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Spatiotemporal pattern and isoforms of cadherin 23 in wild type and *waltzer* mice during inner ear hair cell development

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Abstract

Mutant alleles of the gene encoding cadherin 23 are associated with Usher syndrome type 1 (USH1D), isolated deafness (DFNB12) in humans, and deafness and circling behavior in *waltzer* (*v*) mice. Stereocilia of *waltzer* mice are disorganized and the kinocilia misplaced, indicating the importance of cadherin 23 for hair bundle development. Cadherin 23 was localized to developing stereocilia and proposed as a component of the tip link. We show that, during development of the inner ear, cadherin 23 is initially detected in centrosomes at E14.5, then along the length of emerging stereocilia, and later becomes concentrated at and subsequently disappears from the tops of stereocilia. In mature vestibular hair bundles, cadherin 23 is present along the kinocilium and in the region of stereocilia–kinocilium bonds, a pattern conserved in mammals, chicks, and frogs. Cadherin 23 is also present in Reissner's membrane (RM) throughout development. In homozygous v^{6J} mice, a reported null allele, cadherin 23 was absent from stereocilia, but present in kinocilia, RM, and centrosomes. We reconciled these results by identifying two novel isoforms of *Cdh23* unaffected in sequence and expression by the v^{6J} allele. Our results suggest that *Cdh23* participation in stereocilia links may be restricted to developing hair bundles.
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Keywords: Cadherin 23; Stereocilia; Kinocilium; Centrosomes; Reissner's membrane; Usher syndrome; Deafness; *Waltzer* mice; Adhesion proteins

Introduction

During development of the neurosensory epithelium of the inner ear, a complex series of morphological changes give rise to a polarized mosaic pattern of supporting cells and hair cells (Bryant et al., 2002; Forge et al., 1997; Nishida et al., 1998). On the apical surface of hair cells, actin-filled stereocilia emerge and surround a single tubulin-based kinocilium to form immature hair bundles (Frolenkov et al., 2004). The kinocilium originates from one centrosome/basal body of a pair (Alieva and Vorobjev, 2004; Beisson and Wright, 2003; Hagiwara et al., 2004), and its ultimate position on one side of the hair cell is thought to define hair bundle polarity. In mature hair bundles, stereocilia are organized in rows of increasing height (Tilney et al., 1988; Denman-Johnson and Forge, 1999; Forge and Wright, 2002) and fine filaments connect the upper part of the kinocilium with the tallest neighboring stereocilia (Ernstson and Smith, 1986; Goodyear and Richardson, 2003; Hillman, 1969; Tsuprun et al., 2004). A variety of link types interconnect stereocilia to one another (Goodyear and Richardson, 1992, 1999, 2003) and some of these may be formed by adhesion molecules required for the formation and maintenance of the stereocilia bundle as a cohesive structure (Frolenkov et al., 2004).

Several adhesion proteins are essential for normal development and maintenance of the auditory system (Kelley, 2003) including protocadherin 15 (Ahmed et al., 2003; Alagramam et al., 2001) and cadherin 23, members of a family of membranous adhesion glycoproteins characterized by a variable number of calcium dependent

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extracellular (EC) domains (Hirano et al., 2003). The full-length isoform of *CDH23* comprises 69 coding exons and is predicted to encode a protein with 27 EC domains, a trans-membrane domain, and a unique cytoplasmic domain (Bolz et al., 2001; Bork et al., 2001). Two splice isoforms of the cytoplasmic domain of the human and mouse cadherin 23 have been reported, with and without exon 68 (Bork et al., 2001; Di Palma et al., 2001b). Mutant alleles of *Cdh23* cause deafness and circling behavior in *waltzer* (*v*) mice, which have disorganized cochlear and vestibular hair cell stereocilia and misplaced kinocilia (Di Palma et al., 2001a). In humans, mutant alleles of *CDH23* are either associated with deaf–blindness, Usher syndrome type 1 (Bolz et al., 2001; Bork et al., 2001), or non-syndromic deafness (Bork et al., 2001).

In situ hybridization analyses of the mouse inner ear revealed *Cdh23* mRNA in sensory hair cells and Reissner's membrane (RM) (Wilson et al., 2001). Immunohistochemical analysis showed cadherin 23 in the photoreceptor layer of the retina and in stereocilia of immature mouse cochlear and vestibular hair bundles (Boëda et al., 2002; Siemens et al., 2002). Recently, cadherin 23 was proposed to be a component of the tip link (Siemens et al., 2004; Sollner et al., 2004) and was reported to be present in adult mouse cochlear hair cell stereocilia, at odds with the previously reported disappearance of cadherin 23 from stereocilia of mature hair bundles (Boëda et al., 2002).

To elucidate this discrepancy, we performed a systematic investigation of the temporal and spatial pattern of cadherin 23 expression during hair bundle development. We show that cadherin 23 is initially expressed in centrosomes, followed by its expression in RM, stereocilia, and kinocilia. Furthermore, while cadherin 23 disappears from stereocilia of mature hair bundles, it is retained in centrosomes, RM, and along kinocilia particularly in the region of stereocilia–kinocilium bonds. Cadherin 23 expression pattern in centrosomes and hair bundles is conserved in mammals, chicks, and frogs, and its expression pattern in RM is also conserved among mammals. We present evidence for novel isoforms of cadherin 23 that are present in late gestation and these isoforms are unaffected by the v^{6J} *waltzer* allele. Taken together, these data support the hypothesis that cadherin 23 has multiple functions which are evolutionally conserved during hair bundle development of the inner ear.

Materials and methods

Expression constructs

cDNA fragments encoding the cytoplasmic domain of mouse cadherin 23 with (GenBank accession NM_023370 nucleotides 9322–10052) and without (nucleotides 9322–9633, 9739–10052) exon 68 were cloned into pGEX-5X-1 (Amersham-Pharmacia Biotech), pMAL-c2 (New England Biolabs), and pEGFP-C2 vectors (Clonetech). GST-CDH23(+68) and MBP-CDH23(+68) fusion proteins were expressed in Rosetta *E. coli* (Novagen) and purified according to the manufacturer's instructions.

Cadherin 23 antigens and antisera

Three New Zealand white rabbits were immunized with purified GST-CDH23(+68) fusion protein producing polyclonal antibodies TF7, TF8, and TF9. Purified MBP-CDH23 protein was used to affinity-purify rabbit antisera using an AminoLink Plus Immobilization Kit (Pierce).

Transfection of CDH23-GFP into HeLa cells

We evaluated the specificity of our antibodies raised against the cytoplasmic domain of cadherin 23 by performing a co-localization assay. Using Lipofectamine 2000 (Invitrogen), a GFP-CDH23(+68) expression vector was transfected into HeLa cells, which do not endogenously express cadherin 23. After incubation for 24 h at 37°C and 5% CO_2 in DMEM with 10% FCS (Invitrogen), transfected cells were fixed with 4% paraformaldehyde in PBS for 15 min and processed for immunostaining (Ahmed et al., 2003; Belyantseva et al., 2003) using a 1:500 dilution (~0.5 mg/ml stock) of antibodies TF7 and TF8.

