Otoferlin interacts with myosin VI: implications for maintenance of the basolateral synaptic structure of the inner hair cell

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Otoferlin has been proposed to be the Ca\(^{2+}\) sensor in hair cell exocytosis, compensating for the classical synaptic fusion proteins synaptotagmin-1 and synaptotagmin-2. In the present study, yeast two-hybrid assays reveal myosin VI as a novel otoferlin binding partner. Co-immunoprecipitation assay and co-expression suggest an interaction of both proteins within the basolateral part of inner hair cells (IHCs). Comparison of otoferlin mutants and myosin VI mutant mice indicates non-complementary and complementary roles of myosin VI and otoferlin for synaptic maturation: (i) IHCs from otoferlin mutant mice exhibited a decoupling of CtBP2/RIBEYE and Ca\(_V\)1.3 and severe reduction of exocytosis. (ii) Myosin VI mutant IHCs failed to transport BK channels to the membrane of the apical cell regions, and the exocytotic Ca\(^{2+}\) efficiency did not mature. (iii) Otoferlin and myosin VI mutant IHCs showed a reduced basolateral synaptic surface area and altered active zone topography. Membrane infoldings in otoferlin mutant IHCs indicated disturbed transport of endocytotic membranes and link the above morphological changes to a complementary role of otoferlin and myosin VI in transport of intracellular compartments to the basolateral IHC membrane.

INTRODUCTION

Otoferlin, a protein with several C2 domains present in cochlear sensory cells termed inner (IHC) and outer hair cells (OHC) was found to be mutated at various positions in an autosomal recessive deafness form, DFNB9 (1–4). The name otoferlin derives from its homology with the ferlin (Fer-1) protein family, which led to the suggestion for its possible role in Ca\(^{2+}\)-triggered docking and fusion of vesicles with plasma membranes (4). This assumption has recently been strengthened by the observation that exocytosis in IHCs from otoferlin-deficient mice is nearly abolished (5) and that otoferlin exhibits a Ca\(^{2+}\)-dependent interaction with syntaxin-1, SNAP-25 and Ca\(_V\)1.3 (5,6).

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Several findings question the view of otoferlin being the Ca\textsuperscript{2+} sensor controlling the intrinsic exocytotic Ca\textsuperscript{2+} dependence in hair cells: (i) a widespread distribution of otoferlin in central neurons and nerve fibers as revealed by immunohistochemistry and in situ mRNA detection (7); (ii) maintained exocytosis in an animal model without otoferlin expression (8); (iii) changes in the exocytotic Ca\textsuperscript{2+} sensitivity as a function of age and cochlear location, without any obvious variation in otoferlin patterning (9); (iv) otoferlin has been shown to affect exocytosis in cochlear but not vestibular hair cells (5); for a recent review see (10).

In the present study, using yeast two-hybrid and co-immunoprecipitation studies, we identified myosin VI, the gene responsible for deafness and vestibular dysfunction in the Snell’s waltzer (sv) mouse (11,12), as a binding partner of otoferlin. Myosin VI is an unconventional myosin originally reported in porcine and rodent species and is widely expressed, including the cochlea (11,13,14). The corresponding human gene, MYO6, is located on chromosome 6q13 and maps with the deafness locus DFNA22 and DFNB237 (14–16), and its mutation is associated with dominant (16) and recessive (15) deafness in humans.

In the present study, a mouse line with a missense mutation of otoferlin in exon 10, which causes a non-conservative amino acid exchange of isoleucine to asparagine in a conserved region in the C2B domain of the protein and which is described as deaf5/deaf3 mutant (17,18) was analyzed. Missense mutations in otoferlin C2 domains underlie several forms of human deafness, including the P490Q and I515T amino acid exchange of isoleucine to asparagine in a conserved region in the C2B domain of the protein and which is described as deaf5/deaf3 mutant (17,18) was analyzed. Missense mutations in otoferlin C2 domains underlie several forms of human deafness, including the P490Q and I515T mutations in the C2C domain (2). The phenotype observed in IHCs from otoferlin mutant mice was compared with that described in Snell’s waltzer mouse (11,12).

Data are discussed in the context of non-complementary and complementary roles of myosin VI and otoferlin for synaptic function and maturation: (i) Otoferlin could play a role in the positional priming of synaptic vesicles at the IHC release site. (ii) Myosin VI controls the targeting of apical hair cell proteins and maturation of exocytosis. (iii) Both otoferlin and myosin VI are likely to control the topographical development of IHC active zones by appropriate targeting of intracellular compartments to the basolateral IHC membrane.

