Rab8b GTPase, a protein transport regulator, is an interacting partner of otoferlin, defective in a human autosomal recessive deafness form

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Abstract

Mutations within *OTOF* encoding otoferlin lead to a recessive disorder called DFNB9. Several studies have indicated otoferlin’s association with ribbon synapses of cochlear sensory hair cells, as well as data showing the protein’s presence in neurons, nerve fibers and hair cells, suggesting a more ubiquitous function. Otoferlin’s co-localization not only with ribbon synaptic proteins, but also with additional endosomal (EEA1) or Golgi proteins (GM130) were motivation for a search for further binding partners of otoferlin by a yeast two-hybrid screen in a rodent cochlear cDNA library (P3-P15). This screen identified Rab8b GTPase as a novel interacting partner, substantiated by transient co-expression and co-localization in HEK 293 cells and co-immunoprecipitation of the complex using tagged proteins *in vitro* and native proteins from cochlea. This finding implies that otoferlin could be a part of components contributing to trans-Golgi trafficking.

Introduction

Hearing deficiency is a frequent congenital neurosensory disorder resulting from impairment of various cellular components of the hearing apparatus, the hereditary forms act via different genetic mechanisms. Quite commonly, the defects are associated with damage in the Organ of Corti that is localized within the cochlea and responsible for mechanotransduction. A protein with several so-called C2 domains, being one of the many components in cochlear receptor cells, termed inner and outer hair cells, was noted to have mutations at various positions in an autosomal recessive deafness form, DFNB9 (1-4). Its homology to Ferlin (Fer-1) led to the name “otoferlin” and a role in Ca\(^{2+}\)-triggered fusion of vesicles with plasma membranes was suggested (2). Most recent investigations yielded data that either support this hypothesis (5, 6) or assigned to otoferlin a more ubiquitous role in endosome trans-Golgi dynamics (7). Co-immunoprecipitation of otoferlin with syntaxin1 and SNAP25 was shown and used as an argument for the ribbon synapse connection (5) while immunohistochemistry and *in situ* mRNA
detection argued for a more widespread distribution and demonstrated otoferlin’s presence in neurons, nerve fibers and hair cells and extending beyond the regions of synaptic fusion (7). Using presynaptic markers, a distinct spatial distribution for otoferlin was discernible, thus assigning it to localization beyond the regions of synaptic vesicle fusion. A hypothyroid animal model lacking otoferlin in the hair cells but displaying exocytosis also contradicts the idea of otoferlin as the major \( \text{Ca}^{2+} \) sensor-triggering membrane fusion protein at the inner hair cell (IHC) ribbon synapse (8). Its co-localization not only with ribbon synaptic proteins, but also with additional endosomal (EEA1) or Golgi proteins (GM130) encouraged our search for further binding partners of otoferlin by a yeast two-hybrid screen using otoferlin fragments representing various domains as baits in a rodent cochlear cDNA library. This screen and complementary approaches identified Rab8b GTPase as a novel interacting partner.

Transient co-expression and co-localization of otoferlin and Rab8b in an experimental cell system (HEK 293 cells) and reciprocal co-immunoprecipitation using proteins from the cell system as well as native cochlea substantiated the original observation of temporal co-expression of both proteins in isolated inner hair cells of the cochlea. Since Rab8 has been shown to regulate traffic along the trans-Golgi-network/recycling endosome/plasma membrane pathway (9) and appears to be involved in vesicular movement of membrane proteins into cilia (10), the newly found otoferlin-Rab8b interaction now links otoferlin to this basolateral endocytic and secretory trafficking.

Results

Identification of Rab8b GTPase as an interacting partner for otoferlin

We used the yeast two-hybrid system to screen a cochlea-specific cDNA library (from mice P3-P15) with several otoferlin domains as bait (for details see Materials and Methods). Bait 3 contains a C2 domain and bait 5 the cytoplasmic COOH-terminus of otoferlin. Approximately 150 independent transformants were screened, of which 80 clones were positive for \( \alpha \)-
galactosidase expression. To choose candidate clones for further study, two supporting criteria were applied: (a) expression of the respective gene in hair cells and (b) temporal co-expression with otoferlin during the developmental stages. One of the interacting clones that met these criteria was identified using bait 3 and coded for Rab8b GTPase (NM_173413).