Western blot and dot blot analysis

For Western blot analysis, the MBP-CDH23(+68) fusion protein was expressed and partially purified from *E. coli* and denatured in sample buffer (6.25 ml 1 M Tris–HCl pH 7.8, 1 ml 10% SDS, 0.5 ml glycerol, 0.25 ml β-mercaptoethanol, 0.125 ml ddH_2O, 10 mg bromophenol blue) for 3 min at 95°C, then separated on 4–20% Tris–Glycine precast Novex gels (Invitrogen). Western blot analyses were performed as previously described (Ahmed et al., 2003). We used a 1:1000 dilution of antibodies TF7 and TF8, and a 1:10,000 dilution of alkaline phosphatase-conjugated anti-rabbit secondary antibody (Promega). For Western dot blot analysis, 0.5 μg of an exon 68 peptide and 0.1 μg of MBP-CDH23(+68) fusion protein were applied to the membrane. For tissue Western blot analysis, cochleas of postnatal day 7 (P7) WT (B10.A-$H2^{h4}$/(4R)SgDvEg × C57BL/6)F1 and adult homozygous v^{6J} (B10.A-$H2^{h4}$/(4R)SgDvEg × C57BL/6)F1 mice were dissected and 25 μg of protein sample was separated on 3–8% Novex Tris-acetate gel (Invitrogen) and processed using TF7 (1:1000 dilution, ~0.5 mg/ml stock) according to Ahmed et al. (2003).

Immunofluorescence study of mouse inner ear sensory epithelia

(B10.A-$H2^{h4}$/(4R)SgDvEg × C57BL/6)F1 mice were mated and offspring were euthanized according to NIH animal protocol 1126-03, and the inner ears removed by dissection. Primary antibodies were diluted 1:1000 for TF7

and TF8, 1:200 for monoclonal anti-β-tubulin-FITC conjugate (SIGMA), and 1:200 for pericentrin MAb (BD Biosciences). Rhodamine phalloidin and Alexa Fluor 633 phalloidin (Molecular Probes) were diluted 1:100. Anti-rabbit Ig, fluorescein conjugated (Amersham Biosciences), and Alexa Fluor 594 goat anti-rabbit (Molecular Probes) secondary antibodies were used at 1:200 dilution as previously described (Ahmed et al., 2003). For color consistency, in Figs. 2–6, cadherin 23 is shown in green and β-tubulin in red, regardless of the secondary antibody. The control experiments were performed with substitution of pre-immune sera for immune sera, omission of the primary antisera, exclusion of permeabilizing detergents, and preincubation of cadherin 23 antisera with GST-CDH23(+68) fusion protein.

Northern blot analysis

A human multiple tissue northern blot (Poly A+ RNA, MTN Human Blot; BD Bioscience) was hybridized with a probe generated from the *CDH23* cytoplasmic domain and processed as previously described (Bork et al., 2001).

Novel human and mouse isoforms of cadherin 23

FirstEF software (Davuluri et al., 2001) was used to predict candidate first exons between exons 40 and 68 of human *CDH23.* Forward PCR primers were designed in the five predicted sequences of putative exons while reverse primers were designed from the sequence of exons 68 and 69 of *CDH23.* Human retina cDNA (Genemed Biotechnologies, Cambridge UK) was used as template for PCR amplification using LA-Taq polymerase (PanVera). The primers are listed in Supplementary Table 1.

Mouse *Cdh23* isoform B was identified by BLAST analysis of the unique sequence of human *CDH23* isoform B against the mouse genome. A forward primer designed within one of the candidate novel *Cdh23* first exon sequences and a reverse primer from mouse *Cdh23* exon 69 (Supplementary Table 1) were used to amplify isoform B from cDNA, which was synthesized from inner ear mRNA of P45–P50 WT and P60 homozygous v^{6J} mice using a Poly(A)Pure kit (Ambion). 5′ RACE reactions extended the sequence to a likely transcription start site.

EST CD675842 has 32 unique bases upstream of *CDH23* exon 66. A forward primer designed within this 32 bp of sequence and a reverse primer from exon 69 of *CDH23* (F: 5′ CTAGCCGCCCTCCCCACT; R: 5′ CCAGTGAAATGGAGAGAATGCTTGT) were used to PCR amplify isoform C which was subcloned and sequenced. Human retina cDNA was used as template. In addition, a 5′ RACE primer (5′ TTGGGGTGTGAGGCCCTGGTGCCTGTGGTGCA) was designed within the unique 32 bp region of EST CD675842 which was used to amplify a product that was subcloned and sequenced. A nested primer was designed for the second round of 5′ RACE (5′ ATGTCAGTCTGGATTGCCTTTGGTGCAGAG). A new set of primers (Supplementary Table 1) amplified the full-length isoform C. The mouse ortholog of this novel human isoform C was identified and primers were designed for cDNA amplification (Supplementary Table 1) using mouse cochlea and retina cDNA.

Results

Specificity of antisera

Twenty-four hours after transfection of HeLa cells with a GFP-CDH23(+68) expression vector, the staining pattern obtained with antibodies TF7 and TF8 overlapped with the green fluorescence from GFP-CDH23(+68) (Figs. 1A–H) and from GFP-CDH23(−68) (data not shown). In cells transfected with the GFP expression vector alone, cadherin 23 immunoreactivity was not detected (Figs. 1I–L). We also evaluated the ability of the antibodies to recognize cadherin 23 cytoplasmic domain and the amino acid sequence of exon 68 by Western blot analysis. TF7 and TF8 recognized the MBP-CDH23(+68) fusion protein expressed and purified from *E. coli* lysate (Fig. 1M). Antiserum TF7 does not recognize an exon 68 only encoded peptide while antiserum TF8 does recognize this peptide (Fig. 1N). In the immunohistochemical studies, antibodies TF7 and TF8 were used interchangeably and gave identical results unless specified.

Cadherin 23 in stereocilia of immature hair cells

Consistent with the progressive development from base to apex of the neurosensory epithelium of the cochlea (Lim and Anniko, 1985), cadherin 23 expression was first observed in the mouse at embryonic day 16.5 (E16.5) along the length of stereocilia of inner and outer hair cells in the basal but not in the apical turns of the cochlea (Figs. 2A, B), which is in agreement with observations reported by Boëda and coworkers (2002). By E18.5, cadherin 23 is expressed in stereocilia of inner and outer hair cells of the basal and apical turns of the cochlea (Figs. 2C, D). At P4–P5 in mouse and rat cochlea (Figs. 2E–G), cadherin 23 immunoreactivity is present in the distal portion of inner and outer hair cell stereocilia. In mouse outer hair cells, immunoreactivity of cadherin 23 is more intense in the region of contact between the kinocilium and the upper portion of the adjacent tallest stereocilia (Fig. 2E). Subsequently, the intensity of cadherin 23 immunofluorescence diminished in hair cell stereocilia, and from P16 we could not detect cadherin 23 in mouse or rat cochlear stereocilia (Figs. 2H–J). At P16, cadherin 23 appears in the pericuticular necklace (Fig. 2H).

This same pattern of cadherin 23 expression during development was also seen in hair cell stereocilia of the basilar papilla (BP) of the embryonic chicken inner ear and the adult bullfrog macula sacculi. At E19, when hair bundles

