{3+}, or subtilisin

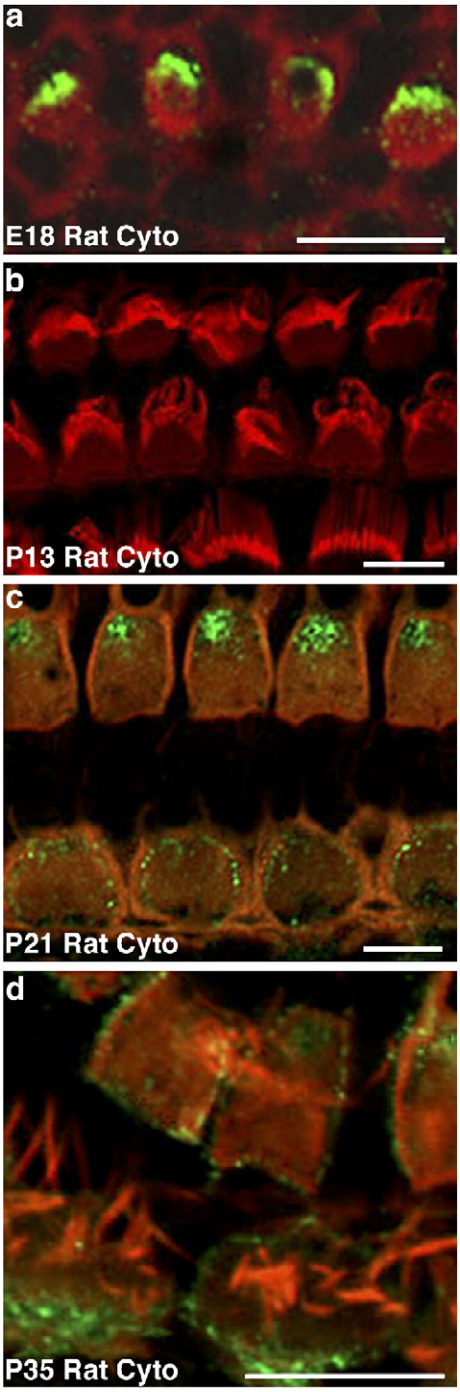

Fig. 4. Expression of Cyto epitopes during cochlear development. Wholemount preparations of the rat cochlea double labeled with TRITC phalloidin (red channel) and affinity purified Cyto antibody (green channel). Cyto labels IHC hair bundles at E18 (a). Cyto labeling cannot be detected at P13 (b), appears in the apical pericuticular region of IHC and OHC at P21 (c), and can still be detected at P35 (d). Images are single confocal sections. Scale bars = 10 μm.

to examine the relative sensitivity of the cadherin 23 epitopes recognized by the N1, the Ela3N, and the Cyto antibodies, and those recognized by the E1 antibodies directed against the intracellular domain of recombinant human cadherin 23 (Figs. 7 and 8). The epitopes