Otoferlin and Rab8b co-expression in hair cells

Rab8, originally a transforming gene (MEL) from human melanoma cells (NK14), was reported to have orthologues in canine and rodent species (11, 12). Its expression was noted in various cell types but its function was mainly studied in epithelial cells (13, 14), a finding which we could substantiate (Fig 1. lanes 1-3). To confirm its validity as a potential binding partner for otoferlin, we tested Rab8b expression in the cochlea of mice at times of pre- and post-hearing onset. RT-PCR with RNA material representing an in toto cochlear mRNA (Fig. 1, lane 4 and 5) and with mRNA isolated separately from the inner (IHC) and outer hair cells (OHC; Fig. 1, lanes 6-9), respectively, demonstrated the presence of Rab8b mRNA in immature and mature hair cells. In addition, using rabbit polyclonal otoferlin and mouse monoclonal Rab8b antibodies, Western blot analysis confirmed presence of otoferlin (Fig. 2, lane 1) and Rab8b (Fig. 2, lane 2) polypeptides of the appropriate size. In line with Yasunaga and co-workers (2), a ~230 kDa and a ~240 kDa otoferlin polypeptide were detected in adult rat cochleae. Transcripts encoding the short isoform of otoferlin, which were found in humans (2) but to date not in rodents, were not detected in the rat cochlea. This finding is also in line with Yasunaga and co-workers who detected only transcripts encoding the long isoforms (2). Moreover, a monoclonal Rab8b-specific antibody recognized a ~23 kDa band, corresponding to the full-length protein (NP_775589). These data also demonstrated that otoferlin and Rab8b co-exist in an identical time frame within the same specific cells.

Co-localization of otoferlin and Rab8b in HEK 293 cells

Using mammalian HEK 293 cells for transfection with both plasmids encoding the tagged pro-
teins, co-expression of Rab8b and otoferlin could be demonstrated (Fig. 3). The Rab8b gene was cloned as a DsRed-tagged fusion protein, while fragment 3 of the otoferlin gene (for details see Materials and Methods) was cloned as an EGFP-tagged fusion protein. Transfection of the empty pEGFP- (Fig. 3A, green) and pDsRed (Fig. 3B, red) vector resulted in the typical ubiquitous distribution of EGFP and DsRed, respectively. Shown exemplarily for two independent transfection assays, co-transfection with Rab8b-cDNA (Fig. 3C and D, red) and with otoferlin-cDNA (Fig. 3E and F, green) produced a strong immunofluorescence within the cell membrane (Fig. 3C-F, open arrow), with some signal within the endoplasmic reticulum (Fig. 3C-F, closed arrow). Co-localization was validated upon an overlay of both fluorescent expression patterns (Fig. 3G and H, merge, open and closed arrow). Transfection of otoferlin-cDNA without co-transfection with Rab8b-cDNA also resulted in immunofluorescence within the cell membrane (Fig. 3E insert). Untransfected HEK 293 cells gave no fluorescent signal (Fig. 3 I and J). The specificity of Rab8b expression in HEK cell membranes was confirmed using a Rab8b monoclonal and an Alexa 488 secondary antibody to detect transfected Rab8b protein. Strong Rab8b-positive fluorescent signal could be visualized in the membrane of transfected HEK cells, shown for two cells from different experiments (Fig. 3K, green), whereas no positive signal was noted when the primary antibody was omitted (Fig. 3L) or when untransfected cells were used.