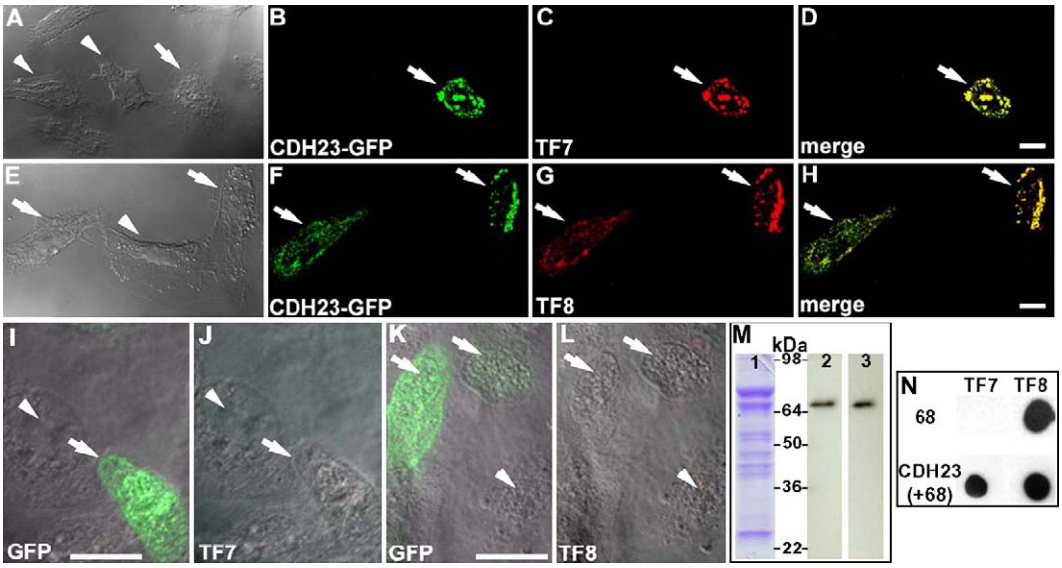

Fig. 1. Validation of cadherin 23 antisera. (A–L) Arrows indicate transfected cells, arrowheads indicate non-transfected control cells. (A, E) Nomarski images of HeLa cells 24 h after transfection with an expression vector encoding a GFP-tagged cadherin 23 cytoplasmic domain (B, F, green). Staining of the cells with antibodies TF7 and TF8 (C, G, respectively, red) overlapped with GFP fluorescence (D, H). (I–L) HeLa cells transfected with the GFP expression vector alone (I, K, green) revealed that subsequent immunolabeling with TF7 and TF8 antisera (J, L, respectively, red) produced no detectable levels of immunofluorescence. (M) Western blot of partially purified lysate from *E. coli* expressing the MBP-CDH23 fusion protein. Lane 1, the lysate was separated on a 4–20% Tris–Glycine gel (Novex, Invitrogen) and stained with Coomassie blue. The lysate was diluted 1:1000 in lane 2 and 1:100 in lane 3. TF7 (lane 2) and TF8 (lane 3) antibodies recognized a single band of approximately 69 kDa (42.7 kDa MBP plus 26.7 kDa cadherin 23 cytoplasmic domain). (N) Dot blot with 0.5 μg of peptide 68, (CLEDYLRLKKLFAQRMVQASSCHSSISE, Princeton Biomolecules) corresponding to amino acid residues 3219 to 3246 encoded by exon 68 (GenBank accession number AAG52817), and 0.1 μg of MBP-CDH23(+68) fusion protein. Antibody TF7 does not recognize the exon 68 peptide, while TF7 and TF8 recognize the expressed cytoplasmic domain encoded by exons 65–69. Scale bars, 5 μm.

at the base of the BP have reached maturity (Tilney et al., 1988), cadherin 23 is present in stereocilia of hair cells located at the extreme apex and the apex but is not detected in hair cells at the base of the BP (Figs. 2K–M). In the macula sacculi of the adult bullfrog, cadherin 23 was detected along the length of stereocilia of nascent hair bundles, located at the periphery of this tissue (Fig. 2N).

In vestibular hair cells of the mouse, rat, and chick, localization of cadherin 23 within stereocilia depends upon the stage of hair bundle development. At E14.5, cadherin 23 is detected along the length of stereocilia of nascent mouse ampullar hair bundles (Fig. 3A). At P3, when mature bundles are present in the center of sensory epithelia, and immature stereocilia bundles can be identified at the periphery (Denman-Johnson and Forge, 1999; Sans and Chat, 1982), cadherin 23 is detected along the length of the stereocilia of the immature bundles (Figs. 3B, D). At P16 in rat ampulla, the most intense cadherin 23 staining was observed along the length of stereocilia in immature hair bundles and in the region closer to the top of stereocilia in maturing hair cell bundles from the periphery of the ampulla. As hair bundles continue to develop, the intense immunoreactivity of cadherin 23 gradually disappears from the top of stereocilia (Figs. 3C, E). Similarly, in the chicken ampulla at E10, cadherin 23 was localized along the length of nascent stereocilia (Fig. 3F), and at E19 was no longer observed in stereocilia of mature hair bundles (data not shown).

Cadherin 23 in the kinocilium, Reissner's membrane, and centrosomes

In E14.5 mouse ampullar hair cells, the kinocilium has migrated to the side of the immature stereocilia bundle. At this stage, cadherin 23 staining appears along the length of stereocilia and in the kinocilium at the point of contact with the stereocilia (Fig. 4A). By E16.5, cadherin 23 is detected along the length of kinocilia of mature mouse ampullar hair bundles (Fig. 4B), where it persists in the rat at P21 and is visible both along the length and in more concentrated patches of staining, possibly in the region of contact between the tallest stereocilia and the kinocilium (Fig. 4C). Immunoreactivity of cadherin 23 was observed in kinocilia of mouse utricle and saccule, and along the length of hair cell kinocilia in the chick and guinea pig as well, where concentrated patches of immunostaining in the kinocilium and in the tallest stereocilia in the region of stereocilia–kinocilium bonds were detected (data not shown). This same pattern of cadherin 23 localization is seen in the adult bullfrog utricle and macula sacculi, chick basilar papilla, and in the rat ampulla (Figs. 4D–G).

In the mouse organ of Corti, cadherin 23 expression in hair cell kinocilia is first detected at P1 in the basal turn of the cochlea (Figs. 4H–J). The kinocilium is stained along its entire length, with a brighter signal in the region of contact with adjacent stereocilia. By P4, cadherin 23 expression in hair cell kinocilia extends from the base to the apex (Figs.