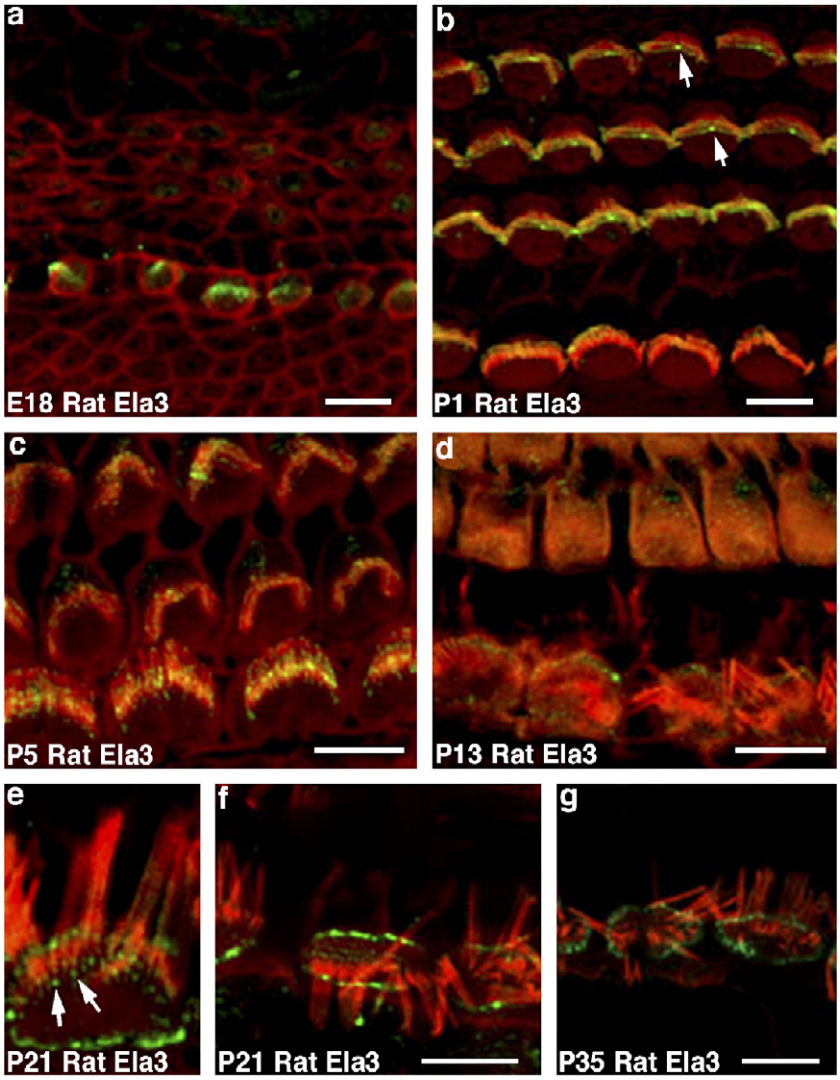

Fig. 5. Expression of Ela3 epitopes during cochlear development. Wholemount preparations of the rat cochlea double labeled with TRITC phalloidin (red channel) and a mixture of antibodies, Ela3, directed against all 3 peptide epitopes (green). Ela3 labels the hair bundles of IHCs at E18 (a), and the distal tips of inner and outer hair cell bundles at P1 (b). Note the strong labeling of the kinocilia (arrows) on the OHCs. Hair bundle staining becomes diminished by P5 (c) and labeling can only be observed in the cytoplasm at P13 (d). At P21 and P35, prominent staining is observed in the pericuticular necklace of the IHCs (e–g). Images are single confocal sections. Scale bars = 10 μm.

recognized by the Ela3N antibody are retained on the hair bundle surface following treatment with either BAPTA or La^{3+} (Figs. 7d and m), but lost following exposure to subtilisin (Fig. 7g). Identical results were obtained using the N1 antibody (Fig. 8). The epitopes recognized by the Cyto antibody are resistant to treatment with BAPTA, La^{3+}, or subtilisin, although some slight reduction in stain intensity was observed following La^{3+} treatment (Figs. 7e, h, n and 8). Results similar to those observed with Cyto were also obtained with E1 (Figs. 7f, i, and o). The cadherin 23 ectodomain epitopes also remained associated with the hair bundles when cultures were treated with BAPTA in the absence of Mg^{2+}, or with 5 mM EDTA (data not shown).

Mechano-electrical transducer currents were elicited in neonatal apical coil OHCs by alternating inhibitory and excitatory sinusoidal mechanical stimuli (Géléoc et al., 1997; Kros et al., 1992). Large mechano-electrical transducer currents could be recorded in response to a fluid jet stimulus (Fig. 9a), and these were rapidly abolished when 5 mM BAPTA was superfused over the apical surface of the cochlear epithelium at P3 (Fig. 9b) and P6. BAPTA abolished transducer currents irrespective of the presence or absence of Mg^{2+} (0.9 mM) in the extracellular solution. In all experimental conditions, the transducer current was strongly reduced within a minute of the onset of BAPTA superfusion. Mechano-electrical transducer currents were also recorded from OHCs that had been exposed to 50 μg/ml subtilisin for 15 min at room temperature (Fig. 9c). The average amplitudes of the transducer currents measured in OHCs before the superfusion of BAPTA or following subtilisin treatment at