Rab8b co-immunoprecipitates with otoferlin

Differently epitope-tagged otoferlin and Rab8b were co-expressed in HEK 293 cells and presence of otoferlin-EGFP and Rab8b-DsRed was examined by directly immunoblotting lysates from these cells with antibodies against EGFP- (Fig. 4A, lane 1) and DsRed tag (Fig. 4A, lane 2), respectively. We confirmed protein expression using anti-Rab8b monoclonal antibody (Fig. 4A, lane 3) in the same lysate. The estimated size of ~44 kDa of the EGFP fusion protein of the otoferlin fragment was detected (Fig. 4A, lane 1) using anti-GFP antibody. The estimated size of ~51 kDa of the DsRed fusion protein of Rab8b was shown using either anti-DsRed
(Fig. 4A, lane 2) or anti-Rab8b antibody (Fig. 4A, lane 3). These data (Fig. 4A, lanes 1-3, input) thus confirmed the expression of both proteins of interest in the cell lysate used for the co-immunoprecipitation. Next to validate Rab8b as an interacting binding protein of otoferlin, the same lysates were used for co-immunoprecipitation. Using anti-Rab8b antibody for precipitation, we demonstrated co-immunoprecipitated otoferlin (Fig. 4A, lane 4). In reciprocal immunoprecipitation experiments using anti-GFP antibody for precipitation, we confirmed that Rab8b-DsRed co-immunoprecipitated with otoferlin-EGFP (Fig. 4A, lane 5). When the same approach was performed using untransfected HEK 293 cells neither anti-Rab8b nor anti-GFP showed cross-reactivity (Fig. 4A, lane 6 and 7). Additional controls were performed by immunoblotting lysates of HEK 293 cells, which were transfected with either empty pDsRed-Monomer-N1 or pEGFP-N1 vector resulting in expression of the corresponding tag proteins. The estimated size of ~25 kDa of the EGFP protein and ~27 kDa of the DsRed protein was recognized upon application of anti-GFP (Fig. 4A, lane 8) or anti-DsRed antibodies (Fig. 4A, lane 9), respectively. These results strongly support that otoferlin and Rab8b interact with each other in an in vitro cell system.

Aiming at validating the interaction of otoferlin with Rab8b in vivo, the co-immunoprecipitation strategy was used with proteins isolated from native mouse cochlea (P5-P8). First, using anti-otoferlin antibody we showed that in cochlear lysate otoferlin of the estimated size of 240 kDa and 230 kDa (compare Fig. 2, lane 1) is in fact expressed. Second, anti-Rab8b antibody demonstrated presence of the Rab8b protein of the expected size of 23 kDa in cochlear lysate (Fig. 4B, lane 1, input; compare also Fig. 2, lane 2). Finally, the same lysate was used for co-immunoprecipitation. When applying anti-otoferlin antibody for precipitation of otoferlin, we detected co-immunoprecipitated Rab8b (Fig. 4B, lane 2).

To confirm the observed co-localization of otoferlin and Rab8b in inner hair cells, a rabbit polyclonal anti-Rab8b antibody was combined with mouse monoclonal anti-otoferlin antibody to study expression of both proteins in IHCs. Shown for a mouse cochlea at postnatal day (P) 7
and dependent on the focus, otoferlin was visualized in the upper part (Fig. 5A, closed arrow) or in the basal part of the IHCs (Fig. 5D, open arrow). The Rab8b antibody stained similar subcellular areas in the IHCs (Fig. 5B and E, open arrows). Areas with overlapping expression of both proteins are outlined by yellow staining when photographs were merged (Fig. 5C and F, arrows). These results further support the finding of a direct cooperation of otoferlin with the small GTPase Rab8b in the cochlea.

