Carcinoembryonic antigen-related cell adhesion molecule 16 interacts with α-tectorin and is mutated in autosomal dominant hearing loss (DFNA4)

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We report on a secreted protein found in mammalian cochlear outer hair cells (OHC) that is a member of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family of adhesion proteins. *Ceacam16* mRNA is expressed in OHC, and its protein product localizes to the tips of the tallest stereocilia and the tectorial membrane (TM). This specific localization suggests a role in maintaining the integrity of the TM as well as in the connection between the OHC stereocilia and TM, a linkage essential for mechanical amplification. In agreement with this role, CEACAM16 colocalizes and coimmunoprecipitates with the TM protein α-tectorin. In addition, we show that mutation of *CEACAM16* leads to autosomal dominant nonsyndromic deafness (ADNSHL) at the autosomal dominant hearing loss (DFNA4) locus. In aggregate, these data identify CEACAM16 as an α-tectorin–interacting protein that concentrates at the point of attachment of the TM to the stereocilia and, when mutated, results in ADNSHL at the DFNA4 locus.

cochlea | deafness genes

In the mammalian cochlea, the sound-induced vibratory pattern of the basilar membrane (BM) is sensed and transduced by hair cells, which are subclassified as either inner hair cells (IHC) or outer hair cells (OHC). These epithelial cells are incorporated into the cellular matrix of the organ of Corti, which is sandwiched between the tectorial membrane (TM) and the BM. IHC have a rich afferent innervation and function as auditory sensory receptors conveying sound-related information to the central nervous system. OHC, in contrast, have motor capabilities associated with the voltage-sensitive molecular motor prestin (1). In response to receptor potential changes, OHC change length (2), thereby providing mechanical feedback and significant amplification of BM motion. Without feedback from OHC, mammals suffer an ∼50–60 dB hearing loss (3).

The mechano-sensitive input organelles of hair cells are the stereociliary bundles, which comprise a cross-linked actin-filament core, extracellular links to promote bundle integrity, and protein complexes responsible for mechano-transduction and mechano-adaptation (4). The extracellular links are formed by a number of different transmembrane proteins including cadherin 23 (CDH23) (5), protocadherin 15 (PCDH15) (6), the very large G protein-coupled receptor-1 (VLGR1) (7), usherin (8), and a receptor-like inositol lipid phosphatase (Ptprq) (9). Mutation of any one of these proteins causes hearing impairment (10). Because OHC participate in a mechanical feedback loop, their tallest stereocilia are attached firmly to the overlying TM, an essential coupling for OHC motor function (11). Disrupting this connection opens the feedback loop and results in loss of amplification (12). IHC are the sensors of vibratory patterns established by complex interactions among the BM, TM, and organ of Corti. Because both morphological and physiological evidence indicates that the IHC stereocilia are not firmly connected to the TM (13, 14), they are not participants in the cochlear amplification feedback loop but rather are its beneficiaries.

The tips of the tallest rows of OHC stereocilia insert into shallow pits on the underside of the TM (14). The connection is stronger than a mere insertion because upon mechanical removal of the TM, stereocilia occasionally are avulsed and maintain their TM attachments (14). Although the molecular basis of this attachment is obscure, it is pertinent that the bottom layer of the TM, which is in contact with OHC stereocilia, is different from its main collagenous body. Referred to as "Hardesty's membrane" (15) or "Kimura's membrane" (14), this region is constituted mainly of tectorin proteins coded for by the *Tecta* and *Tectb* genes (16). These proteins are made in and secreted from supporting cells that surround IHC and OHC but are not detectable within the hair cells themselves (17). *Tecta*-null mice lack α-tectorin, and in these animals the residual TM is detached from the organ of Corti. As a consequence, the ability of OHC to operate as efficient amplifiers of BM motion is compromised (12, 18). Electron microscopy resolves attachment links that form a TM-attachment crown around the tips of the tallest OHC stereocilia, connecting them to the undersurface of the TM (19). Although stereocilin may be associated with the crown (20), other possible TM crown components have not been identified. It is known, however, that the crown is susceptible to digestion by subtilisin but is not sensitive to calcium chelation with 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) (21).

Carcinoembryonic antigen-related cell adhesion molecule 16 (CEACAM16) is an adhesion protein that differs from all reported cochlear adhesion proteins. Based on bioinformatics analysis, CEACAM16 belongs to the CEACAM family, a group of Ig-related glycoproteins with diverse functions that include tissue architecture/homeostasis, cell growth and differentiation,

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angiogenesis, and tumor suppression (22). The function of CEACAM16 has not been described. Bioinformatic analysis (23) predicts that CEACAM16 is a secreted glycoprotein with no recognizable transmembrane domain or GPI anchor. It has two Ig-variable (IgV)-like domains at its N and C termini (shown in yellow in Fig. 1*A*) and two Ig-constant (IgC)-like domains of subtype A and B in the middle (shown in green in Fig. 1*A*). Of the 22 *Ceacam* genes found in mice, *Ceacam16* is one of only five conserved among mice, rats, and humans (24). In humans *CEACAM16* is located on 19q13.31, where DFNA4, an autosomal dominant nonsyndromic hearing loss (ADNSHL) locus was mapped in an American family (Family 1070) (25). Persons in this family have hearing loss that begins in early adolescence and progresses to ~50 dB across all frequencies, consistent with loss of OHC-mediated amplification (3, 26).