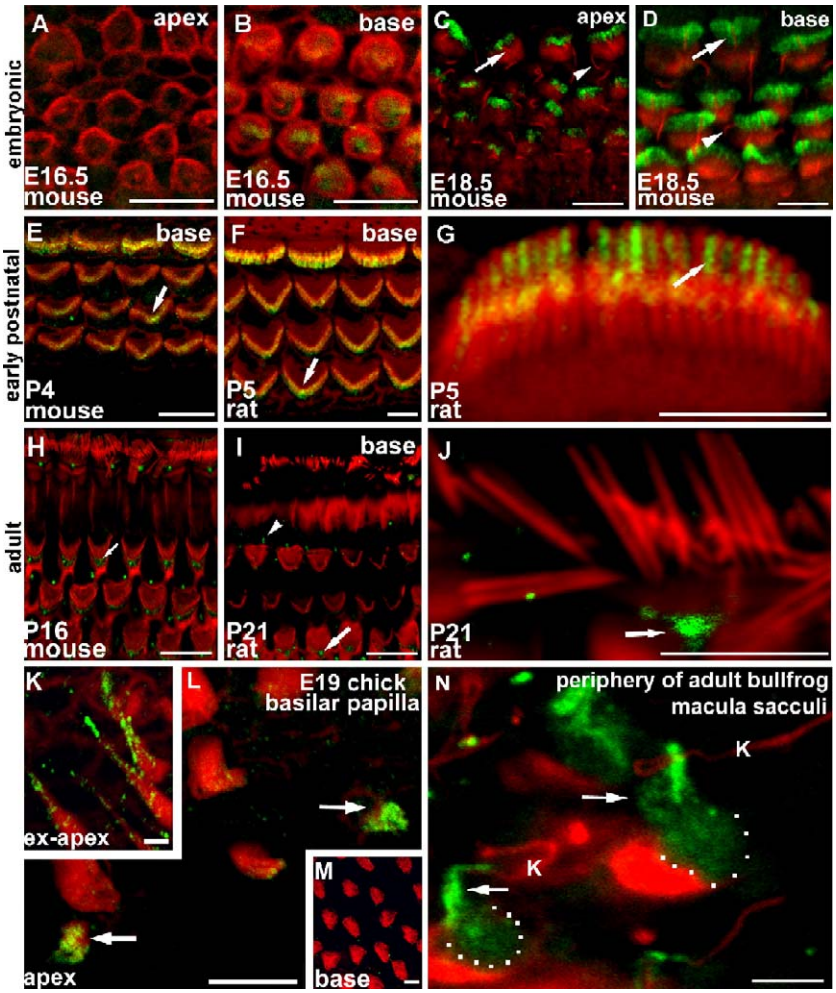

Fig. 2. Temporal and spatial expression pattern of cadherin 23 (shown in green) in stereocilia of mouse and rat organ of Corti, and in stereocilia of the chick basilar papilla and frog saccule, the homologs of the mammalian organ of Corti. (A) At E16.5 in the apical turn of the cochlea, stereocilia have not yet emerged and cadherin 23 is not detected. (B) Cadherin 23 (green) is detected at E16.5 in stereocilia (stained for F-actin, red) of hair cells from the basal turn of the cochlea. (C, D) At E18.5, cadherin 23 is detected along the entire length of stereocilia of hair cells from the apical and basal turns of the mouse cochlea. Kinocilia of hair cells (arrows) and primary cilia of Dieters cells (arrowheads) are stained for β-tubulin (red). In basal cochlear hair cells of the P4 mouse (E) and P5 rat (F), cadherin 23 (green) is localized near the top of stereocilia (stained for F-actin, red). Staining for cadherin 23 appears more intense in outer hair cells in the region occupied by the kinocilium (arrows) both in the mouse and rat. (G) In inner hair cell stereocilia of the P5 rat, visualized using a 100× Zeiss objective, N.A. = 1.45, cadherin 23 (green) is localized at the upper portion of stereocilia and seen as a patch between the actin core of neighboring stereocilia. At P16 in mouse (H) and P21 in rat (I, J), cadherin 23 is no longer detected in stereocilia and is detected in the pericuticular necklace (arrow). (K–M) In E19, chick basilar papilla cadherin 23 (green) is distributed along stereocilia of immature bundles from the extreme apex (indicated ex-apex in panel K) and apex (arrows, panel L) but is not detected in stereocilia of basal hair cell bundles (M). (N) In adult bullfrog saccule stained for cadherin 23 (green) and β-tubulin (red), cadherin 23 is present along stereocilia of immature hair bundles located at the periphery of the macula sacculi (arrows). The base of the stereocilia bundle is marked with white dots. Scale bars, 5 μm.

4K–M) of the cochlea. Cadherin 23 was also detected in mouse RM as early as E16.5 (Fig. 5A) where it persists in the adult mouse (Fig. 5B), guinea pig (Fig. 5C), and rat (data not shown). In addition, using antisera TF7 (but not TF8, data not shown), cadherin 23 was detected in the centrosome/basal body where it localizes but does not overlap with pericentrin (Fig. 5D), which labels the centrosome tube (Ou and Rattner, 2000). Cadherin 23 was also detected in the centrosomes using antisera TF9, which was also generated against the cytoplasmic domain of cadherin 23 and showed identical immunoreactivity to TF7 (data not shown). In centrosomes, cadherin 23 is initially detected at E14.5, before stereocilia emerge on the apical surface of hair cells, and persists throughout mouse development. Cadherin 23 is detected in centrosomes of auditory and vestibular sensory epithelial cells and in centrosomes of cells of the greater epithelial ridge in the cochlea. Cadherin 23 was also detected in centrosomes of cells of the auditory and vestibular sensory epithelia of chick and frog (data not shown).

Cadherin 23 expression in v^{6J} mice

The v^{6J} allele of *Cdh23* is a G to T transversion in exon 9, encoding the third EC domain, and is predicted to

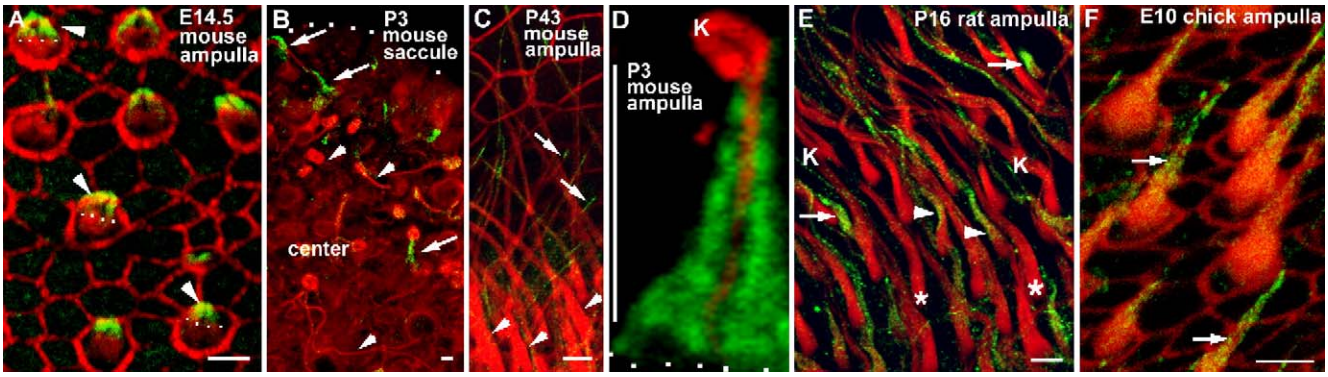

Fig. 3. Immunolocalization of cadherin 23 (shown in green) in stereocilia of immature vestibular hair bundles from mouse, rat, and chick. (A) Cadherin 23 (green) is distributed along the length of stereocilia (stained for F-actin, red) of immature bundles (arrowhead) in an E14.5 mouse ampulla. The base of the stereocilia bundle is marked with white dots. (B) In a P3 mouse saccule, cadherin 23 (green) is detected along the length of stereocilia of immature hair bundles (arrows) located at the periphery of the saccule (marked with white dots), but not in the center. Kinocilia are stained for β-tubulin (red, arrowheads). (C) In a P43 mouse ampulla, cadherin 23 (green) is present in kinocilia (arrows) but absent from stereocilia (arrowheads) (stained for F-actin, red). (D) In P3 mouse ampulla, cadherin 23 (green) is present along the length of stereocilia of immature hair bundles located at the periphery of ampullar tissue. The base of the stereocilia bundle is marked with white dots, and the kinocilium (K), stained for β-tubulin (red), is localized at the side of the stereocilia bundle. Some co-localization of cadherin 23 and β-tubulin is visible along the length of the stereocilia in the region of contact between the kinocilium and the stereocilia. (E–F) Cadherin 23 (green) and F-actin (red). In the periphery of ampullar tissue of P16 rat (E), where immature, almost mature, and mature hair bundles are present, cadherin 23 is distributed along stereocilia (arrows) of immature bundles and towards the top of stereocilia of more mature developing hair bundles (arrowheads). Cadherin 23 is not detected in stereocilia of mature bundles (indicated with asterisks located at the base of mature hair bundles). (F) In the E10 chick ampulla, cadherin 23 is distributed along stereocilia of the immature bundles (arrows). Scale bars, 5 μm (A, C–F) and 1 μm (B).

create a nonsense mutation (premature translation stop codon). Thus, v^{6J} was reported to be a functional null allele (Di Palma et al., 2001a,b). As expected, cadherin 23 immunoreactivity was absent from stereocilia of P5 homozygous v^{6J} mice (Figs. 6A–D). However, we detected cadherin 23 in vestibular (Figs. 6E–G) and cochlear hair cell kinocilia (Figs. 6H–J), in RM of young (Fig. 6K) and adult (Fig. 6L) mice, and in the centrosomes of cells from auditory and vestibular epithelia of v^{6J} homozygotes (Fig. 6M). This suggested to us that not all isoforms of *Cdh23*, encoding the cytoplasmic domain to which our antisera are directed, are affected by the v^{6J} nonsense mutation.