**Discussion**

Otoferlin has been shown to be an essential component of a functioning hearing apparatus and its defect leads to hearing impairment in both patients and mouse models (2, 3, 5, 15). For otoferlin, a role in Ca\(^{2+}\)-triggered fusion of vesicles with plasma membranes was assumed (2) and successive co-immunoprecipitation indicated an interaction of otoferlin with syntaxin1 and SNAP25, data which argue for the ribbon synapse connection (5, 6). Using a hypothyroid animal model lacking otoferlin in hair cells but displaying exocytosis indicates that an exclusive function of otoferlin for exocytosis may be questionable (8). Based on distribution of otoferlin close to Golgi membranes and presence in neurons and nerve fibers, a more general role within the exocytosis/endocytosis cycle was suggested (7). The present study demonstrates Rab8b GTPase to emerge as one of the strongest binding candidates in a yeast two-hybrid study when using otoferlin fragments as bait. Rab proteins are small monomeric members of the Ras GTPase family, which in total comprises about 100 distinct Rab proteins (16, 17). Rab8 was described as a key regulator of constitutive polarized vesicle transport to the dendrites in the neurons or to the basolateral membrane in epithelial cells (18-20) but also reported in intra-flagellar transport and ciliopathies (10). Rab8 was at first noted in kidney epithelia as a regulator of basolateral protein sorting, possibly acting at the level of trans-Golgi-network, the recycling endosomes or the plasma membrane, or on the pathway linking these components (21). Rab8 appears to be functionally associated with BBS1, a member of a protein complex playing a role in intracellular transport. Accordingly, defective Rab8 leads to impaired cilio-
genesis subsequent to disturbed delivery of basolateral cargo (18) resulting in polycystic kidneys or the Bardet-Biedl syndrome (22-24). Rab8 was moreover documented to be essential for post-Golgi transport of rhodopsin to retinal photoreceptors, Rab’s disruption leads to retinal degradation (22). More recently, Rab8 was also shown to be involved in sorting and/or storage of neural recycling endosomes (25). The role of Rab8 for apical or basolateral transport in polarized cells is still controversial. In vitro data indicate Rab8 to regulate basolateral secretory traffic at the level of endosomes (9), however neonatal mice lacking Rab8 revealed impaired distribution of apical proteins leaving basolateral proteins unchanged (26). In yeast, the Rab8 homologue Sec4 targets vesicles to sites of exocytosis at the cell surface (27).

While the role of Rab8a in vesicle traffic has been elucidated, the function of Rab8b, a closely related isoform, remains unclear. Rab8b has an overall amino acid identity of 80% with Rab8a. The NH2 termini of both isoforms are highly conserved but the carboxy-terminal domain of Rab8b is substantially divergent from Rab8a. Recent data support the notion that Rab8b, similar to Rab8a, is involved in vesicle secretion (28). The role in secretion, however, may be not shared or substituted by Rab8a (28).

While we could confirm Rab8b synthesis in colonic epithelial cells and also noted its presence in rodent brain, the substantial new finding is Rab8b’s presence in ciliated cells of the cochlea. It occurs in this neurosensory tissue within the same time frame as does otoferlin. Several lines of evidence, presented in this study, demonstrate an interaction of both proteins. Otoferlin and Rab8b were found to be co-localized in the apical region of IHCs where recent data also describe a co-localization of otoferlin with GM130 immunopositive trans-Golgi-network (7). Considering the special rapid vesicle replenishment of inner hair cells upon an endocytosis pathway (29), Rab8b/otoferlin complexes may thus play a role within the recycling of endosomes. In line, based on the co-localization of the protein with trans-Golgi network proteins, clathrin-coated endosomal structures, including Rab8, have been shown to play a crucial role for basolateral transport of vesicles to sites of exocytosis (30). Therefore, Rab8b and otoferlin
on cargo vesicles may be required for the delivery of recycling endosomes to vesicles that are transported to the basolateral plasma membrane where tethering of vesicles occurs before fusion. While our data suggest a role of otoferlin/Rab8b complexes for recycling endosomes and proper basolateral transport of vesicles to the individual active zones where exocytosis takes place they do not exclude otoferlin’s role as a protein that senses calcium for the vesicle fusion process.

Rab8 GTPase mutants (Rab8−/−) with deleted Rab8a isoform die 3-4 weeks after birth (13). Considering the substantial sequence difference at the carboxy-terminal between Rab8a and Rab8b as being responsible for individual roles in secretion that are not shared or substituted (28) further elucidation of the function of Rab8b will require Rab8b specific transgenic mouse models, a challenge for future studies.

**Materials and Methods**

**Animals and tissue preparation**

Wistar rats and NMRI mice were used for this study. Before decapitation, animals were asphyxiated with carbon dioxide. Cochleae of postnatal rats were then isolated and dissected as previously described (31). For RNA and protein isolation, cochleae were dissected, immediately frozen in liquid nitrogen and stored at −70°C before use.