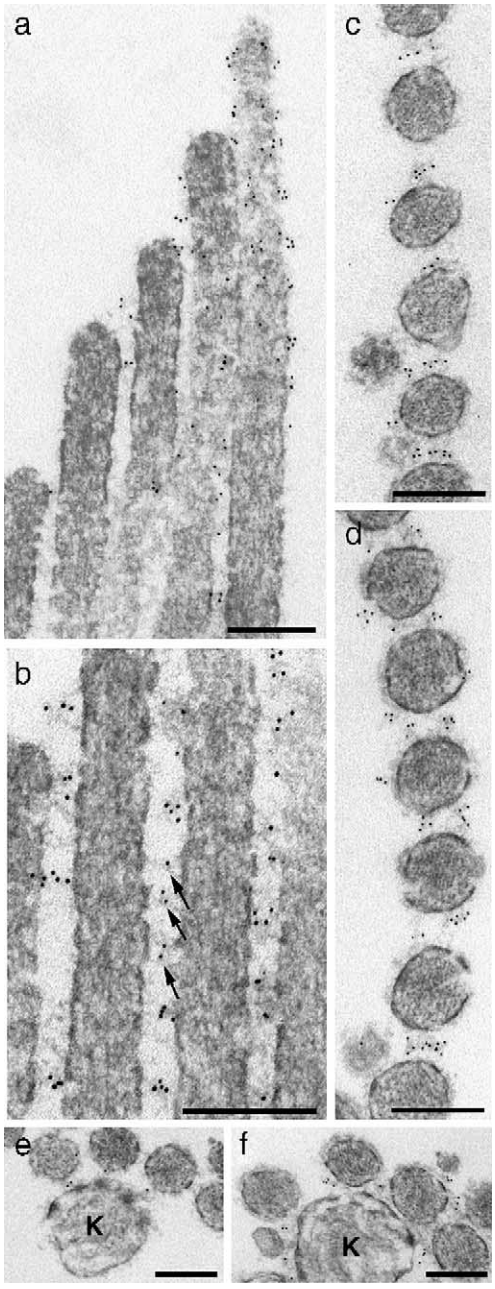

Fig. 6. Distribution of N1 and Ela3N in developing mouse cochlear hair bundles. Thin sections of mouse cochlear cultures labeled with N1 (a–d and f) or Ela3N (e) followed by 5-nm gold-conjugated anti-rabbit antibodies. Longitudinal profiles of the hair bundles (a and b) reveal cadherin 23 is concentrated at the distal end of the hair bundle (a) where it is associated with links running between the rows of stereocilia (b). Horizontal cross sections of the hair bundle (c and d) show that cadherin 23 labeling is also associated with the links that connect stereocilia within a row. Cadherin 23 is additionally associated with the links found between the kinocilium and the immediately adjacent stereocilia (e and f). The kinocilia are nested centrally within the inverted 'v' on the outer edge of the 'W'-shaped hair bundle. Scale bars = 200 nm; K, kinocilium.

different membrane potentials were similar (Fig. 9d) and comparable to those previously recorded from aged-matched control OHCs (Gale et al., 2001; Géléoc et al., 1997; Kros et al., 1992).

Discussion

The results of this study show that cadherin 23 is associated with links that interconnect the stereocilia of the developing hair bundle and with links connecting the kinocilium to the adjacent stereocilia. Cadherin 23 is initially expressed at high levels over the entire surface of the emerging hair bundle, and as development proceeds, it disappears from the base of the hair bundle and becomes progressively restricted to the distal tip. These observations provide strong support for the hypothesis (Boëda et al., 2002) that cadherin 23 plays a critical role, as a component of the transient lateral links, in ensuring the cohesion of the stereocilia during the early stages of hair bundle development. These transient lateral links may maintain adhesive forces within the hair bundle during the initial stages of its growth. The results also predict at least three 'isoforms' of cadherin 23 are expressed in the cochlear epithelium during the early stages of hair bundle development; two transmembrane forms differing by the presence or absence of the peptide encoded by exon 68 in the cytoplasmic domain, and a cytosolic form. This latter form does not have a predicted transmembrane or ectodomain and may not be membrane anchored. It is unlikely to function as an adhesion molecule although it may well modulate the interaction of the transmembrane forms with their cytoplasmic partners.

Cadherin 23 could not be detected in the hair bundles of the mature cochlea with antibodies directed against peptide epitopes in either the intra- or the extracellular domains of cadherin 23. This is a surprising finding, but one that is not necessarily inconsistent with the hypothesis that cadherin 23 is a component of the tip link (Siemens et al., 2004; Söllner et al., 2004). Indeed, within the tip link complex, these peptide epitopes may be masked by posttranslational modifications or the presence of associated proteins. Moreover, the antibodies used in this study may well recognize a single epitope per peptide antigen, thus the signal may be weaker than that obtained with antibodies derived from an immune serum raised to a large recombinant fragment encompassing the entire cytoplasmic domain, including the peptide encoded by the alternative exon 68 (Siemens et al., 2004). We were, however, also unable to detect cadherin 23 in mature rodent cochlear hair bundles with antibodies raised to the recombinant intracellular domain of human cadherin 23 containing the exon 68 peptide (data not shown). The relative concentration of the protein in the tip link, where there may be no more than four cadherin molecules (Corey and Sotomayor, 2004; Tsuprun et al., 2004), may be considerably less than that present along a growing stereocilium or within the vesicles of the pericuticular necklace, and this also could account for our inability to detect tip link staining.

There was a difference between the stage at which we could first detect cadherin 23 in the pericuticular region of hair cells with antibodies to the cytoplasmic and extracellular domains of cadherin 23; the Cyto antibody stained from P21 while the N1 antibody only stained at 3 months of