RESULTS

Myosin VI is an interaction partner for otoferlin

The yeast two-hybrid system was used to screen a cochlea-specific cDNA library (from postnatal days 3–15 mice) with several otoferlin protein domains as bait (Fig. 1A and Materials and Methods). Using bait 3, which covered part of the otoferlin C2D domain, we found an interacting protein identified as myosin VI (Fig. 1B: 184 amino acids, 624–807 of 1262 amino acids; NM_001039546, NP_001034635). The mRNA products collected from the whole cochlea or isolated IHCs or OHCs (Fig. 1C) were amplified using RT–PCR primers that covered the unique 53 amino acids ‘reverse gear’ insert in the motor domain of myosin VI (Fig. 1B) (19,20). The ‘reverse gear’ insert allows myosin VI to move towards the minus end of actin filaments (21). So far, no other minus-end-directed myosin has been identified, for review see (19,22). The motor protein myosin VI has been shown to be involved in different intracellular processes including the formation of synaptic vesicles during endocytosis (23). Positively amplified products from mRNA were collected from total cochlea (Fig. 1C, lanes 1 and 2, CO), isolated IHCs (Fig. 1C, lanes 3 and 4) and OHCs (Fig. 1C, lanes 5 and 6) at indicated postnatal age. Initially, primers flanking the region of the myosin VI tail domain containing the inserts that generate the known four alternatively spliced myosin VI isoforms (Fig. 1B) were used in control brain tissue. We have found that all isoforms with either no insert (NI), the small insert (SI), the large insert (LI) or both the small and large insert (SI+LI) were amplified at least to some degree (see examples in Fig. 1D: Brain, lanes 2 and 3). In the mature organ of Corti (Fig. 1D, CO, lane 4) and in isolated IHCs (Fig. 1D, IHC, lanes 5 and 6), myosin VI transcripts without or with the small insert were amplified, whereas the longer myosin VI fragment was never found in either the cochlear tissue or isolated IHCs.

Co-immunoprecipitation of otoferlin and myosin VI and co-expression in IHCs

A critical step in the evaluation of yeast two-hybrid interaction partners is the co-localization of both proteins. Co-localization studies using antibodies against otoferlin and myosin VI were performed in immature and mature IHCs from both otoferlin and myosin VI wild-type and mutant mice. In wild-type mice, the two proteins were found to co-localize in the synaptic region and in the lateral membrane up to the supranuclear level of both P9 (Fig. 2A–C) and P20 IHCs (Fig. 2D–I, closed arrowheads). Myosin VI was additionally expressed in the apical pole of IHCs (Fig. 2D–F, open arrowhead); see also (12). Analysis of otoferlin expression in GFP-positive IHCs from a GFP reporter mouse, using whole-mount preparations and confocal microscopy, demonstrated that the expression of otoferlin was associated with the IHC basolateral membrane and not with the whole cytoplasm (Fig. 2J and K). Immunostaining for otoferlin and myosin VI was absent in IHCs from their respective mutant mice (Fig. 2L and M), indicating specificity of the two antibodies. This further confirms that the otoferlin missense mutation has a very severe effect on protein conformation or stability, which results in degradation (18).

Co-immunoprecipitation experiments with cochlear protein lysates were performed in mice and rats to confirm the interaction between the two above proteins. In P7 rat cochlear lysates, otoferlin antibodies cross-reacted with polypeptides of the estimated size of ~230 and ~240 kDa (Fig. 2N, lane 1), which represent the two long otoferlin isoforms detected in the cochlea of rats and mice (7), as well as with myosin VI of the expected size of ~148 kDa (Fig. 2N, lane 2) corresponding to the full-length protein (NP_001034635). Upon co-immunoprecipitation with anti-otoferlin antibody, myosin VI was co-immunoprecipitated (Fig. 2N, lane 3). These data strongly suggest that otoferlin and myosin VI are co-expressed in the basolateral but not in the apical part of IHCs.

Expression and targeting of BK channels to the IHC neck region requires myosin VI but not otoferlin

Because myosin VI is involved in the sorting and targeting of cargo into different cellular compartments, for review see...
(19), we tested this in IHCs, and also whether the targeting of proteins requires an interaction with otoferlin. For these experiments, we have chosen the α-subunit of the voltage- and Ca\(^{2+}\)-activated K\(^+\) channel (BK\(\alpha\)), known to be expressed in the apical-lateral (neck) membrane of adult IHCs (24–26), as an example for apical targeting. The surface expression of BK\(\alpha\) protein in IHCs from wild-type mice (Fig. 3A) was absent in age-matched cells from myosin VI mutant mice (Fig. 3B). In contrast, BK expression in IHCs from wild-type and otoferlin mutant mice was normal (Fig. 3C and D), indicating that myosin VI, independently from otoferlin, is involved in the delivery of BK\(\alpha\) proteins to the apical pole of IHCs.