Cadherin 23 isoforms B1 and B2

Previously, we mentioned but did not characterize a smaller isoform of *CDH23* (approximately 1.35 kb; Fig. 7B) based on observations from a Northern blot analysis of human tissue using a 488 bp probe from the cytoplasmic domain of human *CDH23* (exons 66–69) (Bork et al., 2001). Using primers complementary to predicted additional exons of *CDH23*, we identified cDNAs that represent isoforms B1 and B2 (GenBank accession nos. AY563165 and AY563166, respectively), which encode products of 4044 bp and 3939 bp (without exon 68), respectively. Isoform B has stop codons in all three reading frames upstream of a predicted translation start codon, which has a favorable Kozak consensus sequence (Kozak, 1996) around the AUG initiation codon located in exon 46a. Isoform B is predicted to encode a protein with seven EC-domains, a transmembrane domain and a cytoplasmic domain with and without the amino acid sequence encoded by exon 68. Similarly, the mouse *Cdh23* isoforms B1 and B2 (AY563163 and AY563164, respectively) have a translation start codon in Kozak consensus at position 443 and are 3787 bp and 3682 bp long (without exon 68) (Fig. 7C).

Cadherin 23 isoforms C1 and C2

Human isoforms C1 and C2 encode products of 1793 bp (exons 66–69) and 1678 bp (exons 66, 67 and 69) (AY563161 and AY563162). The mouse *Cdh23* isoforms C1 and C2 are similarly constructed (AY563159 and AY563160). Isoform C encodes the cytoplasmic domain of cadherin 23 but does not encode any EC-domains nor a transmembrane domain. In both human and mouse isoform C, there is a novel exonic sequence, 65a (Fig. 7A). The translation initiation codon for isoform C is present at nucleotide 410 (AY563161 and AY563162) in human and at nucleotide 443 in mouse (AY563159 and AY563160). Isoform C in humans has 23 unique amino acids of which a stretch of 7 residues (six identical, one conserved) are present in the mouse. All three *Cdh23* isoforms (A, B, and C) with and without exon 68 are expressed in wild type mice, while isoforms B and C are also expressed in the retina and cochlea of homozygous v^{6J} mice (Figs. 7C, D).

Western blot analysis of P7 WT and adult v^{6J} cochleae (Fig. 7E) revealed a band at approximately 370 kDa corresponding in size to the full-length isoform A in the WT, which is absent in the homozygous v^{6J} mice. In the WT, two bands just below and above the 111 kDa size marker are present, one of which may correspond to isoform B with the expected size of ~120 kDa. The smallest band of ~41 kDa was observed in the WT and v^{6J} mice, possibly

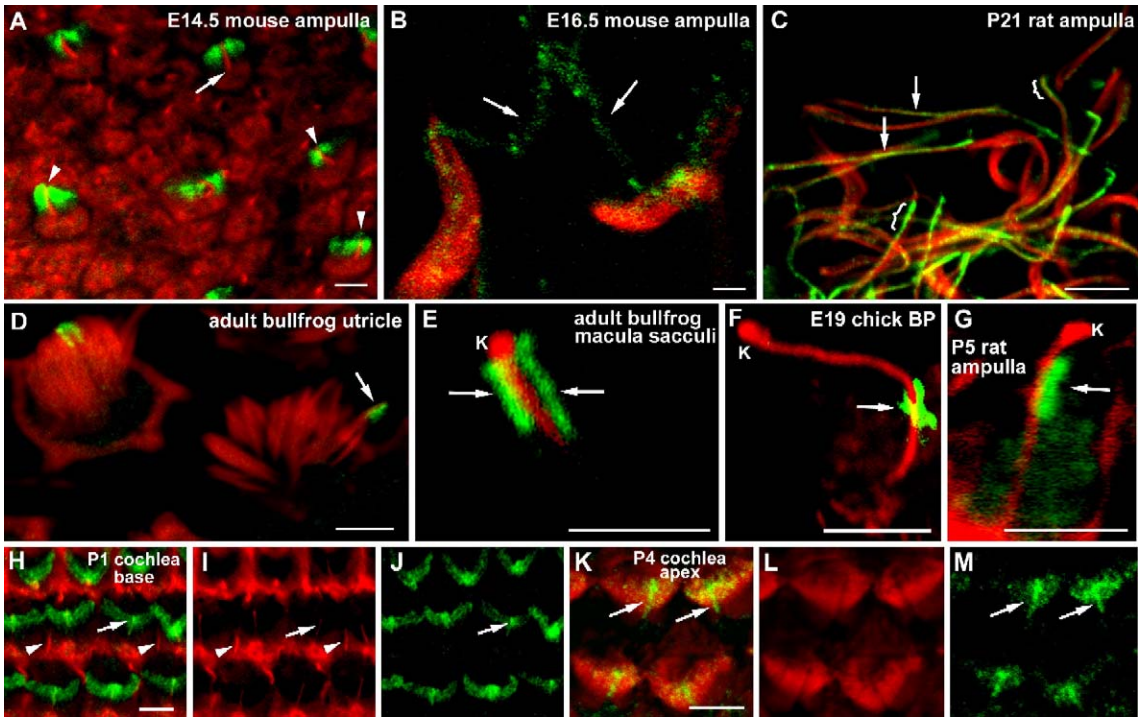

Fig. 4. Immunolocalization of cadherin 23 (shown in green) in hair cell kinocilia of mouse, rat, and bullfrog. (A) At E14.5, in immature mouse ampullar hair bundles, cadherin 23 (green) is localized along the length of stereocilia and is already detected in the top of the kinocilium (stained for β-tubulin—arrow, red) in the region of contact with the stereocilia (arrowhead). (B) At E16.5, in mouse ampulla, cadherin 23 (green) is detected along the length of the kinocilium (arrows) of mature hair bundles. (C) In a P21 rat ampulla, cadherin 23 (green) is detected along the length of mature hair cell kinocilia (arrows) and is also detected with a brighter signal in the regions that may be the location of the stereocilia–kinocilium bonds (brackets). (D) In the adult bullfrog utricle, cadherin 23 (green) is detected near the top of the tallest stereocilia (stained for F-actin-red) which are in contact with the kinocilium, in the region of the stereocilia–kinocilium links. (E) In the adult bullfrog macula sacculi, cadherin 23 (green, arrows) is localized at the top of the kinocilium (stained for β-tubulin—red, and indicated with K) in the region of the stereocilia–kinocilium bonds. (F) In hair bundles from the apex of the E19 basilar papilla, cadherin 23 (green, arrows) is localized in the region of the stereocilia–kinocilium bonds. The kinocilium is stained for β-tubulin (red), and is indicated with K. (G) The same was observed in the P5 rat ampulla where some staining of cadherin 23 is detectable in stereocilia, but the intense staining for cadherin 23 is visible in the region of the stereocilia–kinocilium bonds (arrow). In P1 mouse cochlea (H–J) stained for β-tubulin (red, I) and cadherin 23 (green, J), cadherin 23 is detected in stereocilia and in kinocilia of hair cell bundles from the basal turn of the cochlea (H, J). The kinocilia are stained along their length with the more intense staining in the region of the stereocilia–kinocilium links (arrows). Dieters cells primary cilia (H, I arrowheads) are not stained for cadherin 23. At P4 (K–M), cadherin 23 (green) is detected in stereocilia (stained for F-actin—red, L) and in kinocilia (arrows, K, M) of the apical turn of the cochlea. Scale bars, 5 μm.

corresponding to isoform C with the expected size of ~30 kDa. Additional bands are visible, which may represent other isoforms of cadherin 23 such as GenBank accession no. AK039126. Moreover, cadherin 23 is a predicted glycoprotein and glycosylation may alter the migration of the protein in the gel matrix.