Myosin heavy-chain 14, non-muscle (*MYH14*) also maps to the DFNA4 locus, and mutations in this gene have been identified in several DFNA4 families reported subsequent to the initial mapping (25, 27). However, mutation screening of *MYH14* in Family 1070 failed to identify any mutations, and haplotype reconstruction with SNPs in this region narrowed the original DFNA4 interval and excluded *MYH14* by 15 kB, providing firm evidence of a second DFNA4 gene (28). Here we show that *Ceacam16* mRNA is expressed in OHC and that CEACAM16 protein probably is associated with the TM. We demonstrate that CEACAM16 and α-tectorin interact, providing a mechanism for producing a connection between stereocilia and Hardesty's membrane and suggesting that CEACAM16 may be a component of the attachment crown. Finally, we show that mutation of *CEACAM16* is responsible for the dominant form of human deafness, DFNA4, in Family 1070 (25).

Results

CEACAM16 Is Secreted in Multiple Oligomeric Forms. CEACAM16 has seven cysteines (marked with asterisks in Fig. 1*A*) and seven predicted N-glycosylation sites (shown in red Fig. 1*A*). Bioinformatic analysis also indicates that CEACAM16 is a secreted protein because it has a signal peptide at its N terminus with a proteolytic cleavage site between the 22nd and 23rd amino acids (24). To test this prediction, full-length *Ceacam16* (~1,300 bp) cloned from the cochlea was inserted into a mammalian expression vector with a V5-His epitope downstream of *Ceacam16*. V5-*Ceacam16* was transiently transfected into mammalian cell lines [opossum kidney (OK) and HEK293T cells], and cell media used to culture these cells were collected separately and centrifuged to remove cell fragments and possible exosomes (29). As shown in Fig. 1 and Fig. S1, CEACAM16 was found in both OK and HEK cell pellets (Fig. 1*B* and Fig. S1*C*) and media (Fig. 1*C* and Fig. S1*C*), suggesting that it is a secreted protein, a result consistent with an earlier prediction for CEACAM16 (24). In addition, subtilisin degraded CEACAM16 bands (Fig. 1*B*). Western blots from both cell pellets and medium showed that CEACAM16 proteins formed ladders of monomers (~50–75 kDa), dimers (~150 kDa), and tetramers (>250 kDa) (Fig. 1 *B* and *C* and Fig. S2*B*). Treatment of samples from OK (Fig. S2*B*) and HEK293T (Fig. 1*C*) cells with DTT reduced oligomers to monomers, suggesting that CEACAM16 forms disulphide cross-linked dimers and tetramers.

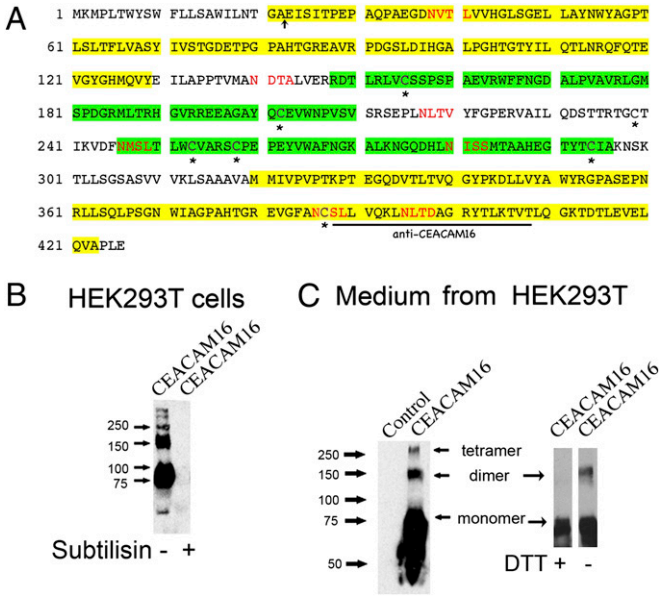

Fig. 1. (*A*) CEACAM16 amino acid sequence and structural domains. Yellow indicates IgV-like domains; green indicates IgC-like domains. Predicted N-glycosylation sites are in red. Asterisks indicate cysteines; arrow shows the proteolytic cleavage site. Amino acids used to generate anti-CEACAM16 are underlined. (*B*) CEACAM16 forms oligomers in the cell pellet and is degraded by subtilisin. (*C*) CEACAM16 oligomers also are found in the cell-culture medium collected from *Ceacam16*-transfected HEK293T cells (CEACAM16) but not from untransfected cells (control). DTT reduces CEACAM16 oligomers to monomers.