Care and use of the animals and experimental protocols were reviewed and approved by animal welfare commissioner and the regional board for scientific animal experiments in Tübingen.

**Yeast two-hybrid**

To identify otoferlin interacting partners, the Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, USA) was used. We screened a mouse cochlear cDNA library isolated from P3-P15 (kind gift of Prof. Richard J.H. Smith, The University of Iowa, Department of
Otolaryngology) with five different fragments of the otoferlin protein. For this purpose, five different expression vectors pGBK7T with *Otof* were generated. We subcloned fragments of *Otof* cDNA (NP_114081) isolated from the rat cortex into the pGBK7T yeast expression vector in frame with the DNA-binding domain (DNA-BD). The otoferlin fragments we used correspond to following amino acids of mouse otoferlin (NP_114081): bait 1 (100-197), bait 2 (603-887), bait 3 (904-1025), bait 4 (1299-1417), bait 5 (1917-1981). The sequence integrity of the otoferlin-baits was confirmed through DNA sequencing. The expression of the otoferlin-GAL4 fusion proteins was further verified by Western blot analysis using a monoclonal antibody against the c-myc epitope (Clontech). The pGBK7T vector carries a yeast TRP1 marker for auxotrophic selection on -Trp medium. The cochlear cDNA library contained inserts cloned into pAD-GAL4-2.1 (Stratagene, Amsterdam, Netherlands). This vector carries a LEU2 transformation marker for selection on -Leu medium and a transcriptional activation domain (AD) under the control of GAL4 promoter. Interaction between a target library-encoded protein fused with the AD, and otoferlin fused with DNA-BD resulted in the reconstruction of the novel transcriptional activator with binding affinity for GAL upstream activating sequences (UAS) of the reporter gene. Three reporter genes, ADE2, HIS3, and MEL1 (or *LacZ*), under the control of distinct GAL4 UAS and TATA boxes integrated in the genome of the AH109 yeast strain, made an interaction phenotypically detectable. If the proteins did not interact with each other, the reporter genes were not transcribed.

The AH109 host strain (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1 TATA-lacZ) was first transformed with the pGBK7T plasmid containing otoferlin constructs, carrying the LacZ reporter and stored in SD/-Trp medium/50% glycerol for further experiments. These transformants were then used for the mating procedure with the pAD-GAL4-2.1 library plasmids. After mating, cells were plated on a minimal synthetic dropout -Trp/-Leu-SD
medium that selected for both pGBK7 and the AD/library plasmid, but not for the interaction directly. Positive interactions were identified by the ability of yeast to grow on leucine-tryptophan-histidine triple-selective plates (SD-LTH). Surviving colonies were replicated on leucine-tryptophan-histidine-adenine quadruple-selective plates (SD-LTHA) containing X-α-Gal. Galactosidase expression was indicated by the blue color observed in the presence of X-α-Gal for detecting protein-protein interaction.

mRNA isolation and RT-PCR

For RT-PCR analysis, mRNA from rat cochlea was isolated using DYNAbeads (DYNAbeads mRNA Direct Kit, DYNALE, Oslo, Norway), and total RNA from rat brain tissue was isolated using the RNeasy Minikit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. After reverse transcription using iScript cDNA synthesis kit (Bio-Rad, München, Germany), PCR was performed with PuReTaq Ready-To-Go PCR beads (GE Healthcare, Freiburg, Germany). For single cell RT-PCR analysis, IHCs and OHCs were collected with micropipettes (32) and frozen in liquid nitrogen. Synthesis of cDNA was performed using Superscript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). A 600 bp fragment of Rab8b was amplified by RT-PCR with the oligonucleotides 5’-ATGGCGAAGACGTACGATTATC-3’ and 5’-GAGCCGGTCTAAGAAGACCAGT-3’ (annealing temperature: 58 °C, 35 cycles, or 50 cycles for single cell RT-PCR). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers were used as a control: 5’-TCTACTGGCGTCTTACCCA-3’ and 5’-AGGAGACAACCTGGTCCTCAGT-3’ to amplify a 550 bp long fragment (annealing temperature: 55 °C, 35 cycles, or 50 cycles for single cell RT-PCR). The resulting PCR products were analyzed on agarose gels and stained with ethidium bromide. The PCR product was sequenced for confirming Rab8b specificity. The analysis was performed in triplicate.