Discussion

Developmental profile of cadherin 23 in stereocilia

During development, we first detected cadherin 23 at E16.5 along the entire length of hair cell stereocilia at the

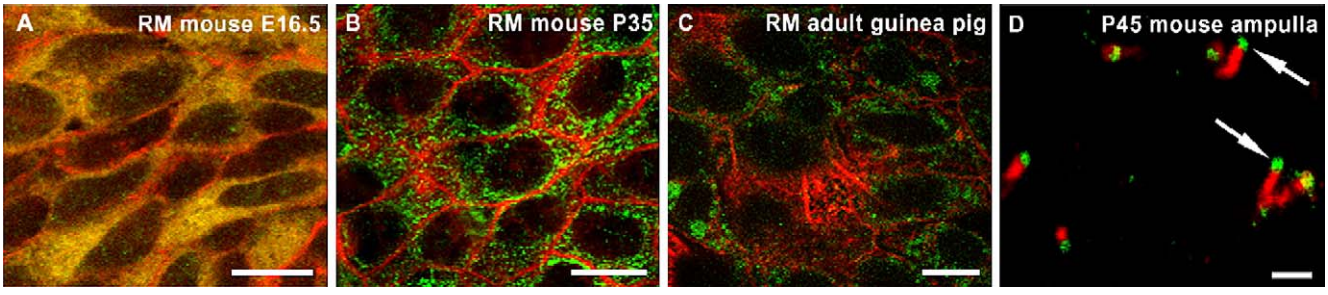

Fig. 5. Immunolocalization of cadherin 23 (shown in green) in Reissner's membrane and in the centrosomes. (A–C) Double staining for cadherin 23 (green) and F-actin (red) revealed cadherin 23 in RM of E16.5 (A) and P35 (B) mice, as well as in RM cells of adult guinea pig (C), where it appears to co-localize with F-actin in a cytoplasmic staining pattern. (D) In P45 mouse ampullar sensory epithelial cells, cadherin 23 (arrows, green) is detected with TF7 antibody and appears as green dots on the centrosomal tube stained for pericentrin (red). Scale bars, 5 μm.

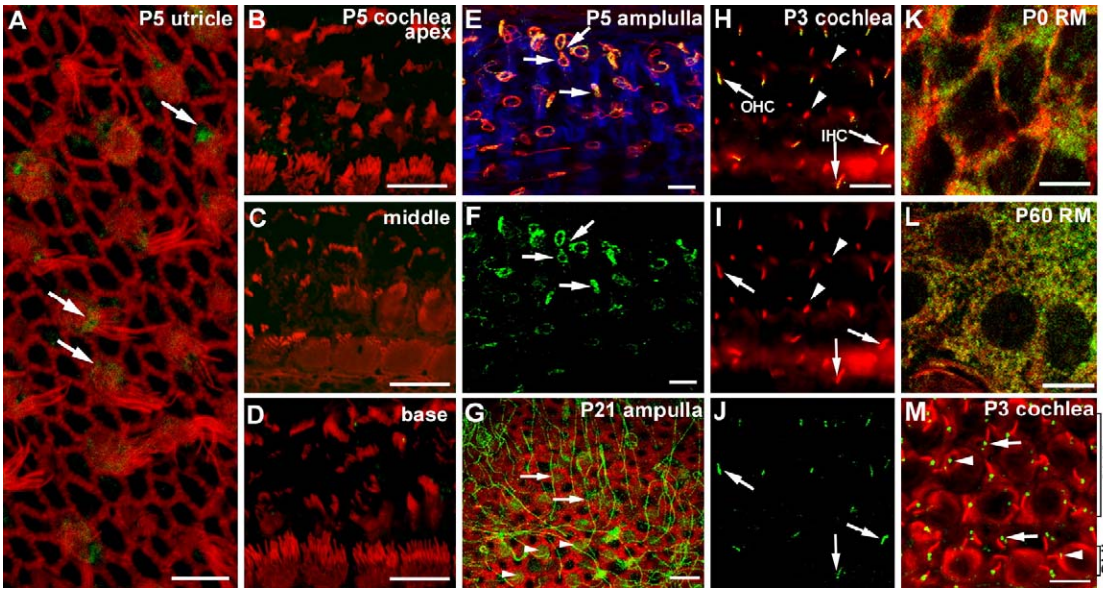

Fig. 6. Immunolocalization of cadherin 23 (shown in green) in the inner ear of homozygous *waltzer* v^{6J} mice. (A) Cadherin 23 (green) was not detected in utricle stereocilia (stained for F-actin, red) but was detected in the cuticular plate of hair cells (arrows) of the same focal plane. Cadherin 23 immunofluorescence was not detected in hair cell stereocilia from the apical (B), middle (C), or basal (D) turns of the P5 cochlea. (E) Triple staining of P5 ampulla for β-tubulin (red), F-actin (blue), and cadherin 23 (green) revealed that cadherin 23 is localized in ampullar hair cell kinocilia (F) indicated by arrows (E, F). (G) At P21, double staining for F-actin (red) and cadherin 23 (green) revealed cadherin 23 in kinocilia (arrows) of ampullar hair cells and in the cuticular plate of hair cells of the same focal plane (arrowheads). (H) Double staining for β-tubulin (red) and cadherin 23 (green) revealed cadherin 23 in hair cell kinocilia of inner (IHC) and outer (OHC) hair cells (arrows, H–J). Cadherin 23 is not detected in Dieters cells primary cilia (arrowheads, H–I). (K–L) In double staining for F-actin (red) and cadherin 23 (green), cadherin 23 appears to localize in the region of F-actin in RM cells of P0 (K) and P60 (L) v^{6J} mice. (M) Cadherin 23 (green) is localized in centrosomes (arrowheads) of inner (IHC) and outer (OHC) hair cells (also stained for β-tubulin, red) and in centrosomes of Dieters cells (arrows) of a P3 v^{6J} cochlea. Scale bars, 5 μm.

basal turn of the mouse cochlea. As stereocilia elongate, cadherin 23 becomes concentrated near their top and can be seen as complimentary patches adjacent to the actin core on the sides of neighboring stereocilia (Fig. 2G). This observation is consistent with the hypothesis that cadherin 23 is a component of the filamentous extracellular links between stereocilia in mammals present early in development (Di Palma et al., 2001a; Furness et al., 1989; Holme and Steel, 2002). These links appear to be essential for normal development of the stereocilia bundle and it seems likely that splaying of stereocilia in the *waltzer* mouse (Di Palma et al., 2001a) is due to the absence of cadherin 23 isoform A1 and/or A2 (Fig. 7A) mediated transient links connecting stereocilia.

Cadherin 23 is detected along the length of stereocilia before tip links can be distinguished from other stereocilial links and before mechanotransduction is obvious (Géléoc and Holt, 2003; Géléoc et al., 1997). In maturing hair bundles, cadherin 23 staining becomes less prominent and later, when the staircase architecture of stereocilia bundles develop, some of these transient links disappear, as does cadherin 23 (Denman-Johnson and Forge, 1999; Furness et al., 1989; Goodyear and Richardson, 1999; Pickles et al., 1991). From P16 onward, in mature hair cells of the mouse and rat organ of Corti (Figs. 2H–J), as in mature vestibular hair cells (Fig. 3D), our antibodies no longer detect cadherin 23 near the top of hair cell stereocilia using fluorescent immunolocalization and a LSM510 confocal light microscope. This observation is in agreement with previously reported data (Boëda et al., 2002).

Our antibodies do not reveal cadherin 23 in regions consistent with a tip link localization between the short and tallest stereocilia in mature hair cells as proposed by Siemens et al. (2004). Rather, our antibodies show cadherin 23 only near the tops of the tallest stereocilia, in the region of stereocilia–kinocilium links of the various species studied. We can reproduce Fig. 2C from Siemens et al. (2004) and we can detect cadherin 23 along the length of the kinocilium without raising excessively the gain of the confocal microscope, and without using image processing software or an intermediate antibody to amplify the primary signal. What then accounts for the discrepancy in detection of cadherin 23 at the tips of stereocilia? It may be that the antibody used in the study by Siemens et al. (2004) recognizes an epitope of cadherin 23 not detected by our antisera. Alternatively, the Siemens et al. (2004) antibody may be multi-specific (James et al., 2003) and is cross-reacting with an unrelated protein at the tips of stereocilia.