CEACAM16 Protein Is Located at the Top of the Tallest Stereocilia of OHC in Adult Mice. According to reported RT-PCR results, *Ceacam16* is less widely expressed in tissue than *Ceacam1* (24). However, *Ceacam16* mRNA is expressed abundantly in cochleae collected from embryonic day 17 (E17) to adolescent mice based on quantitative RT-PCR (qRT-PCR) results (Fig. S3). In situ hybridization experiments showed that a negative-control probe produced no staining (Fig. 2*A*) in postnatal day 42 (P42) mouse cochlear tissue, whereas the positive *Ceacam16* RNA probe stained OHC but not supporting cells or IHC (Fig. 2*B*, brown). These results demonstrate that *Ceacam16* mRNA is expressed in OHC.

To study the location of CEACAM16 protein, we raised antiserum against an epitope near the C terminus of mouse CEACAM16 (Fig. 1*A*). Specificity of the anti-CEACAM16 was tested in mammalian cells transiently transfected with a plasmid encoding V5-CEACAM16. As shown in immunofluorescent images (Fig. S2*A*), the same cells were labeled by both anti-V5 (red) and anti-CEACAM16 (green). In addition, both anti-V5 and anti-CEACAM16 recognize the same protein bands in Western blots of HEK293T cells expressing CEACAM16 (Fig. S2*B*). These results suggest that anti-CEACAM16 recognizes CEACAM16 protein. We then performed immunofluorescence on whole-mount cochlear samples. As shown in Fig. 2*C*, CEACAM16 is located at the tip of OHC but not IHC bundles, a result consistent with our in situ data (Fig. 2 *A* and *B*). Using the Z-section mode of the confocal microscope, we followed CEACAM16 and actin staining from the top of the tallest stereocilia downward for 3.3 μm at intervals of 0.15 μm. The 21 images were combined to make a 3D movie (Movie S1). We noticed that CEACAM16 staining was not always the same size. In some cases, large dots were associated with stereocilia tips (Fig. 2*C*), as if CEACAM16 were pulling material out of the TM. This observation suggests that CEACAM16 may bind TM protein(s).

We also examined the biochemical properties of CEACAM16 in vivo. Cochleae were dissected from adult mice for both immunofluorescence and Western blot. In Fig. 3 *A*–*F*, immunofluorescent images are shown for both untreated (Fig. 3 *A*–*C*) and subtilisin-treated (Fig. 3 *D*–*F*) cochlear whole mounts. Comparison of the two rows of the figure shows that the green staining of CEACAM16 in untreated OHC stereocilia completely disappears after subtilisin treatment. Similarly, Western blot experiments using cochlear material show subtilisin-induced degradation of CEACAM16 bands (Fig. 3*G*). Similar to data collected from HEK293T cells (Fig. 1), CEACAM16 appears to form oligomers in the cochlea, and these oligomers can be converted to monomers by adding the reducing reagent DTT

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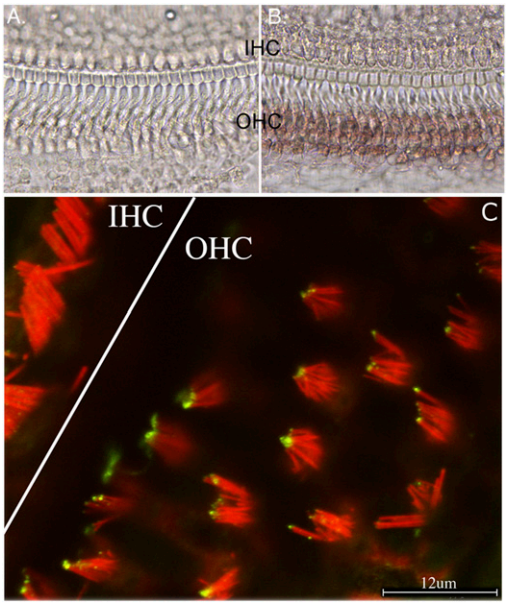

Fig. 2. *Ceacam16* mRNA is present in OHC. (*A*) In situ hybridization of cochlear tissue derived from P42 129/C57BL6 mice hybridized with a negative RNA probe shows no staining. (*B*) Brown staining was observed in OHC only when tissue was hybridized with an anti-sense *Ceacam16* RNA probe. (*C*) CEACAM16 (anti-CEACAM16, green) is localized at the tip of OHC but not IHC stereocilia. Red staining for actin helps identify the stereocilia core.