Figure 1. Yeast two-hybrid screen with otoferlin baits revealed myosin VI as a new interaction partner. (A) Diagram of the domain organization of the otoferlin protein (NP_114081), mouse long isoform modified from (4). The position of the five baits with different fragments of otoferlin, used for the yeast two-hybrid screen, is displayed. Using bait 3, myosin VI was identified as otoferlin binding partner. Boxes indicate the predicted domains of the protein. Black boxes, C2 domains; crosshatched box, transmembrane domain. (B) Scheme of the domain organization of myosin VI protein, modified from (19,20). (C) RT–PCR with primers covering the 53 amino acids insert in the motor domain of myosin VI, which is unique for myosin VI and comprises the ‘reverse gear’ of myosin VI. Expression of myosin VI in the total cochlea (lanes 1 and 2) and in separately isolated inner (IHC) and outer hair cells (OHC) before (P5) and after (P14–22) onset of hearing. (D) RT–PCR analysis of RNA samples from adult rat brain, mouse cochlea (P15) and mouse IHCs (P21) with primers (5'-TCCCTGCCCAGGACTTGAG-3', 5'-CAGTAACCGACTTGGGAGC-3') flanking the region of the tail domain containing the inserts that generate four alternatively spliced isoforms. For more information see Materials and Methods.

Reduced exocytosis and absence of co-localization between ribbon synapses and Ca\(_{\text{V}}\)\(_{1.3}\) channels in otoferlin mutant IHCs

Synaptic ribbons and presynaptic Ca\(_{\text{V}}\)\(_{1.3}\) channels were stained using double-labeling with anti-CtBP2/RIBEYE in combination with anti-Ca\(_{\text{V}}\)\(_{1.3}\) antibodies. The spatial organization of active zones was analyzed in adult (P20–P30) IHCs from wild-type and otoferlin mutant mice (Fig. 4). In wild-type IHCs, about 17 ± 2.2 (mean ± SEM) CtBP2/RIBEYE-positive dots were closely co-localized with Ca\(_{\text{V}}\)\(_{1.3}\) channel clusters (Fig. 4A: green and red, respectively) as indicated by overlapping immunoreactivity (Fig. 4B and C, yellow). Such co-localization was absent in P20 (Fig. 4D and E) and P9 (data not shown) otoferlin mutant IHCs. As shown in Figure 4F, the number of overlapping spots was significantly reduced to 6.1 ± 1.8 (mean ± SEM; P < 0.001) in adult (P20–P30) otoferlin mutant IHCs.

We performed exocytotic measurements in otoferlin mutant IHCs from mice carrying an otoferlin missense mutation (17,18) at immature stages (P7–P9). In mature mutant IHCs (P17–P20), the nucleus was more mobile than normal, quickly moved towards the pipette tip and clogged it. This disturbed intracellular integrity prevented stable recordings from immature otoferlin mutant IHCs. Depolarization-induced Ca\(^{2+}\) currents (I\(_{\text{Ca}}\)) and exocytosis were measured in IHCs using 5 mM extracellular Ca\(^{2+}\) (Fig. 5). A typical example of the
Ca\(^{2+}\) current \((I_{\text{Ca}})\) recorded in response to 8 ms depolarizing voltage steps from a wild-type and otoferlin mutant IHC is shown in Figure 5A. Average current–voltage \((I–V)\) curves, calculated during the last ms of \(I_{\text{Ca}}\) responses, showed slight reduction in the peak current \((P_{\text{0.05}})\) in otoferlin mutant IHCs (Fig. 5B) compared with wild-type cells. The voltage-dependence of \(I_{\text{Ca}}\) activation was found to be similar among the two genotypes (data not shown). Ca\(^{2+}\) current traces evoked by 100 ms voltage steps to 0 mV and corresponding changes in membrane capacitance \((\Delta C_m)\), reflecting exocytosis, are shown in Figure 5C for a wild-type (black traces) and an otoferlin mutant IHC (gray traces). On average, \(\Delta C_m\) was significantly \((P<0.0001)\) reduced from 11.6 \(\pm\) 3.7 fF in 11 wild-type IHCs to 2.0 \(\pm\) 1.8 fF in 9 otoferlin mutant IHCs (Fig. 5D). Despite the fact that the Ca\(^{2+}\) charge flowing during the 100 ms voltage pulse was similar between wild-type and mutant mice (Fig. 5E), the exocytotic Ca\(^{2+}\) efficiency, defined as the ratio between \(\Delta C_m\) and Ca\(^{2+}\) charge, was significantly \((P<0.0001)\) reduced in otoferlin mutant IHCs (Fig. 5F).