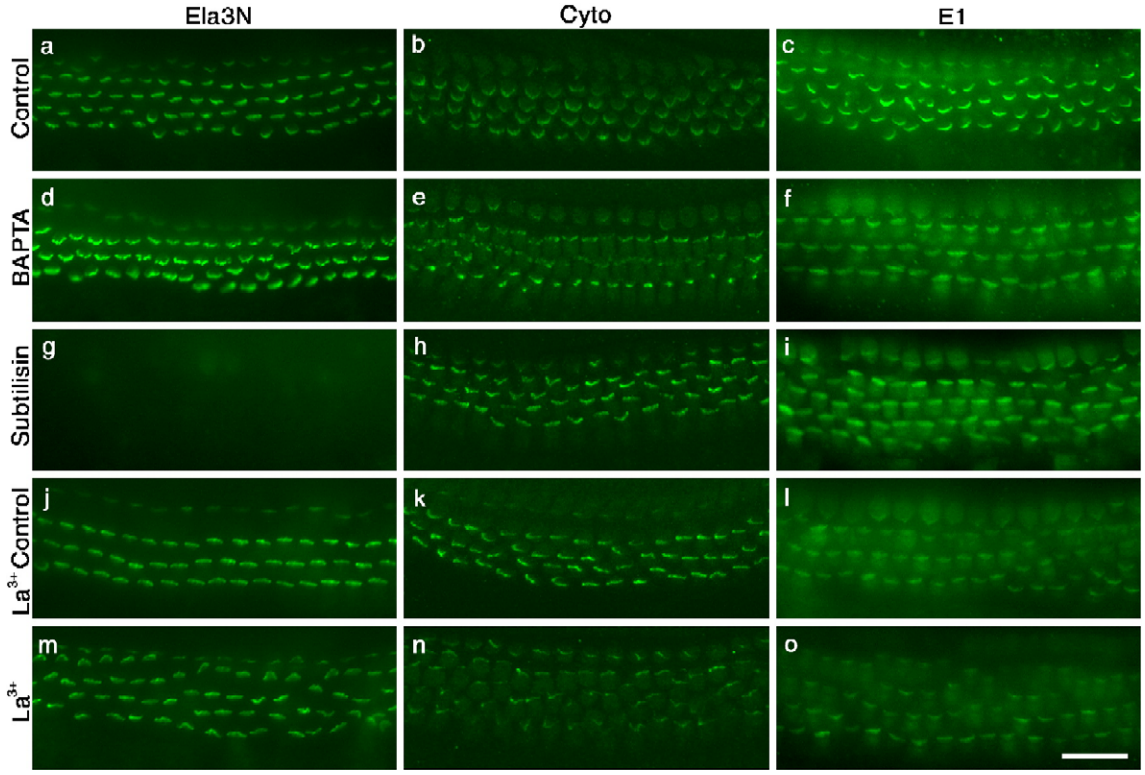

Fig. 7. Sensitivities of the Ela3N, Cyto, and E1 epitopes to BAPTA, subtilisin, and La^{3+}. Images from apical coil mouse cochlear cultures labeled with Ela3N (a, d, g, j, and m), Cyto (b, e, h, k, and n), or E1 (c, f, i, l, and o) following incubation in HBHBSS (Control, a–c), Ca^{2+}-free HBHBSS with 5 mM BAPTA (BAPTA, d–f), subtilisin (Subtilisin, g–i), La^{3+} control saline (La^{3+} Control, j–l), or 5 mM La^{3+} (La^{3+}, m–o). The ectodomain epitope recognized by Ela3N is retained on the surface of the hair bundle following exposure to BAPTA (d) or La^{3+} (m), but lost on exposure to subtilisin (g). Epitopes in the cytoplasmic domain are unaffected by exposure to BAPTA (e and f), subtilisin (h and i), and partially lost following La^{3+} treatment (n and o). Non-confocal images. Scale bar = 20 μm.