(Fig. 3*G*). Besides the tip of OHC stereocilia, strong CEACAM16 (green) staining was found in the main body of the TM where α-tectorin protein is enriched (Fig. 3*I*). TM staining was not observed when the primary antibody was replaced with nonspecific rabbit Ig proteins (Fig. 3*H*). These results suggest that CEACAM16 is a secreted protein that is a part of the TM.

Relationship Between CEACAM16 and α-Tectorin. Because Hardesty's membrane into which the tallest OHC stereocilia insert is composed of tectorin proteins (17), we explored the potential relationship between CEACAM16 and α-tectorin by transfecting OK cells with plasmids of *Ceacam16* and *Tecta* cDNA. As shown in Fig. 4*A* for CEACAM16 and in Fig. 4*B* for α-tectorin, there is evidence for colocalization as indicated by the yellow color in the merged image (Fig. 4*C*). These results suggest a potential direct interaction between α-tectorin and CEACAM16. As a negative control, *Ceacam16* was cotransfected with a plasmid encoding GFP-prestin, which is an OHC-specific membrane protein. No colocalization between GFP-prestin and CEACAM16 was seen (Fig. S4).

To confirm that CEACAM16 and α-tectorin interact, coimmunoprecipitation experiments were performed using either HEK293T or OK cells. Proteins from nontransfected host cells or cells transfected with a single plasmid did not coimmunoprecipitate with CEACAM16 (Fig. S5). In Fig. 4 *D* and *E*, OK cells were cotransfected with plasmids of either V5-*Ceacam16*/*GFP-prestin* (CEACAM16+GFP-prestin) or V5-*Ceacam16*/*myc-Tecta* (CEACAM16+α-tectorin). Forty-eight hours after transfection, cells were lysed and subjected to anti-V5 immunoprecipitation. Cell lysates (input) and eluted proteins (output) were separated on a 4–20% gradient gel followed by blotting with anti-myc, anti-GFP, and anti-V5 for detecting α-tectorin, prestin, and CEACAM16 proteins. As shown in Fig. 4 *D* and *E* (*Left*), CEACAM16, α-tectorin, and prestin protein bands were detected in the cell lysates (input) as expected, whereas output myc–α-tectorin coimmunoprecipitates only with CEACAM16 (Fig. 4*E*, *Right*) and not with prestin (Fig. 4*D*, *Right*). These data demonstrate that CEACAM16 is associated with α-tectorin. Because α-tectorin is a large, secreted TM protein, it may be sticky and bind other proteins

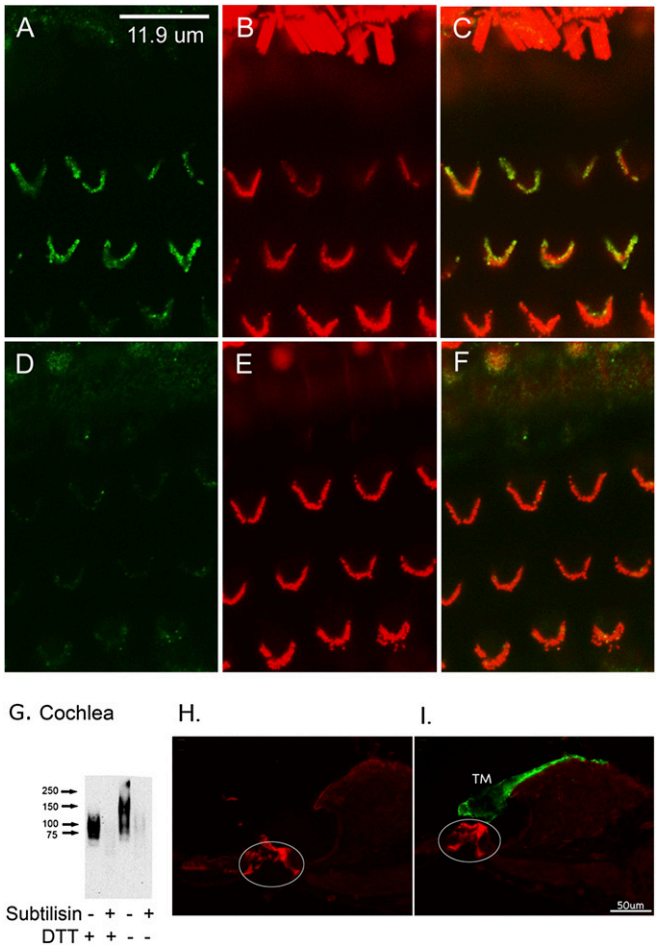

Fig. 3. (*A–G*) CEACAM16 protein in adult cochleae is degraded by subtilisin. (*A–C*) Immunofluorescent images of an adult mouse cochlea stained with anti-CEACAM16 (*A*, green) and Texas Red-X phalloidin (*B*, red). Merged image is shown in *C*. (*D–F*) Similar to *A–C*, but samples were treated with subtilisin. (*G*) The CEACAM16 band also is degraded by subtilisin. Cochlear samples were separated on NEXT-PAGE and blotted with anti-CEACAM16. DTT reduced CEACAM16 oligomers to monomers. (*H–I*) Immunofluorescent images show the location of CEACAM16 in the organ of Corti. (*H*) Cross-section of the negative-control mouse cochlea. Anti-CEACAM16 is replaced with rabbit Ig control (2.5 μg/mL). (*I*) Immunofluorescent image shows that CEACAM16 (green) is present in the TM. The organ of Corti (indicated by circles) is visualized by actin staining.