**Myosin VI mutant mice show exocytosis with an immature Ca\(^{2+}\) efficiency**

The spatial organization of active zones was also analyzed in adult (P20–P30) IHCs from wild-type and myosin VI mutant mice (Fig. 6A) and myosin VI mutant mice (Fig. 6A). In contrast to otoferlin mutant mice, myosin VI mutants did not show any obvious mutant mice (P20), respectively. In this and following figures, nuclei are stained in blue with DAPI. Scale bars, 10 \(\mu\)m. (N) Co-immunoprecipitation of cochlear otoferlin and myosin VI. Lanes 1 and 2, respectively, confirm the expression of otoferlin with the estimated size of \(\sim 230\) and \(\sim 240\) kDa and the expression of myosin VI with the estimated size of \(\sim 148\) kDa in the cochlear lysate (Input). Lane 3, myosin VI was co-immunoprecipitated from cochlear lysate by the otoferlin-specific antibody.
apical IHCs, an age at which the exocytotic Ca²⁺ zones indicated by open arrowheads in Fig. 6A, main panel. Nevertheless, active zones appeared to form clusters (marked by the closed arrowhead compared with normally sized active zone clusters rather than a reduction in their relative abundance in mutant IHCs (Fig. 6D and F), voltage steps to 0 mV evoked similar changes in membrane capacitance in both myosin VI mutant and wild-type cells (Fig. 6E). Therefore, the resulting exocytotic Ca²⁺ efficiency was severely reduced to 42% in myosin VI mutant compared with wild-type IHCs (Fig. 6G) and was reminiscent of that of immature IHCs (8,27), see also Figure 5F (wild-type).

**Otoferlin and myosin VI mutant mice exhibit a reduced basolateral pole in IHCs due to presumptive disturbed targeting of endocytotic compartments to the basolateral membrane**

Analyzing the expression profile of myosin VI in otoferlin mutant IHCs (Fig. 7B) and of otoferlin in myosin VI mutant IHCs (Fig. 7D), it became evident that both proteins could be expressed without the corresponding interaction partner. A common feature observed in IHCs from both mutants was a significant reduction in the basolateral volume (Fig. 7A–D), which progressed starting from around the onset of hearing (P12 in most rodents). A more detailed analysis revealed that the average distance between the nucleus and most distant ribbons at the active zones was reduced to 71% in otoferlin mutants and to 68% in myosin VI mutants at the age of P20–P30. The possible reduction in the membrane area of myosin VI mutant IHCs was confirmed by the observation that their cell membrane capacitances were significantly (P < 0.0005: Fig. 7F, P17–P19) smaller than that measured in age-matched cells. We found that the number of both CtBP2/RIBEYE and Cav1.3-positive spots was largely reduced (Fig. 7G and H) in myosin VI mutant IHCs of apical cochlear turns (P17–P20) that showed normal exocytosis (Fig. 6E). The normal capacitance increases, together with the unusual enlargement of both CtBP2/RIBEYE and Cav1.3-positive spots (see Fig. 6A main panel, compare the size of spots indicated by closed and open arrowheads), suggest the formation of active zone clusters rather than a reduction in their relative number. Also in otoferlin mutant IHCs the number of ribbons and Cav1.3 channels was reduced (Fig. 7G and H).

Considering the known role of myosin VI for regulation and trafficking of intracellular endocytotic or secretory vesicles (29), we hypothesized a disturbance of intracellular membrane or vesicle transport in otoferlin and myosin VI mutant IHCs. In line with this assumption, we found a reduced level of otoferlin expression in the trans-Golgi region (TGN) in myosin VI mutants (Fig. 7J and L). The decline in otoferlin expression in the TGN was correlated with a reduction in positive staining of VGLUT3, a synaptic vesicle marker, in the basolateral part of myosin VI mutant IHCs (Fig. 7L), suggesting a presumptive role of myosin VI in targeting intracellular membrane compartments to the basolateral membrane. Otoferlin mutant IHCs also showed disturbed intracellular membrane transport (Fig. 7M–P), suggesting a possible common role to that of myosin VI. Using a membrane marker protein (Fig. 7M and
N, red, open arrowhead), membrane infoldings became visible at the supranuclear level and the basal part of P9 otoferlin mutant IHCs (Fig. 7N, closed arrowheads). The origin of the infolded membrane is likely to be due to disturbed endocytosis, as the intracellular lumen of the infoldings is free from any VGLUT3-immunopositive vesicles (Fig. 7P, arrowheads) as shown with confocal microscopy.

**DISCUSSION**

In the present study, we have shown for the first time a possible functional interaction between myosin VI and the transmembrane protein otoferlin, the presumptive Ca\(^{2+}\) sensor of exocytosis in IHCs (4,5), and both common and distinct roles are suggested for the two proteins. (i) Otoferlin may control tight coupling of ribbons and CaV1.3 channel clusters, the failure of which could be responsible for the reduced exocytosis in otoferlin mutant IHCs (Fig. 7N, closed arrowheads). The origin of the infolded membrane is likely to be due to disturbed endocytosis, as the intracellular lumen of the infoldings is free from any VGLUT3-immunopositive vesicles (Fig. 7P, arrowheads) as shown with confocal microscopy.