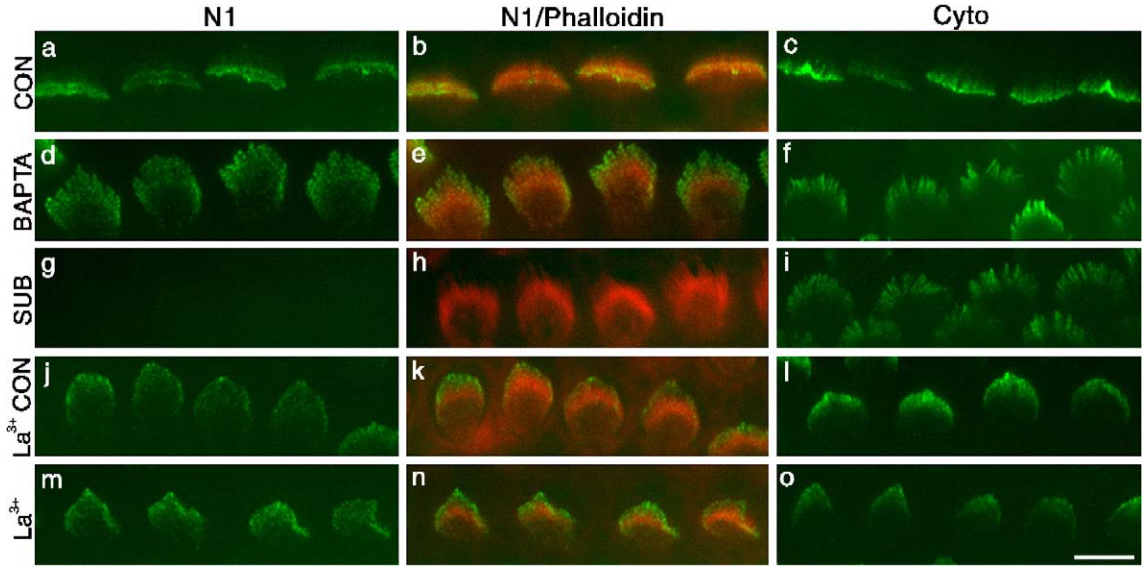

Fig. 8. Sensitivities of the N1 and Cyto epitopes to BAPTA, subtilisin, and La^{3+}. High magnification images of hair bundles from apical coil mouse cochlear cultures labeled with N1 (a, d, g, j, and m), N1 and phalloidin (b, e, h, k, and n), or Cyto (c, f, i, l, and o) following incubation in HBHBSS (CON, a–c), Ca^{2+}-free HBHBSS with 5 mM BAPTA (BAPTA, d–f), subtilisin (SUB, g–i), La^{3+} control saline (La^{3+}CON, j–l), or 5 mM La^{3+} (La^{3+}, m–o). The ectodomain epitope recognized by N1 is retained on the surface of the hair bundle following exposure to BAPTA (d and e) or La^{3+} (m and n), but lost on exposure to subtilisin (g and h). The Cyto epitope is unaffected by exposure to BAPTA (f), subtilisin (i), and reduced following La^{3+} (o). Non-confocal images. Scale bar = 5 μm.

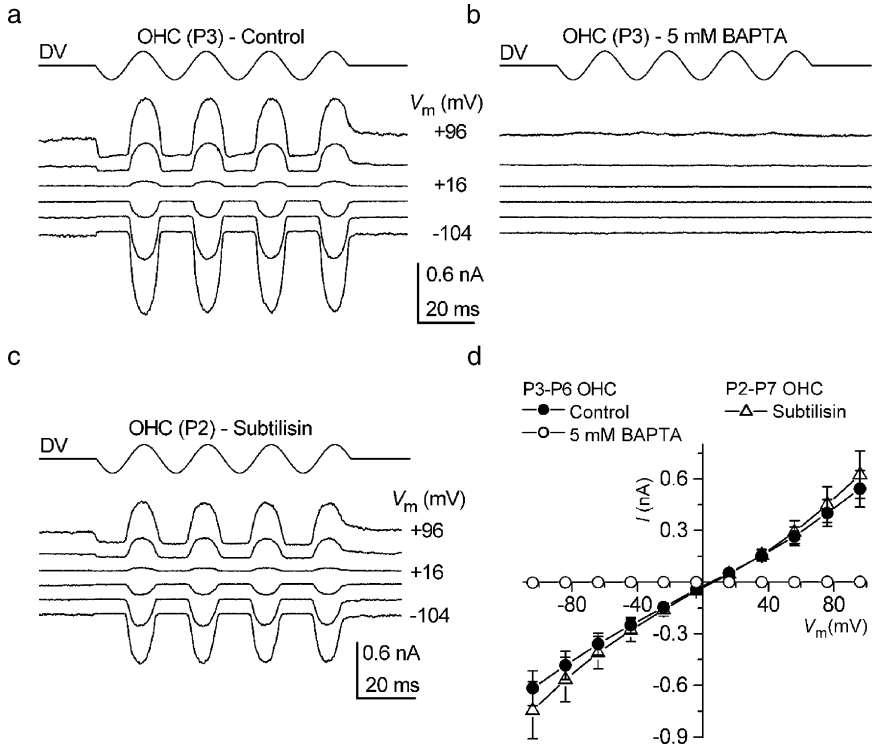

Fig. 9. Effects of BAPTA and subtilisin on mechano-electrical transduction in OHCs. (a and b) Transducer current recorded from a P3 OHC (P1 + 2 days in vitro) before (a, control) and during (b) superfusion of a solution containing 5 mM BAPTA without Mg^{2+}. BAPTA suppresses the transducer current 25 s after the start of the superfusion. The membrane potential was stepped between −104 mV and +96 mV in 20-mV increments from a holding potential of −84 mV. Membrane potentials are shown next to some of the traces. For clarity, only responses to every other voltage step are shown and are offset so that the zero-transducer current levels (responses to inhibitory stimuli) are equally spaced. Driver voltage signal (45 Hz) to the jet (DV; amplitude, 35 V) is shown above the currents. Positive deflections are excitatory. Recordings are single traces. C_m = 6.2 pF; R_s = 1.4 MΩ. (c) Mechano-electrical transducer currents in a P2 (P1 + 1 day in vitro) OHC recorded after it had been bath-perfused for 15 min with subtilisin (50 μg/ml) and then transferred into the recording chamber containing normal extracellular solution. Recordings in c were made about 25 min after the onset of subtilisin treatment. Experimental recording conditions as in a and b. Recordings are the average of 2 repetitions. C_m = 6.4 pF; R_s = 1.4 MΩ. (d) Average transducer currents recorded before and during superfusion of 5 mM BAPTA (n = 5) and after subtilisin treatment (n = 5). An additional 3 apical coil OHCs were investigated between 10 min and 1 h after the superfusion of the organ of Corti with BAPTA. In these cells, transducer current was also absent.