nonspecifically. Hence, additional coimmunoprecipitation experiments were performed between α-tectorin and other proteins. As shown in Fig. 4, plasmids encoding CEACAM16 and oncomodulin (an OHC cytoplasmic protein) were cotransfected with the *Tecta* plasmid into HEK293T cells. CEACAM16 coimmunoprecipitates with α-tectorin (Fig. 4*G*, *Right*), whereas oncomodulin does not (Fig. 4*F*, *Right*). Thus, CEACAM16 can pull down α-tectorin using anti-V5 (Fig. 4*E*) but not prestin (Fig. 4*D*). α-Tectorin can coimmunoprecipitate with CEACAM16 using anti-myc (Fig. 4*G*) but not oncomodulin (Fig. 4*F*) or prestin (see Fig. 6*D*).

We also investigated whether α-tectorin can interact with other CEACAM proteins by performing coimmunoprecipitation between α-tectorin and CEACAM1, which is the most widely expressed CEACAM protein (24). OK cells were cotransfected with plasmids encoding α-tectorin and CEACAM1. As shown in Fig. 4*H*, both CEACAM1 and α-tectorin were found in the cell lysate (input), but the α-tectorin band was not detected in the output, suggesting that CEACAM1 does not bind α-tectorin. These results confirm that CEACAM16 binds to the TM protein α-tectorin.

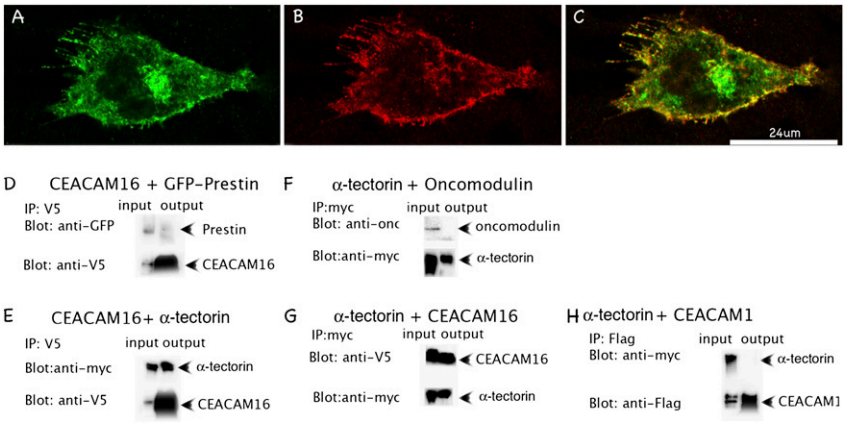

Fig. 4. (*A–C*) Immunofluorescent images show colocalization of CEACAM16 (*A*, green, polyclonal anti-V5) with α-tectorin (*B*, red, anti-myc) in OK cells cotransfected with myc-*Tecta*+V5-*Ceacam16*. Images are merged in *C*. (Scale bar: 24 μm.) (*D–H*) Coimmunoprecipitation experiments. Cell lysates (input) and eluted proteins (output) were separated on 4–20% gradient gel. Anti-V5, anti-myc, anti-oncomodulin, anti-Flag, and anti-GFP were used to identify CEACAM16, α-tectorin, oncomodulin, CEACAM1, and prestin. (*D* and *E*) A plasmid encoding CEACAM16 was cotransfected into OK cells with GFP-*prestin* (CEACAM16+GFP-prestin) and *Tecta* (CEACAM16+*Tecta*) plasmids, respectively. CEACAM16 is pulled down with 2 μg of anti-V5/protein A Sepharose. α-Tectorin coimmunoprecipitates with CEACAM16, but prestin does not. (*F* and *G*) Plasmids encoding α-tectorin were cotransfected into HEK293T cells with oncomodulin (α-tectorin+oncomodulin) and CEACAM16 (α-tectorin +CEACAM16). Cell lysates were subjected to coimmunoprecipitation by using 2 μg of anti-myc protein A Sepharose (pulling down α-tectorin). CEACAM16 coimmunoprecipitates with α-tectorin but not with oncomodulin. (*H*) Plasmid encoding α-tectorin was cotransfected into OK cells with CEACAM1 (α-tectorin +CEACAM1). Cell lysates were subjected to coimmunoprecipitation by using 2 μg of anti-Flag/protein G Sepharose (pulling down CEACAM1). CEACAM1 did not coimmunoprecipitate with α-tectorin.