Otoferlin controls tight coupling of CaV1.3 channels and ribbons

Neurotransmitter release from IHCs is controlled by Ca\(^{2+}\) influx through CaV1.3 Ca\(^{2+}\) channels (30,31), known to cluster at the hair cell’s presynaptic site (32) and to be closely coupled to ribbons (9,32–35). The present study demonstrated the existence of a clear separation between CaV1.3 and CtBP2/RIBEYE staining in otoferlin mutant IHCs. As we used fluorescence microscopy to analyze the spacing in between CaV1.3 channels and ribbons, the resolution was limited to \(\sim 500\) nm that is far beyond the nano-domain (50–100 nm) characteristic for tight coupling of Ca\(^{2+}\) channels at the active zones (36,37).

This nano-domain coupling has also been suggested for IHCs from normal wild-type animals (9,35). However, the distance between non-overlapping fluorescent dots in otoferlin mutant IHCs was on the order of \(\sim 1\) \(\mu m\), thus approaching a microdomain scenario (38). Recent studies have suggested that otoferlin is unlikely to be the Ca\(^{2+}\) sensor of exocytosis in cochlear IHCs as previously suggested (5). In immature IHCs exocytosis depends non-linearly (power of 3) on the Ca\(^{2+}\) current, whereas it becomes a linear function in mature cells (9,27). This developmental change appears not to be associated with differences in the functional coupling between Ca\(^{2+}\) channels and ribbons at the active zones (\(\sim 40–50\) nm) or ribbon morphology (9).

More importantly, the high-order (immature IHCs) and linear (adult IHCs) Ca\(^{2+}\) dependence of exocytosis was not correlated with otoferlin expression pattern (5,9), suggesting that additional still unknown Ca\(^{2+}\) sensing proteins such as synaptotagmins (39) are likely to be expressed in IHCs. Therefore, otoferlin could be essential for other functions in hair cell exocytosis.

The positioning of ‘molecular primed’ vesicles with respect to nearby Ca\(^{2+}\) channels is a crucial Ca\(^{2+}\)-dependent step for
exocytosis. Although being perfectly release competent, nevertheless vesicles could be located too far away from Ca\(^{2+}\) channels at the active zones (40,41), leading to a substantial reduction (factor of ten) of release (42); for review see (38), as observed in otoferlin mutant IHCs (Fig. 5). Therefore, we propose that, in addition to a suggested function for otoferlin for vesicle replenishment (see below), otoferlin is an essential component required for promoting the co-localization of CaV1.3 channels with ribbons at the IHC presynaptic active zones. This conclusion is in agreement with recent findings showing that CaV1.3 channels interact with otoferlin through the C2D domain (6). It will be challenging for future studies to test whether otoferlin plays a direct role for synaptic vesicle priming, a possible role suggested by the observation that in otoferlin mutant IHCs the second (slower) but not the first (fastest) vesicle release component was rescued by flash-released caged Ca\(^{2+}\) (5).

Myosin VI controls the targeting of apical membrane proteins

Previous findings have suggested that myosin VI is involved in anchoring the apical hair cell membrane to the underlying actin-rich cuticular plate (12). Outside of the inner ear, myosin VI has been implicated in endocytotic trafficking, traveling backwards towards the minus-end of actin filaments (19). In the present study, we have demonstrated that multiple roles of myosin VI in mammalian IHCs extend well beyond the transport of essential stereociliary proteins (43).

Fibroblasts from myosin VI mutant mice exhibited a 40% reduction of the Golgi complex (44), which correlated with a failure of myosin VI-dependent targeting of endocytotic compartments and secretion from the trans-Golgi complex (29). In line with a recent study demonstrating the involvement of myosin VI in the transport of endocytosed material away from the periphery of the hair cell (45), the subsequent depletion of TGN would necessarily lead to reduced sorting of apical membrane proteins, as shown here for the proper BK\(\alpha\) surface expression and in previous studies for stereociliary proteins (12,43).

In contrast to otoferlin mutant IHCs, we found that exocytosis in myosin VI mutant cells persists despite their immature characteristics. The data in the present study point to a crucial role of myosin VI for BK expression and proper maturation of IHC exocytosis. It may be intriguing to consider whether myosin VI is also essential for the developmental decline of Ca\(^{2+}\) channels in IHCs.