age. Possibly, the Cyto antibody recognizes nascent molecules in Golgi-derived vesicles and those in the endocytotic or early endosomal compartments, whereas the N1 antibody only recognizes those that are in the latter compartment and denatured such that an epitope normally masked is accessible for immunostaining. Alternatively, the Cyto antibody may recognize the cytosolic form of cadherin 23 predicted to exist from RT-PCR experiments at this stage, and this cytosolic form may appear in the pericuticular region before the transmembrane forms detected by antibody N1. Furthermore, a mixture of antibodies directed against all three peptides (the two extracellular domain peptides, N1 and N2, and the Cyto peptide) detected staining in the pericuticular region from P13 onwards, suggesting there may be additional isoforms that cannot be detected by the N1 and Cyto antibodies alone. The full significance of the labeling observed in the pericuticular region remains to be determined. While it may be derived from molecules that have been retrieved from the stereocilia and are en route for degradation in the endosomal/lysosomal pathway, it is unclear why they should accumulate at high concentrations in this region. Alternatively, or additionally, the signal may be from a pool of molecules that are awaiting incorporation into the plasma membrane at the base of each stereocilium. These molecules could be required to maintain the normal rate of tip-link turnover, or even be a reserve pool maintained to support the synthesis of lateral links required for hair bundle repair following acoustic overstimulation.

Following BAPTA treatment, tip links cannot be observed on hair bundles by scanning or transmission electron microscopy, and transduction is rapidly abolished (Assad et al., 1991; Goodyear and Richardson, 1999). Tip links and transduction are sensitive to La^{3+} (Baumann and Roth, 1986; Kachar et al., 2000). The protease subtilisin is very frequently used to remove the otolithic membrane from mechanosensory epithelia prior to measuring transduction currents in the hair cells, and tip links are readily observed on hair cells that have been treated with this protease (Goodyear and Richardson, 1999, 2003; Jacobs and Hudspeth, 1990). In the mature hair bundles of the frog saccule, tip and kinocilial link staining with antibodies to the intracellular domain of cadherin 23 disappears following EGTA or La^{3+} treatment, consistent with concept that tips links are shed or rapidly

removed from the cell surface following the chelation or displacement of Ca^{2+} (Siemens et al., 2004). In the present study, we found that the cadherin 23 expressed in the developing hair bundles has properties that are the opposite of those expected for a core component of the tip link. The ectodomain epitopes recognized by the N1 and Ela3N antibodies were resistant to BAPTA or La^{3+} treatment and lost on exposure to subtilisin. Consistent with this, we found that the intracellular domain epitopes recognized by Cyto and E1 were also not sensitive to either BAPTA or La^{3+}, although a previous study in the frog (Siemens et al., 2004) has found that the intracellular domain of cadherin 23 disappears from the hair bundle following EGTA or La^{3+} treatment. The subtilisin insensitivity of the cadherin 23 epitopes recognized by the Cyto antibodies is not surprising, as the enzyme is not expected to have access to the cytoplasmic compartment. Mechano-electrical transducer currents could be recorded from early postnatal mouse cochlear hair cells that had been exposed to similar concentrations of subtilisin, and disappeared rapidly following BAPTA treatment. This is consistent with the involvement of tip links in transduction at this stage of development and also indicates that the transient lateral links labeled by cadherin 23 at the early postnatal stages of development are unlikely to mediate mechano-electrical transduction. The possibility that the tip link could be an unknown variant of cadherin 23 with a sensitivity to BAPTA and subtilisin that is different to that of transient lateral links cannot be entirely excluded.

In conclusion, the results of this study show that cadherin 23 is associated with a subset of the links that transiently interconnect the stereocilia and the kinocilium of the developing hair bundle. The intracellular domain of cadherin 23 can interact with isoforms of the PDZ domain protein, harmonin (Boëda et al., 2002; Siemens et al., 2002), especially with harmonin b, a harmonin subclass with actin-bundling properties that is located at the tip of the developing hair bundle. It is therefore likely that the links with which cadherin 23 associates are anchored via harmonin to the actin core of the stereocilium. Harmonin binds to myosin VIIa (Boëda et al., 2002), and the cytoplasmic domain of cadherin 23 interacts with myosin Ic (Siemens et al., 2004), so molecular motors are present that could generate tension between these links and the actin cytoskeleton. These molecular interactions are likely to play a critical role in ensuring the stability of the developing hair bundle and may explain why this structure becomes disorganized and splits into small clusters of stereocilia when the cadherin 23 gene is mutated (Di Palma et al., 2001a; Holme and Steel, 2002). This transient cohesive mechanism may be of particular importance during very early stages of hair bundle formation, before the stereocilia have elaborated the rootlets that anchor them into the cuticular plate, a dense meshwork of actin filaments that lies in the cytoplasm just beneath the hair bundle.

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References

Assad, J.A., Shepherd, G.M., Corey, D.P., 1991. Tip-link integrity and mechanical transduction in vertebrate hair cells. Neuron 7, 985–994.

Bitner-Glindzicz, M., Lindley, K.J., Rutland, O., Blaydon, D., Smith, V.V., Milla, P.J., Hussain, K., Furth-Lavi, J., Cosgrove, K.E., Shepherd, R.M., Barnes, P.D., O'Brien, R.E., Fardnon, P.A., Sowden, J., Liu, X.Z., Scanlan, M.J., Malcolm, S., Dunne, M.J., Aynsley-Green, A., Glaser, B., 2000. A recessive contiguous gene deletion causing infantile hyperinsulinism, enteropathy and deafness identifies the Usher type 1C gene. Nat. Genet. 26, 56–60.