Mutation of CEACAM16 in a DFNA4 Family. The DFNA4 interval as defined by linkage mapping of American Family 1070 is an ∼20 Mb region on chromosome 19q12-13.4 delimited by markers D19S414 and rs648298. Pure-tone auditory testing revealed that hearing-impaired members of Family 1070 have sensorineural, postlingual, bilateral, moderate hearing loss commencing during adolescence and progressing to ∼50 dB in adulthood (25). Visual and vestibular problems are absent.

Based on chromosomal location and predicted function, we considered *CEACAM16* a candidate for the DFNA4 gene in this family. Mutational screening of its six coding exons revealed a heterozygous c.418A→C transition in exon 3 predicting a threonine-to-proline change at conserved amino acid 140 (p.T140P; Fig. 5 *A* and *B*). Sequence alignment results show that threonine is highly conserved at this position in multiple mammalian species (Fig. 5 *C* and *D*). Mutation at T140 disrupts a conserved motif for the N-glycosylation site (-N-X-T/S-). This change segregates in all hearing-impaired members of Family 1070 and was not found in 200 ethnically matched normal-hearing controls (400 chromosomes). To test whether T140P actually affects the glycosylation of CEACAM16, we made the relevant human mutation T140P in mouse *Ceacam16* at position 142: T142P. When CEACAM16 and the P142 mutant mouse proteins were compared on 10% NEXT-PAGE gels, P142-CEACAM16 appeared to run as a monomeric band of smaller mass than WT CEACAM16 (Fig. 5*E*), indicating that the proline change at position 142 affects glycosylation.

To exclude variants in other genes in the DFNA4 interval as the cause of the hearing loss in this family, we performed whole-exome sequencing, focusing on the linked DFNA4 interval. The region between base pairs 31,308,160 and 51,308,188 (University of California, Santa Cruz hg19/GRCh37, February 2009 human reference sequence) contains 715 genes, of which 50 are expressed in the inner ear (National Center for Biotechnology Information database of EST; http://www.ncbi.nlm.nih.gov/projects/dbEST/). Exomic capture and sequencing were completed on affected family member III-3 (Fig. 5*B*) using the SureSelect Human All Exon 50Mb Kit and the SOLiD v4 system.

One quadrant of a SOLiD slide generated 6.56 Gb of 50-bp reads. Alignment of sequence reads showed that we were able to target 98.2% of the requested DFNA4 interval, with 70.2% of the region covered by at least 10 high-quality reads. By using this depth of coverage as a minimum threshold and filtering for changes observed in ≥35% of reads, we identified 30 variants. Eighteen variants were excluded by filtering against dbSNP130 or because they were present in sequenced exomes of unrelated white individuals from our series. Of the remaining 12 variants, six were missense changes, two were nonsense changes, one was a splice-site variant, and three were indels (Table S1). All 12 variants were excluded as the cause of DFNA4 hearing loss in Family 1070 because they either were not verified by Sanger sequencing or did not segregate with the hearing loss. These results suggest that the CEACAM16 p.T140P mutation is causally related to the hearing loss segregating in Family 1070.

Comparison of WT and Mutated CEACAM16. To understand the influence of T142 glycosylation on CEACAM16's function, we

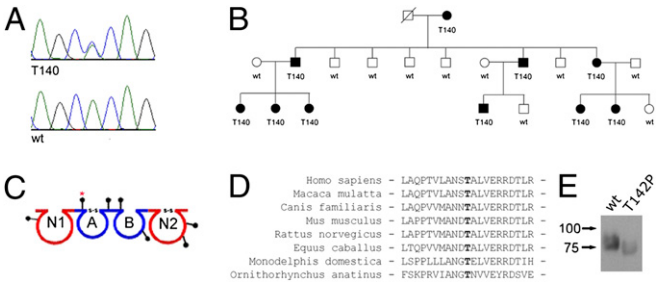

Fig. 5. T140P mutation of CEACAM16 is found in DFNA4. (*A* and *B*) Sequence results (*A*) and pedigree (*B*) showing that the heterozygous T140P mutation segregates with the dominant deafness in family 1070. (*C*) Diagram showing CEACAM16 structural domains. Potential N-glycosylation sites are marked by lollipops. [Modified from Zebhauser et al. (24), copyright (2005), with permission from Elsevier]. The T140P mutant is marked by a red asterisk. (*D*) Sequence alignment showing conservation of T140 (in bold) in mammalian species. (*E*) Western blot of WT and P142-CEACAM16. Proteins were separated on 10% NEXT-PAGE gels and blotted with anti-V5. P142-CEACAM16 appears to have a smaller monomer band than WT CEACAM16.

investigated the mutant protein expression pattern in various cells transiently transfected with both WT and T142P-*Ceacam16*. There was no apparent difference in expression patterns (Fig. S1 *A* and *B*); both proteins associated with intracellular/plasma membranes and accumulated as punctae, which probably include membranous elements of the secretory and/or endocytotic pathways. These observations suggest that the T142P mutation does not significantly change protein distribution patterns. Because CEACAM16 is a secreted protein, we also compared WT CEACAM16 and P142-CEACAM16 mutant samples collected separately both from cell pellets and from culture media. Similar to WT CEACAM16, P142-mutant bands were found in both cell pellet and medium, indicating that the T142P mutation does not interfere significantly with secretion and/or oligomerization (Fig. S1 *C* and *D*).