Both otoferlin and myosin VI appear to control the targeting of intracellular membrane compartments towards the basolateral membrane

The reduced basolateral volume and synaptic surface area were found in IHCs from both otoferlin and myosin VI...
mutants, indicating a common function of both proteins in maintaining the normal cell surface area. Considering recent findings showing the importance of myosin VI in maintaining a normal Golgi complex in fibroblasts (44), it is possible that in mutant IHCs the secretion from the TGN to the basolateral plasma membrane is disturbed. Otoferlin (7) and Rab8 (46) are co-localized with the Golgi marker GM130 (47) in the region of the TGN (48). The reduction of otoferlin in the TGN of myosin VI mutant IHCs, together with its interaction with the protein involved in vesicle endocytosis, Rab8 (46,49), the reduced basolateral synaptic surface area and persisting membrane infoldings in otoferlin mutants (Fig. 7), strongly support the notion for a complementary role of otoferlin and myosin VI in the normal targeting of intracellular membranes to the basolateral part of the cell. Disturbed secretion of vesicles from the TGN would necessarily lead to gradually disturbed replenishment of precursor vesicles in the base of IHCs (48), a feature notable in altered VGLUT3-immunopositive staining in the base of IHCs of myosin VI mutants. VGLUT3 has recently been shown to be a crucial vesicle membrane protein in IHCs required for normal hearing (50). Disturbed replenishment of vesicle supply from the TGN would also gradually affect the replenishment of the precursor pool for transmitter release (51) and sorting of recycled proteins to the basolateral membrane (52,53). In this context, the reduction of CaV1.3 channels and ribbon proteins should be considered as a presumptive consequence of a disturbed TGN vesicle targeting. Persisting exocytosis in myosin VI mutants, however, strongly suggests that the complementary role of otoferlin and myosin VI in targeting of intracellular membranes may not affect the fast exocytosis process.

MATERIALS AND METHODS

Animals and tissue preparation

We used Wistar rats (Charles River, Sulzfeld, Germany) and NMRI mice, as well as otoferlin (17,18) and myosin VI mutants. IHCs of 10 wild-type and 13 myosin VI mutant mice aged P17–P20 confirm reduced cell size. (G) The number of CtBP2/RIBEYE-positive spots (ribbons) was reduced in 31 otoferlin mutant IHCs compared with 35 wild-type IHCs (P < 0.001) and in 9 myosin VI mutant IHCs versus 13 wild-type IHCs (P < 0.001) at P20–P30. (H) The average number of CtBP2-positive spots was significantly reduced in 16 otoferlin mutant IHCs compared with 14 wild-type IHCs (P < 0.05) as well as in 9 myosin VI mutant IHCs compared with 10 wild-type IHCs (P < 0.001) at P20–P30. (I–L) Three-dimensional deconvoluted image of myosin VI wild-type and mutant IHCs double-labeled with anti-otoferlin (green, open arrowheads) and the synaptic vesicle marker anti-VGLUT3 (red, closed arrowheads) at P17. Myosin VI mutant IHCs (J and L) show reduced presence of otoferlin in the supranuclear region of the trans-Golgi network (J and L, asterisk) compared to wild-type IHCs (I and K, upper open arrowhead) and lack synaptic vesicles in the basal pole (L), whereas in the wild-type IHCs, VGLUT3 marks the basal pole as well (K). (M–P) Confocal double-labeling of immature (P9) otoferlin mutant IHCs, stained with a membrane marker (MM, red, open arrowhead) and the efferent marker anti-synaptobrevin2/VAMP2 (green, M and N) reveals large membrane invaginations / vacuoles in their apical cytoplasm (N, closed arrowhead) that can also be detected in immature otoferlin IHCs stained with anti-VGLUT3 (P, closed arrowheads) but not in otoferlin wild-type IHCs (M and O). Scale bars, in (I–L, O and P) 5 μm; in (M and N), 10 μm.

Figure 7. Reduced basolateral pole volume, reduction of presynaptic proteins and failure of proper targeting of intracellular membranes in inner hair cells from otoferlin and myosin VI mutant mice. (A and B) Expression of myosin VI in P20 wild-type and otoferlin mutant IHCs, and expression of otoferlin in P20 wild-type and myosin VI mutant IHCs (C and D) reveals reduction of the length of the basolateral pole in IHCs of both mutants (B and D). Scale bars, 10 μm. (E) Length of the basolateral pole obtained by measuring the distance between the lower edge of the nucleus and the furthest ribbon as indicated by open arrowheads in (A–D) at the age of P20–P30. Mean basolateral pole length was different for 31 otoferlin wild-type IHCs compared with 30 otoferlin mutant mice (P, 0.001) and for 13 myosin VI wild-type versus 14 myosin VI mutant mice (P < 0.001). (F) Average membrane capacitances of apical turn
mutant mice (11) for this study. Both mutant cell lines were obtained from Jackson Laboratories (Bar Harbor, ME).