Construction of immunofluorescence vectors
The full length cDNA of the Rab8b gene delivered from the two-hybrid screens was cloned into epitope tagging vector that could generate high levels of epitope-tagged protein in mammalian cells. The vector expressing an epitope-tagged protein was then co-transfected into mammalian cells with an expression vector for the bait protein.

Full length of *Rattus norvegicus* Rab8b was cloned into pDsRed-Monomer-N1 vector (Clontech) using a forward primer introducing an *Xho*I restriction site (5’-CCCTCGAGACCATGGGAACAGTACGA-3’) and a reverse primer deleting the stop codon and introducing an *EcoR*I restriction site (5’-GGGAATTCCAAGCAGAGAACAGCGGAAG-3’). Bait 3 of otoferlin was cloned into pEGFP-N1 vector (Clontech) in frame with the EGFP epitope. The sequence of the forward primer introducing a *Xho*I restriction site is 5’-CCCTCGAGACCATGGGAACAGTACGACCAG-3’, respectively the reverse primer introducing an *EcoR*I restriction site is 5’-GGGAATTCCAAGCAGAGAACAGCGGAAG-3’. Correct orientation and reading frame were confirmed by sequence analysis.

Cell culture, transfection of HEK 293 cells and immunohistochemistry

Following construction of both recombinants including the tags, HEK 293 cells were transfected. HEK 293 cells were grown in Gibco DMEM (Invitrogen) supplemented with 10% FCS. Cells were plated at a density of 2x10⁶ cells per 6-cm dish on the day before transfection. Cells were transiently transfected with vector DNA (*Otof*-pEGFP and/or *Rab8b*-pDsRed) using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instruction. Each 6-cm dish received 6 µg of plasmid DNA or 2 x 3 µg of expression vectors when co-transfected. After 48 h incubation, cells were lysed and concentration of proteins was measured by using Bradford reagent (Sigma).

HEK 293 cells were also partly seeded onto sterile glass cover slips pre-coated with poly-D-lysine (Sigma) and the transfection efficiency was determined as follows. Cells were washed in PBS and fixed with 2% formaldehyde in PBS at 4°C for 30 min. Fixed cells were washed in PBS and were mounted on a glass slide in Vectashield DAPI medium (Vector Laboratories...
Burlingame, CA, USA) which marks the cell nuclei.

For staining with antibody, cells were permeabilized in PBS containing 0.1% TritonX-100 (Sigma) at room temperature for 3 min. Permeabilized cells were then washed in PBS, incubated for 30 min at room temperature in a blocking solution (1% BSA, Sigma) and then transferred to 0.5% BSA blocking solution containing the appropriate epitope-specific antibody (Rab8b, mouse monoclonal, BD Biosciences, Heidelberg, Germany) at an appropriate dilution (1:50). After overnight incubation, cover slips were incubated for 60 min in 0.5% BSA blocking solution containing the appropriate secondary antibody (Alexa 488, anti-mouse, Molecular Probes, Leiden, The Netherlands; 1:500). Cover slips were washed in PBS and mounted in Vectashield DAPI medium on a glass slide. Samples were analyzed and photographed using Olympus AX70 microscope equipped with epifluorescence illumination.

Protein isolation from cochlea

For a survey of the presence of Rab8b protein in the cochlear tissue and for co-immunoprecipitation of endogenous proteins, the otoferlin/Rab8b complex was isolated from cochlear tissue.

Cochlear tissue was homogenized and lysed in lysis buffer containing 50 mM Tris-Cl pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100 and 2 mM protease inhibitor (Pefabloc, Sigma). Nuclei and cell debris were pelleted by low-speed centrifugation at 280 g for 5 min at 4°C and the supernatant was used for Western blotting and co-immunoprecipitation. Protein contents were determined by using the Bradford method.