To learn whether P142-CEACAM16 interacts with α-tectorin, we studied expression patterns of P142-CEACAM16 and α-tectorin when both cDNAs were cotransfected into OK cells. P142-CEACAM16 (green in Fig. 6*A*) was colocalized with α-tectorin (red in Fig. 6*B*) as indicated by the yellow color in the merged image (Fig. 6*C*). We confirmed this interaction with coimmunoprecipitation experiments using HEK293T cells cotransfected with V5-*Ceacam16*/myc-*Tecta* (WT+α-tectorin), V5-T142P-*Ceacam16*/myc-*Tecta* (T142P+α-tectorin), or V5-*prestin*/myc-*Tecta* (prestin+α-tectorin). Prestin (SLC26A5) with the same tags as CEACAM16 (30) was used as a negative control. In the input, comparable amounts of α-tectorin (Fig. 6*E*), prestin, WT, and P142-CEACAM16 (Fig. 6*G*) were found in all three samples before coimmunoprecipitation. In the output, V5-tagged WT and P142-CEACAM16, as well as prestin proteins, were present in eluted samples, as expected (Fig. 6*F*). We were able to pull down α-tectorin with V5-tagged WT and P142-CEACAM16 but not with V5-prestin (Fig. 6*D*), a result consistent with an interaction between α-tectorin and both CEACAM16 and its mutant P142 form.

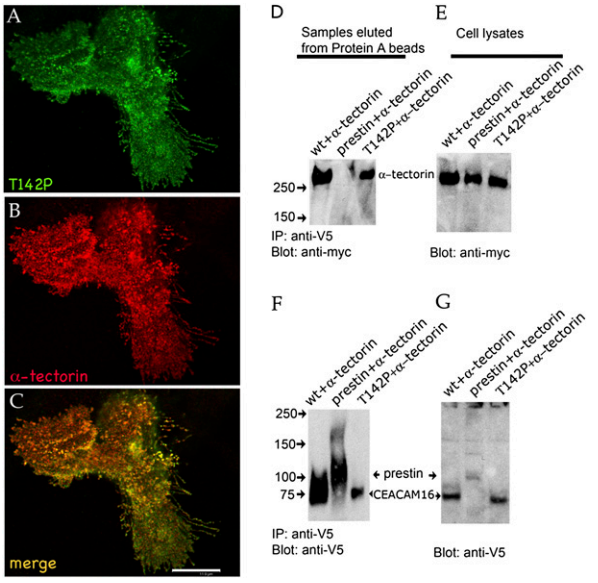

Fig. 6. CEACAM16 with the T142P change is still associated with α-tectorin. (*A*–*C*) Immunofluorescent images show colocalization of the T142P mutant (green, polyclonal anti-V5) with α-tectorin (red, monoclonal anti-myc) in OK cells cotransfected with myc-*Tecta*+V5-T142P-*Ceacam16*. Images are merged in *C*. (Scale bar: 11.9 μm.) (*D*–*G*) HEK293T cell lysates from V5-CEACAM16+myc-α-tectorin–expressing cells (WT+α-tectorin), V5-prestin+myc-α-tectorin–expressing cells (prestin+α-tectorin), and V5-P142-Ceacam16+myc-α-tectorin–expressing cells (T142P+α-tectorin) were subjected to coimmunoprecipitation by using 2 μg of anti-V5 and protein A Sepharose. The eluted proteins were separated on 7.5% NEXT-PAGE. α-Tectorin was visualized by anti-myc. Prestin and CEACAM16 were visualized by anti-V5. (*D*) α-Tectorin precipitated with WT CEACAM16 and P142-CEACAM16, but not with prestin. (*E*) Cell lysates (collected before coimmunoprecipitation) show that α-tectorin was detected in all three samples. (*F* and *G*) CEACAM16 (WT and P142 forms) and prestin proteins were found in samples eluted from protein A beads as expected (*F*) and cell lysates (*G*).

Discussion

The major proteinaceous component of the TM is α-tectorin. It is expressed by supporting cells and can be detected at the mRNA level in the organ of Corti from embryonic day 12.5 to postnatal day 15 (17). This expression time period is noteworthy, because morphological changes to the TM can be observed even in aged animals (31). The molecular basis of these changes is unclear, because *Tecta* mRNA is not detectable in older animals. However, we have demonstrated that *Ceacam16* mRNA is present in OHC and is expressed both contemporaneously with *Tecta* and also in adult animals (embryonic day 17 to postnatal day 42; Fig. 2 and Fig. S3). We also have shown that CEACAM16 proteins are secreted in several reporting systems, suggesting similar secretion by OHC. Hence, CEACAM16 could play an important role in maintaining the integrity of the TM over time.