Baumann, M., Roth, A., 1986. The Ca^{++} permeability of the apical membrane in neuromast hair cells. J. Comp. Physiol., A 158, 681–688.

Bartolami, S., Goodyear, R., Richardson, G., 1991. Appearance and distribution of the 275 kD hair-cell antigen during development of the avian inner ear. J. Comp. Neurol. 314, 777–788.

Boëda, B., El-Amraoui, A., Bahloul, A., Goodyear, R., Daviet, L., Blanchard, S., Perfettini, I., Fath, K.R., Shorte, S., Reiners, J., Houdusse, A., Legrain, P., Wolfrum, U., Richardson, G., Petit, C., 2002. Myosin VIIa, harmonin and cadherin 23, three Usher I gene products that cooperate to shape the sensory hair bundle. EMBO J. 21, 6689–6699.

Bolz, H., von Brederlow, B., Ramirez, E.C., Bryda, K., Kutsche, H.G., Nothwang, M., Seeliger, M., del C-Salcedo Cabrera, M.P., Vila, O.P., Molina, A., Gal, A., Kubisch, C., 2001. Mutations of CDH23, encoding a new member of cadherin gene family, causes Usher syndrome type 1D. Nat. Genet. 27, 108–112.

Bork, J.M., Peters, L.M., Riazuddin, S., Bernstein, S.L., Ahmed, Z.M., Ness, S.L., Polomeno, R., Ramesh, A., Schloss, M., Srisailpathy, C.R., Wayne, S., Bellman, S., Desmukh, D., Ahmed, Z., Khan, S.N., Kaloustian, V.M., Li, X.C., Lalwani, A., Riazuddin, S., Bitner-Glindzicz, M., Nance, W.E., Liu, X.Z., Wistow, G., Smith, R.J., Griffith, A.J., Wilcox, E.R., Friedman, T.B., Morell, R.J., 2001. 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. 68, 26–37.

Corey, D.P., Sotomayor, M., 2004. Hearing. Tightrope act. Nature 428, 901–903.

Corey, D.P., Garcia-Anoveros, J., Holt, J.R., Kwan, K.Y., Lin, S.-Y., Vollrath, M.A., Amalfitano, A., Cheung, E.L.-M., Derfler, B.H., Duggan, A., Géléoc, G.S.G., Gray, P.A., Hoffman, M.P., Rehem, H.L., Tamasaukas, D., Zhang, D.-S., 2004. TRPA1 is a candidate for the mechanosensitive transducer channel of vertebrate hair cells. Nature 432, 723–730.

Di Palma, F., Holme, R.H., Bryda, E.C., Belyantseva, I.A., Pellegrino, R., Kachar, B., Steel, K.P., Noben-Trauth, K., 2001a. Mutations in Cdh23,

encoding a new type of cadherin, cause stereocilia disorganization in waltzer, the mouse model for Usher syndrome type 1D. Nat. Genet. 27, 103–107.

Di Palma, F., Pellegrino, R., Noben-Trauth, K., 2001b. Genomic structure, alternative splice forms and normal and mutant alleles of cadherin 23 (*Cdh23*). Gene 281, 31–41.

Erneston, S., Smith, C.A., 1986. Stereo-kinociliar bonds in mammalian vestibular organs. Acta Otolaryngol. 101, 395–402.

Furness, D.N., Hackney, C.M., 1985. Cross-links between stereocilia in the guinea pig cochlea. Hear. Res. 18, 177–188.

Gale, J.E., Marcotti, W., Kennedy, H.J., Kros, C.J., Richardson, G.P., 2001. FM1-43 dye behaves as a permeant blocker of the hair-cell's mechanotransducer channel. J. Neurosci. 21, 7013–7025.

Géléoc, G.S.G., Lennan, G.W.T., Richardson, G.P., Kros, C.J., 1997. A quantitative comparison of mechanoelectrical transduction in vestibular and auditory hair cells of neonatal mice. Proc. R. Soc. London, B 264, 611–621.

Goodyear, R., Richardson, G., 1992. Distribution of the 275 kD hair cell antigen and cell surface specializations on auditory and vestibular hair bundles in the chicken inner ear. J. Comp. Neurol. 325, 243–256.

Goodyear, R., Richardson, G., 1999. The ankle-link antigen: an epitope sensitive to calcium chelation associated with the hair-cell surface and the calycal processes of photoreceptors. J. Neurosci. 19, 3761–3772.

Goodyear, R.J., Richardson, G.P., 2003. A novel antigen sensitive to calcium chelation that is associated with the tip links and kinocilial links of sensory hair bundles. J. Neurosci. 23, 4878–4887.