Co-immunoprecipitation

Co-immunoprecipitation (co-IP) was performed with HEK cell lysates of transfections with otoferlin-pEGFP and Rab8b-pDsRed constructs. For co-immunoprecipitation, we used Matchmaker co-IP Kit (Clontech). DsRed-tagged Rab8b was immunoprecipitated from cleared lysates (200-300 µg protein) overnight at 4 °C using 1 µg of mouse anti-Rab8b antibody (BD
Biosciences), whereas EGFP-tagged otoferlin was immunoprecipitated by using 1 µg polyclonal goat anti-GFP antibody (Santa Cruz, Santa Cruz, CA, USA, sc-33354) and by incubating reactions with 3 µl of Protein A beads (Clontech) for 90 minutes at room temperature with shaking. After washes according to the protocol of the manufacturer, the protein complexes were analyzed on immunoblots by using XCell II Sure Lock™ Mini-Cell and XCell II Blot Module from Invitrogen. Co-immunoprecipitated DsRed-tagged Rab8b was detected by goat anti-Rab8b antibody (BD Biosciences) and co-immunoprecipitated EGFP-tagged otoferlin was detected by polyclonal goat anti-GFP antibody (Santa Cruz).

The second approach was co-immunoprecipitation studies using protein extracts from cochlear tissue. Rab8b protein was immunoprecipitated using monoclonal anti-Rab8b antibody (BD Biosciences), otoferlin was immunoprecipitated by using polyclonal anti-otoferlin antibody (7) and immunoblotted by using anti-otoferlin and anti-Rab8b antibody, respectively. To confirm expression of the relevant proteins in cell lysates used for the co-immunoprecipitation, we run as a control about 40 µg protein isolated from either transfected HEK 293 cells or cochlea organ.

Electrophoresis and Western blotting

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis using the XCell Sure Lock Mini Cell and NuPage 4-12% Bis-Tris Gels (Invitrogen) according to the manufacturer’s instructions, and were blotted to polyvinylidene difluoride membranes using the XCell II Blot Module (Invitrogen). After incubation with primary, anti-Rab8 (1:2000; BD Biosciences) and anti-otoferlin (1:2000) (7) and subsequently secondary antibodies (ECL peroxidase labeled anti-mouse from sheep and anti-rabbit from donkey, respectively, GE Healthcare) signals were visualized (Enhanced Chemiluminescence Plus Western Blotting Detection Reagent, GE Healthcare, AGFA X-ray film).

Immunohistochemistry on cochlear sections
For standard immunohistochemistry, mouse cochleae of postnatal day 7 were fixed in paraformaldehyde, cryosectioned and stained as described (31, 33). Rabbit polyclonal antibody against human Rab8b (Protein Tech group, Chicago, IL, USA) and mouse monoclonal antibody against otoferlin corresponding to amino acids 1-395 of human otoferlin (Biozol, Eching, Germany) were used at a dilution of 1:100. Primary antibodies were detected with Cy3-conjugated (Jackson ImmunoResearch Laboratories, West Grove PA, USA) or Alexa Fluor 488-conjugated antibodies (Molecular Probes, Eugene, OR, USA). Sections were embedded with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) staining cell nuclei in blue. Specimens were photographed using an Olympus AX70 microscope equipped with epifluorescence illumination.

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References


Figure legends

**Figure 1. Rab8b expression in brain and cochlea**
Lane 1, RNA isolated from mouse colon (C) was used as a positive control; lane 2 and 3, Rab8b mRNA is present in the mouse brain (IC, inferior colliculus; AC, auditory cortex); lane 4 and 5, cochlear tissue at postnatal day 5 (CO P5) and at postnatal day 14 (CO P14); lane 6 and 7, expression of Rab8b in isolated inner (IHC) and lane 8 and 9, expression of Rab8b in outer hair cells (OHC, apical) was shown by isolation single cells from the organ of Corti at P5 and P21/22. Lane 10, no cDNA as negative control (N).

**Figure 2. Expression of otoferlin and Rab8b in the mature cochlea**
Lane 1, using a polyclonal anti-otoferlin antibody, two polypeptides (~230 and ~240 kDa) were recognized in mature (4-month-old) rat cochlea, correspondent to the estimated size of the full-length otoferlin protein (2). Lane 2, using a monoclonal anti-Rab8b antibody, a single polypeptide of ~23 kDa was detected correspondent to the estimated full-length protein.