Care and use of the animals as well as the experimental protocol were reviewed and approved by the animal welfare commission and the regional board for scientific animal experiments in Tübingen.

For RNA and protein isolation, cochleae were dissected as described (54,55) and immediately frozen in liquid nitrogen and stored at −70°C before use. For immunohistochemistry, cochleae were dissected (54,55) and fixed by immersion in 2% paraformaldehyde, 125 mM sucrose in 100 mM phosphate-buffered saline (PBS), pH 7.4, for 2 h. Cochleae of animals older than postnatal day 10 (P10) were decalcified after fixation for 15 min to 2 h in rapid bone decalcifier (Eurobio, Fisher-Scientific, 61130 Nidderau, Germany). After overnight incubation in 25% sucrose in PBS, pH 7.4, cochleae were embedded in O.C.T. compound (Miles Laboratories, Elkhart, IN). Tissues were then cryosectioned at 10 μm thickness, mounted on SuperFrost® plus microscope slides, dried for 1 h and stored at −20°C before use.

**GFP reporter mouse**

Hair cell-specific GFP reporter mice were generated employing a regulatory region of neurotrophin BDNF exons and a GFP sequence upon heterologous recombination technique. This resulted in an insertion of the GFP construct upstream of an up to now unknown hair cell-specific gene.

**Yeast two-hybrid**

To identify otoferlin interacting partners, the Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, CA) was used. We screened a mouse cochlear cDNA library isolated from P3–P15 (kind gift of Professor Richard J.H. Smith, Department of Otolaryngology, The University of Iowa) with five different fragments of the otoferlin protein (Fig. 1A). For this purpose, five different expression vectors with *Otof* were generated in frame with the DNA-binding domain (DNA-BD). Sequence in amino acids for otoferlin baits (NP_114081) were cloned into pGBK7 vector and covered amino acids 100–197 (bait 1), 603–887 (bait 2), 904–1025 (bait 3), 1299–1417 (bait 4), or 1917–1981 (bait 5). For details see (46).

**mRNA isolation and RT–PCR**

For RT–PCR analysis, mRNA from rat cochlea was isolated using DYNAbeads (DYNAbeads mRNA Direct Kit, DYNAL, Oslo, Norway) 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 (28) and frozen in liquid nitrogen. cDNA synthesis was performed using Superscript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). A 426 bp fragment of myosin VI was amplified by RT–PCR with the oligonucleotides 5′-GGAGCCAGCTTCATCCGC TG-3′ and 5′-GACTGACAGTGACACCACCTGGG-3′ (accession no. NM_001039546) (annealing temperature: 59°C, 35 cycles, or 50 cycles for single-cell RT–PCR). To distinguish between the myosin VI isoforms, we used primers flanking the region of the tail domain containing the inserts that generate four alternatively spliced isoforms (5′-TCTTGCCAGAGATAGCAG-3′, 5′-CAGTACCAGC TGGGAGC-3′) (20,56). Hereby, transcripts without insert (NI) exhibit a 306 bp PCR product, transcripts with the large insert (LI, 23–31 amino acids), respectively, a 375 and a 399 bp PCR product, and transcripts with the small insert (SI, 9 amino acids) a 333 bp PCR product. Finally, transcripts with both inserts exhibit, respectively, a 402 and a 426 bp PCR product. The resulting PCR products were analyzed on ethidium bromide agarose gels. The PCR product was sequenced for confirming myosin VI specificity. The analysis was performed in triplicate.