To address the possibility that our antisera may be less sensitive in detecting low levels of the cadherin 23 cytoplasmic domain, we amplified the primary signal using biotinylated secondary antibody and a fluorescent avidin conjugate. This resulted in amplified fluorescence at the

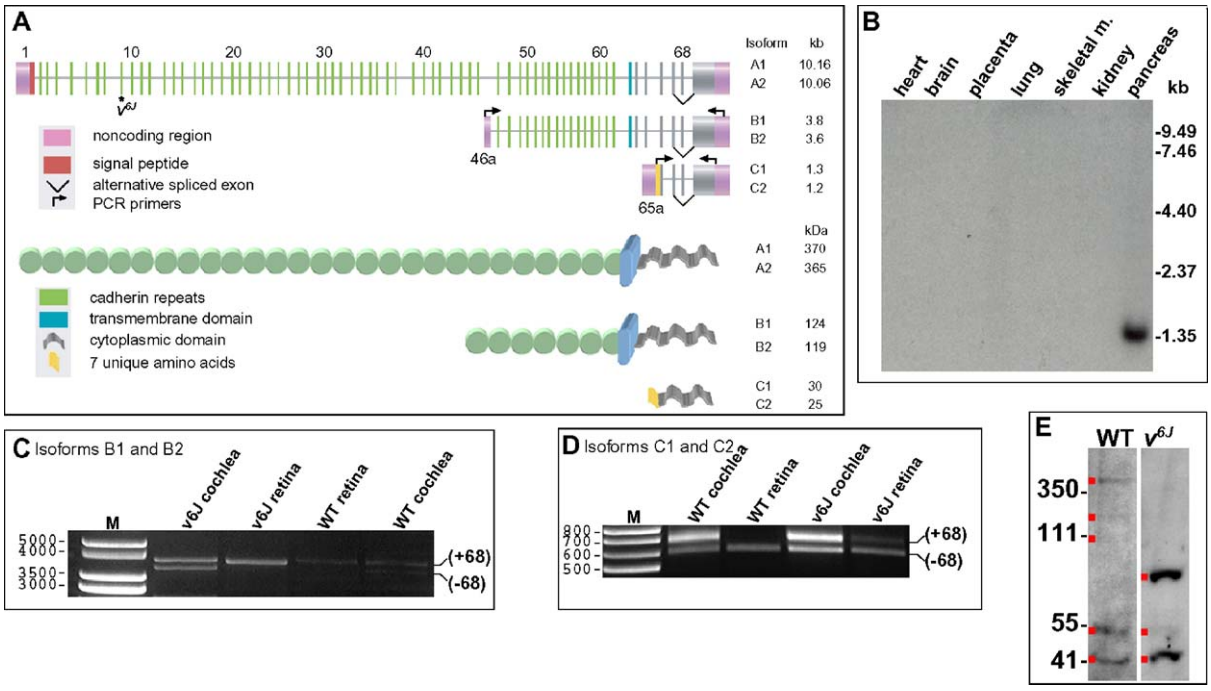

Fig. 7. Novel isoforms of cadherin 23. (A) Schematic illustration of the structure of *Cdh23* and cadherin 23 isoforms A, B, and C, and the location of PCR primers (arrows) used to amplify the transcripts. (B) A human multiple tissue Northern blot was hybridized with a probe from a sequence encoding the cytoplasmic domain of cadherin 23 (exons 66–69). A band of ~1.35 kb was detected in the pancreas. (C) Amplification of full-length isoforms B1 and B2 with primers shown in panel A (right and left pointing arrows) yields products of 3787 bp and 3682 bp, respectively, from the adult wild type mouse (WT) and v^{6J} cochlea and retina. Isoform B1 is present in all lanes whereas isoform B2 (without exon 68) is absent from WT and v^{6J} retina. (D) Amplification of isoforms C1 and C2 yielded products of 724 bp and 619 bp, respectively, from adult wild type (WT) and v^{6J} cochlea and retina. Isoform C1 message was detected in all cDNA samples except from WT retina. Amplification products were subcloned and sequence verified. (E) Western blot analysis of P7 WT and adult v^{6J} cochleae using TF7 revealed five bands in the WT and three bands in the v^{6J} (marked with red squares). The largest band in the WT corresponds in size to isoform A ~370 kDa and is absent in the v^{6J} homozygous mice. The smallest band, ~41 kDa, present in the WT and in the mutant, possibly corresponds to isoform C with the expected size of ~30 kDa. Two bands above and below 111 kDa are present in the WT and absent in the v^{6J} mice one of which may correspond to isoform B with the expected size of ~120 kDa. A band between 111 and 55 kDa is present in the v^{6J} homozygous mice and absent in the WT, and a band of ~55 kDa is present both in the WT and in the v^{6J} mice. These bands may represent other uncharacterized isoforms of cadherin 23 (GenBank accession number AK039123) recognized by our antisera.

upper region of stereocilia of immature hair bundles, in the region of stereocilia–kinocilium links, RM, and centrosomes in immature and mature bundles, as seen without signal amplification. Still, we did not detect cadherin 23 at the tips of stereocilia in mature hair bundles (data not shown).

Cadherin 23 localizes to the kinocilium and centrosomes

The migration of the kinocilium to one side of the developing hair bundle is a step that defines hair bundle polarity, and kinocilial links that connect the kinocilium to the adjacent tallest stereocilia have previously been described (Ernstson and Smith, 1986; Goodyear and Richardson, 2003; Hillman, 1969; Tsuprun et al., 2004). In early stages of hair bundle development, the mammalian vestibular hair cells are in various stages of maturation and the kinocilium is peripherally situated in the more mature hair bundles (Anniko et al., 1983; Denman-Johnson and Forge, 1999). At E14.5, in mouse vestibular hair cells, cadherin 23 is already detected in the region of the stereocilia–kinocilium links and perhaps cadherin 23 plays a role in hair bundle polarity. By E18.5 in the mouse, the majority of vestibular hair cells are mature (Anniko et al., 1983), tip links become evident (Denman-Johnson and Forge, 1999), mechanotransduction of vestibular hair cells can be measured (Géléoc and Holt, 2003; Zhao et al., 1996), and cadherin 23 appears along the kinocilium. The appearance of tip links and the acquisition of mechanotransduction of sensory hair bundles coincide with the immunohistochemical detection of cadherin 23 in hair cell kinocilia but not in primary cilia of other cell types. This raises the question as to whether or not proper bonding between the kinocilium and stereocilia is necessary for initiation of mechanotransduction.

In mature mouse hair bundles, we observed cadherin 23 along the entire length of the kinocilium. It is possible that the sugar components of cadherin 23 may interact with the glycocalyx, which covers the entire length of hair cell kinocilium and stereocilia (Takumida, 2001; Takumida et al., 1988a,b). It is also possible that cadherin 23 participates in links between the kinocilium and the otolithic membrane (rich in glycoproteins) to which the kinocilia in its upper

portion together with the tallest stereocilia are interconnected (Takumida and Bagger-Sjoback, 1991; Takumida et al., 1992; Sobkowicz et al., 1995).

Cadherin 23 was also localized in the centrosome which together with the kinocilium is considered the centrosomal complex (Alieva and Vorobjev, 2004). The expression of cadherin 23 in the centrosomes precedes its expression in stereocilia, persists throughout development, and is evolutionally conserved in mammals, chicks, and frogs. Cadherin 23 expression in the centrosome may have a role not only in the development of the kinocilium but also in the organization of microtubules (Chausovsky et al., 2000).