Goodyear, R.J., Legan, P.K., Wright, M.B., Marcotti, W., Oganesian, A., Coats, S.A., Booth, C.J., Kros, C.J., Seifert, R.A., Bowen-Pope, D.F., Richardson, G.P., 2003. A receptor-like inositol lipid phosphatase is required for the maturation of developing cochlear hair bundles. J. Neurosci. 23, 9208–9219.

Goodyear, R.J., Marcotti, W., Kros, C.J., Richardson, G.P., in press. Development and properties of stereociliary link types in hair cells of the mouse cochlea. J. Comp. Neurol.

Hillman, D.E., 1969. New ultrastructural findings regarding a vestibular ciliary apparatus and its possible functional significance. Brain Res. 13, 407–412.

Hillman, D.E., Lewis, E.R., 1971. Morphological basis for a mechanical linkage in otolithic receptor transduction in the frog. Science 174, 416–419.

Holme, R.H., Steel, K.P., 2002. Stereocilia defects in waltzer (Cdh23), shaker1 (Myo7a) and double waltzer/shaker1 mutant mice. Hear. Res. 169, 13–23.

Jacobs, R.A., Hudspeth, A.J., 1990. Ultrastructural correlates of mechanoelectrical transduction in hair cells of the bullfrog's internal ear. Cold Spring Harbor Symp. Quant. Biol. 55, 547–561.

Kachar, B., Parrakal, M., Kurc, M., Zhao, Y., Gillespie, P.G., 2000. High-resolution structure of hair-cell tip links. Proc. Natl. Acad. Sci. U. S. A. 97, 13336–13341.

Kros, C.J., Rüsch, A., Richardson, G.P., 1992. Mechano-electrical transducer currents in hair cells of the cultured mouse cochlea. Proc. R. Soc. London, B 249, 185–193.

Küssel-Andermann, P., El-Amraoui, A., Safieddine, S., Nouaille, S., Perfettini, I., Lecuit, M., Cossart, P., Wolfrum, U., Petit, C., 2000. Vezatin, a novel transmembrane protein, bridges myosin VIIA to the cadherin–catenins complex. EMBO J. 19, 6020–6029.

Noben-Trauth, K., Zheng, Q.Y., Johnson, K.R., 2003. Association of cadherin 23 with polygenic inheritance and genetic modification of sensorineural hearing loss. Nat. Genet. 35, 21–23.

Pickles, J.O., Comis, S.D., Osborne, M.P., 1984. Cross-links between stereocilia in the guinea pig organ of Corti, and their possible relation to sensory transduction. Hear. Res. 15, 103–112.

Pickles, J.O., von Perger, M., Rouse, G.W., Brix, J., 1991. The development of links between stereocilia in hair cells of the chick basilar papilla. Hear. Res. 54, 153–163.

Reiners, J., Reidel, B., El-Amraoui, A., Boëda, B., Huber, I., Petit, C., Wolfrum, U., 2003. Differential distribution of harmonin isoforms and their possible role in Usher-1 protein complexes in mammalian photoreceptor cells. Invest. Opthamol. Visual Sci. 44, 5006–5016.

Richardson, G.P., Bartolami, S., Russell, I.J., 1990. Identification of a 275-kD protein associated with the apical surfaces if sensory hair cells in the avian inner ear. J. Cell Biol. 110, 1055–1066.

Russell, I.J., Richardson, G.P., 1987. The morphology and physiology of hair cells in organotypic cultures of the mouse cochlea. Hear. Res. 31, 9–24.

Sidi, S., Freidrich, R.W., Nicolson, T., 2003. NompC TRP channel required for vertebrate sensory hair cell mechanotransduction. Science 301, 96–99.

Siemens, J., Kazmierczak, P., Reynolds, A., Sticker, M., Littlewood-Evans, A., Muller, U., 2002. The Usher syndrome proteins cadherin 23 and harmonin form a complex by means of PDZ-domain interactions. Proc. Natl. Acad. Sci. U. S. A. 99, 14946–14951.

Siemens, J., Lillo, C., Dumont, R.A., Reynolds, A., Williams, D.S., Gillespie, P.G., Muller, U., 2004. Cadherin 23 is a component of the tip link in hair-cell stereocilia. Nature 428, 950–955.

Söllner, C., Rauch, G.J., Siemens, J., Geisler, R., Schuster, S.C., Muller, U., Nicolson, T., Tübingen 2000 Screen Consortium, 2004. Mutations in cadherin 23 affect tip links in zebrafish sensory hair cells. Nature 428, 955–959.

Tsuprun, V., Goodyear, R.J., Richardson, G.P., 2004. The structure of tip links and kinocilial links in avian sensory hair bundles. Biophys. J. 87, 4106–4112.

Verpy, E., Leibovici, M., Zwaenepoel, I., Liu, X.-Z., Gal, A., Salem, N., Mansour, A., Blanchard, S., Kobayashi, I., Keats, B.J.B., Slim, R., Petit, C., 2000. A defect in harmonin, a PDZ domain-containing protein expressed in the inner ear sensory hair cells, underlies Usher syndrome type 1c. Nat. Genet. 26, 51–55.