The protein structure and α-tectorin–binding ability of CEACAM16 also make it an excellent candidate as a component of the attachment crown that connects the tips of the tallest OHC stereocilia to the TM. CEACAM16 has both IgV and IgC domains. The former are found at its N and C termini, an arrangement unique among CEACAM proteins with both IgV and IgC domains, because, with the exception of CEACAM16, the IgV domain always is on the N terminus and the IgC domain always is on the C terminus (22). IgV domains mediate cell–cell adhesion (22) and would enable CEACAM16 to link α-tectorin and a currently unidentified membrane-anchored protein in the membrane of the OHC, thus forming the TM attachment crown complex (Fig. S6).

Our genetic data also implicate CEACAM16 as a cause of deafness at the DFNA4 interval. The identified missense mutation in CEACAM16 segregates with all deaf persons in American Family 1070, and exome sequencing and interrogation of all inner-ear–expressed genes at the DFNA4 locus failed to identify other deafness-causing variants. Importantly, the DFNA4 interval was defined originally by this family, and although *MYH14* mutations have been identified in other DFNA4-designated families, *MYH14* lies outside the DFNA4 interval (25, 28). The T140P CEACAM16 mutation alters a highly conserved N-glycosylation site (Fig. 5), which is known to facilitate protein folding, the degradation of misfolded/mutant proteins (32, 33), and protein stability (34). Therefore it is possible that the mutation could affect CEACAM16's stability. The fact that this variant was not found among 200 normal-hearing controls (400 chromosomes) indicates that this glycosylation site may play an important role in hearing.

In summary, we have identified CEACAM16 as a protein secreted by cochlear OHC that localizes to the tips of the tallest stereocilia and the TM and binds to α-tectorin. This distribution suggests that CEACAM16 is a component of the TM attachment crown and may help maintain the integrity of the TM. Consistent with an important cochlear function, we show that a mutation of CEACAM16 in humans results in deafness at the DFNA4 locus.

Materials and Methods

DNA Constructs and Antibodies. Animal care and use procedures were approved by the Northwestern University Institutional Review Board and the National Institutes of Health. RNA was isolated from mice cochleae and used for cloning CEACAM16. CEACAM16 mutant T142P was generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). Details for generating myc-α-tectorin, Flag-CEACAM1, and V5-CEACAM16 plasmids are described in *SI Materials and Methods*. A 22-amino acid peptide (CSLLVQKLNLTDTGRYTLKTVT) corresponding to amino acids near the C terminus of mouse CEACAM16 (Fig. 1) was used to immunize rabbits and to generate affinity-purified anti-CEACAM16

(Covance). Anti-CEACAM16 was used at final concentrations of 3–6 μg/mL for immunofluorescence and 0.3 μg/mL for Western blot. Other commercial antibodies are listed in *SI Materials and Methods*.

Immunofluorescence and in Situ Hybridization. The procedure for cochlear immunofluorescence is described in Homma et al. (35). For subtilisin (Sigma) treatment, cochleae were dissected and incubated with 50 μg/mL subtilisin in Hepes-buffered HBSS solution (21) for 15 min at room temperature before being fixed in 4% formaldehyde. Control cochleae underwent a similar procedure, but subtilisin was omitted. Samples then were incubated with anti-CEACAM16. A similar procedure was used for transfected mammalian cells (30). 3D images were created using image-analysis software Volocity 5X. Detailed procedures for in situ hybridization are described in Sengupta et al. (36) and in *SI Materials and Methods*.

Western Blot and Coimmunoprecipitation. For Western blotting, cochleae and plasmid-transfected cells were collected as described previously (30). Samples were supplemented with either subtilisin or protease inhibitors. Culture medium from *Ceacam16*-transfected cells was collected and successively centrifuged at 10,000 × *g* for 20 min and 100,000 × *g* for 1 h. Proteins in supernatants were precipitated by 5% trichloroacetic acid, resolved by 4–20% gradient gel or 7.5% NEXT-PAGE, and blotted with various antibodies. For coimmunoprecipitation, HEK293T or OK cells cotransfected with various plasmids were harvested, and coimmunoprecipitation experiments were performed as described previously (35). Primary antibody/protein-A or -G beads were saturated with target proteins.

Mutation Screening and Exome Sequencing of Family 1070. Otological and audiological examination was performed as described earlier (25), and peripheral blood samples were obtained from consenting persons via venipuncture. Detailed exome sequencing is described in ref. 37 and in *SI Materials and Methods*.

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