Cadherin 23 in v^{6J} mice and the identification of novel isoforms of cadherin 23

Homozygous v^{6J} mice have disorganized and splayed stereocilia bundles and cadherin 23 is absent from stereocilia (Di Palma et al., 2001a; Siemens et al., 2004), an observation that we confirmed using our antisera directed against cadherin 23 cytoplasmic domain. However, our findings that cadherin 23 is expressed in RM, kinocilia, and centrosomes of homozygous v^{6J} deaf mice (Fig. 6), suggested that, in the inner ear, the temporal and spatial pattern of expression and isoform variants of cadherin 23, and most likely the functions as well, are more elaborate than we and others previously reported (Astuto et al., 2002; Boëda et al., 2002; Bolz et al., 2001; Bork et al., 2001; Di Palma et al., 2001a,b; Siemens et al., 2002, 2004). The originally reported A isoform of cadherin 23 and the B isoform reported here share exons 47–69, which include the cytoplasmic domain (exons 64–69). In the mouse, the novel isoform C shares exons 65–69 with isoforms A and B but has 7 unique amino acids at the amino terminus that are encoded by exon 65a (Fig. 7A). Moreover, all three isoforms are expressed both in retina and inner ear. Isoforms A and B have the same trans-

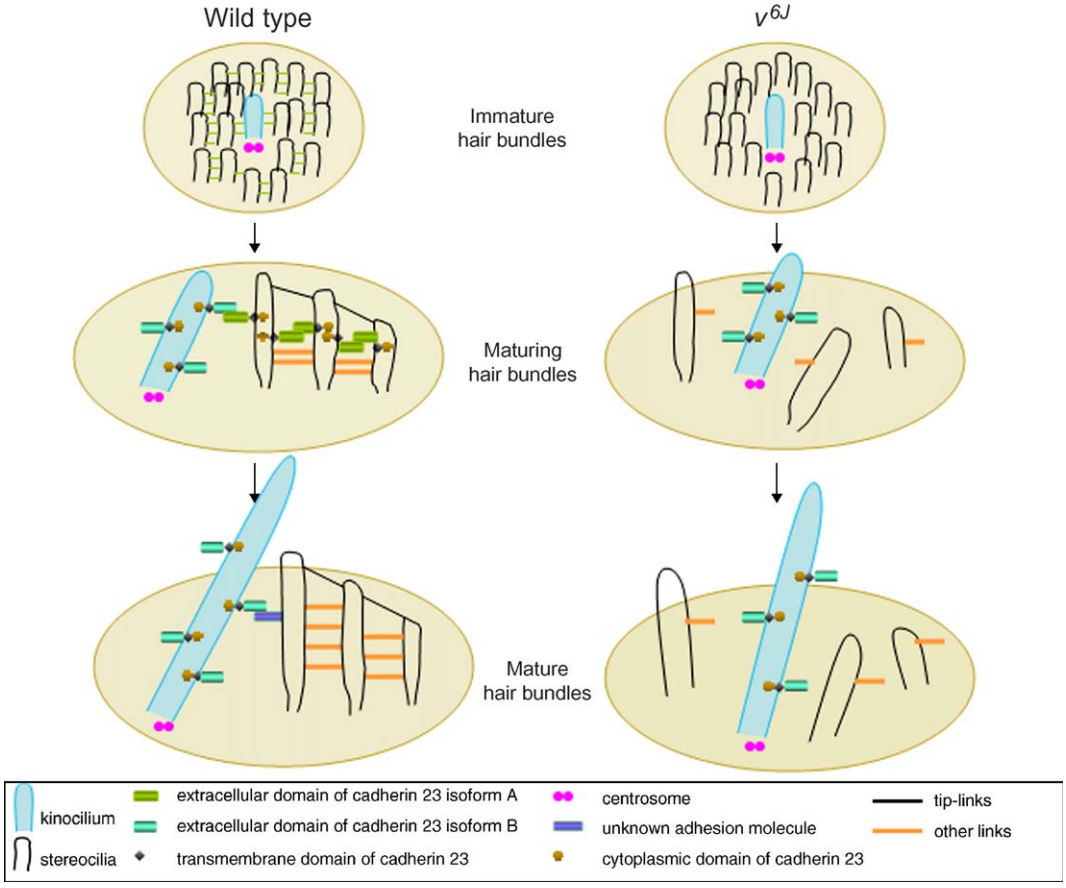

Fig. 8. Schematic representation of cadherin 23 isoforms A and B in stereocilia and stereocilia–kinocilium links in auditory hair cells of wild type and v^{6J} mice. Stereocilia of immature hair bundles are of similar length, and a single centrally positioned kinocilium of the same length accompanies them. During development of the wild type hair bundle, the kinocilium migrates to the periphery of the hair cell and the stereocilia form rows of increasing height where the tallest row is adjacent to the kinocilium. We presume that some of the interstereocilia links are homophilic bonds between cadherin 23 isoform A molecules, and cadherin 23 isoforms A and B participate in stereocilia–kinocilium links. In mature hair bundles, cadherin 23 isoform A disappears from stereocilia, and isoform B is present along the length of the kinocilium and in the region of the stereocilia–kinocilium links. Other links maintain the organized structure of the hair bundle, perhaps protocadherin 15 is one of them (Ahmed et al., 2003; Alagramam et al., 2001). Unlike wild type mice, v^{6J} mutant mice exhibit splayed stereocilia bundles and misplaced kinocilia, and cadherin 23 isoform B and/or C are present along kinocilia. The absence of isoform A from stereocilia may explain the disorganized hair bundles of v^{6J} mice.

membrane domain while isoform C has no predicted transmembrane domain nor EC-domains. Therefore, based simply on amino acid sequence, isoform C cannot function as a classic cadherin molecule (Hirano et al., 2003), but may play a regulatory role as a competitive surface for proteins that interact with the cytoplasmic domain of isoforms A and B of cadherin 23.

The exact function of all three *Cdh23* isoforms remains to be investigated, and we emphasize that the antisera directed against the cytoplasmic domain of cadherin 23 would be expected to recognize cadherin 23 isoforms A, B, and C. At the moment, our antisera and antisera to the cytoplasmic domain of cadherin 23 that have been reported (Boëda et al., 2002; Siemens et al., 2002, 2004) cannot, by immunohistochemical localization studies alone, distinguish between these three protein isoforms encoded by *Cdh23* and *CDH23*.

The loss of immunostaining in hair cell stereocilia of v^{6J} mice does permit conclusions about the localization and possible function of isoform A, which coupled with the *waltzer* phenotype, suggests that in the inner ear, this isoform is important directly or indirectly at least for hair bundle organization and perhaps positioning of the kinocilium as illustrated in our model (Fig. 8). The presence of cadherin 23 in RM, centrosomes, and in the kinocilia of wild type and v^{6J} mice probably represents either or both isoforms B and C, which are not affected by the v^{6J} mutation, and possibly isoform A in the wild type mouse.

Finally, the $Cdh23^{753A}$ allele is associated with age-related hearing loss in the mouse (Noben-Trauth et al., 2003). Since cadherin 23 disappears from stereocilia in the mouse at P16, but is stably retained in the RM of the adult, perhaps age-related hearing loss in the mouse is due to a decline in some function of the RM (Lee and Marcus, 2003) rather than or in addition to its suggested alteration of developing stereocilia (Holme and Steel, 2004). This speculation is strengthened with our report of additional cadherin 23 isoforms B and C and their lifelong persistence in RM and in the kinocilia of wild type and v^{6J} *waltzer* mice. Together, these findings suggest that the various isoforms of cadherin 23 may perform multiple functions within the auditory system in addition to the development of cohesive hair cell stereocilia bundles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.01.015.

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