**Figure 3. Epitope-tagged otoferlin-EGFP and Rab8b-DsRed co-localize in HEK 293 cells**
Transfection of HEK 293 cells with empty pEGFP- (A) and empty pDsRed (B) vector leads to a ubiquitous distribution of the EGFP and DsRed fluorescence signal, respectively. (C-H) HEK cells were co-transfected with otoferlin-pEGFP and Rab8b-pDsRed. Rab8b-DsRed expression (C, D), otoferlin-EGFP expression (E, F), and the merge of both (G, H). Otoferlin-EGFP expression in HEK cells, which were transfected with otoferlin-cDNA without co-transfection with Rab8b-cDNA (E, insert). Untransfected HEK 293 cells gave no fluorescent signal (I, J) Anti-Rab8b-specific antibody visualized with secondary Alexa488 antibody (green) confirmed the expression of Rab8b-DsRed protein in HEK cells (K). (L) Negative control without primary anti-Rab8b antibody. Cell nuclei were counterstained with DAPI (blue). Scale bars indicate 20 μm.

**Figure 4. Co-immunoprecipitation of otoferlin and Rab8b**
(A) Co-immunoprecipitation of transiently expressed otoferlin-EGFP and Rab8b-DsRed in HEK 293 cells. Tagged proteins (Rab8b-DsRed and otoferlin-EGFP) were co-immunoprecipitated (co-IP) using either anti-Rab8b (lane 4) or anti-GFP antibody (lane 5) and were immunoblotted (IB) with anti-GFP or anti-Rab8b antibody, respectively, resulting in expected polypeptide bands of ~44 kDa for otoferlin-EGFP and of ~51 kDa for Rab8b-DsRed. Using five to seven times less protein for Western blot (lanes 1-3, 6-7) than for co-immunoprecipitation (lanes 4, 5) input fractions show anti-GFP (lane 1), anti-DsRed (lane 2), and anti-Rab8b (lane 3) immunoblots, confirming expression of the relevant proteins in the HEK 293 cell lysates used for the co-immunoprecipitations. No band was detectable when untransfected HEK 293 cells as negative control were immunoblotted with anti-Rab8b (lane 6) and anti-GFP antibody (lane 7) HEK 293 cells were transfected with empty pEGFP- (lane 8) and empty pDsRed vector (lane 9) and resulted in the expected polypeptide band of 25 kDa for EGFP and of 27 kDa for DsRed.

(B) Co-immunoprecipitation of cochlear otoferlin and Rab8b. Rab8b was co-immunoprecipitated from cochlear lysate by the otoferlin-specific antibody. Lane 1 confirms the expression of Rab8b with the estimated size of ~23 kDa in the cochlear lysate (input), lane 2 shows the co-immunoprecipitated Rab8b using anti-otoferlin antibody. Proteins amounts like in Fig. 4A.

Figure 5. Co-localization of otoferlin and Rab8b in inner hair cells

Immunohistochemistry using anti-otoferlin and anti-Rab8b antibodies was performed on mice cochlear sections at P7. Otoferlin (A, D, green) and Rab8b (B, E, red) were observed in the apical (A, B, closed arrows) and basal part (D, E, open arrows) of inner hair cells (IHC) as shown in two examples with different focus levels. The co-localization of both proteins becomes evident upon merging of corresponding photographs (C, F, yellow). Cell nuclei were counterstained with DAPI (blue). Scale bars indicate 20 µm.
Figure 3
Figure 4

A. **otoferlin-GFP and Rab8b-DsRed transfection**

Co-IP

HEK cells

IB

- ~51 kDa Rab8b-DsRed
- ~44 kDa otoferlin-GFP

B. **cochlea**

Co-IP

IB

- ~23 kDa Rab8b

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Figure 5

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<th>Rab8b</th>
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**Abbreviations:**

IHC, inner hair cell; OHC, outer hair cell; P, postnatal day