**Immunohistochemistry**

For immunohistochemistry, rat or mouse cochlear sections were stained and imaged as previously described (54,55). Briefly, cochlear sections were thawed and permeabilized either with 0.1% Triton X-100 (Sigma) for 3 min or with 0.5% Triton X-100 for 10 min at room temperature, pre-blocked either with 1% bovine serum albumin (BSA) in PBS or 4% normal goat serum in PBS, and incubated at 4°C with primary antibodies. For double labeling studies, both antibodies were simultaneously incubated for identical time periods. We used the following antibodies: rabbit polyclonal anti-myosin VI (Sigma; M5187; 1:200), rabbit polyclonal anti-otoferlin (7) (1:1000), mouse monoclonal anti-otoferlin (Abcam, 53233; 1:50), rabbit polyclonal anti-BKα (Alomone Labs, Jerusalem, Israel, APC-021; 1:50), mouse monoclonal anti-neurofilament 200 (NF200) (Sigma, N5139; 1:1000), rabbit polyclonal anti-CaV1.3 (Alomone Labs, APC-014; 1:50), mouse monoclonal anti-CtPB2/RIBEYE (BD Transduction Laboratories, CA, 612044; 1:50), mouse monoclonal anti-synaptotubulin2/VAMP2 (Synaptic Systems, 104211, 1:300) and rabbit anti-VGLUT3 antibody (50) (P45-3; 1:100). Antibodies were diluted in PBS containing either 0.5% BSA or 2% NaCl/0.1% Triton X-100/1% FCS. Primary antibodies were visualized with either Cy3-conjugated goat anti-rabbit IgG (0.35 μg/ml; Jackson Immuno Research Laboratories, PA) or with Alexa-Green-conjugated goat-anti-mouse antibodies (1:1500; Molecular Probes, Leiden, The Netherlands). Sections were rinsed, mounted in Vectashield containing the nuclear stain DAPI (Vector Laboratories, Burlingame, CA). All histological examinations and documentations were done using an Olympus AX70 microscope equipped with epifluorescence illumination and motorization in the z-axis. Images were acquired using a CCD camera and the imaging software Cell F (OSIS GmbH, Münster, Germany). For ribbon counts, cryosectioned cochlea were imaged over a distance of 8 μm with the complete coverage of the IHC nucleus and beyond in an image-stack along the z-axis (z-stack). One Z-stack consisted of 30 layers with a z-increment of 0.28 μm, for each layer one image per fluorochrome was acquired. To display spatial protein distribution, z-stacks were three-dimensionally deconvoluted using Cell F’s RIDE module with the Nearest
Statistical analyses of immunohistochemical data

Counting of immunoreactive spots in IHCs of wild-type and mutant otoferlin and myosin VI mice were compared and data expressed as mean percentage of the number of immunoreactive spots in control IHCs (set at 100%) ± SEM. For statistical analyses, an F-test (F) was performed to compare variances of the data obtained from counting. Equal variances were assumed for F > 0.05. Accordingly, unpaired Student’s t-test was performed, with n representing the number of analyzed IHCs. Differences in the number of immunoreactive spots were considered statistically significant for resulting P < 0.05.

SDS–PAGE and western blotting

For western blot analysis, cochlear tissue (P5–P8 and P > 30) was homogenized and lysed in lysis buffer containing 50 mM Tris–HCl, 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 280g 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. SDS–PAGE and western blotting were carried out using the ‘XCell II SureLock’ Mini-Cell and XCell II Blot Module from Invitrogen (Carlsbad, CA). 40 µg per lane of each cochlear sample was loaded on a 4–12% Tris–glycine gel (Invitrogen), resolved and transferred onto PVDF membrane according the manufacturer’s instructions. Blotted proteins were incubated with either rabbit polyclonal anti-otoferlin antibody (1:1000) or rabbit polyclonal anti-otoferlin antibody (1:2000) followed by ECL™ Peroxidase labeled anti-rabbit antibody (1:2500; Amersham Biosciences, Freiburg, Germany). Labeled proteins were detected by chemiluminescence using the ‘ECL Plus Western Blotting Detection Reagents’ from Amersham Biosciences.

Electrophysiological recordings and capacitance measurements

Whole-cell voltage-activated Ca²⁺ currents and capacitance changes were recorded in otoferlin (P7–P9) and myosin VI mutant IHCs (P17–P20) and respective age-matched controls using acute preparations of apical cochlear turns of the organ of Corti as described (8). Voltage-activated capacitance changes were evoked by stepping the IHC for 100 ms to 0 mV, the potential of maximal Ca²⁺ influx. The organ of Corti was bathed in a solution composed of (mM) 85 NaCl, 70 lactobionate-NaOH, 5.8 KCl, 1.3 CaCl₂, 0.9 MgCl₂, 0.7 NaH₂PO₄, 10 HEPES, 5.6 glucose, pH 7.4, 320 mOsm kg⁻¹, and superfused with an extracellular solution consisting of 110 NaCl, 5 CaCl₂, 1 MgCl₂, 5.6 glucose, 10 HEPES, 35 TEA, 15 4-AP, 0.005 XE-991, 0.0003 apamin, pH 7.4, 320 mosmol kg⁻¹. The pipette solution contained (in mM) 120 Cs⁺ methanethionate, 20 CsCl, 10 Na⁺ phosphocreatine, 5 HEPES, 5 EGTA, 4 MgCl₂, 4 Na₂ATP, 0.1 CaCl₂, 0.3 GTP, pH 7.35, 305 mosmol kg⁻¹. All recordings were performed at room temperature (22°C). For analysis of the voltage dependence of ICa activation, current–voltage (I–V) curves obtained by step depolarizations were fitted with the product of a Boltzmann function with exponent 2 and the driving force according to the Goldman Hodgkin Katz equation as described (28). This yielded the half-maximum voltage of activation and the slope factor of the Boltzmann function (28, eqn. 1). Statistical analysis of means was made using Student’s two-tailed t-test with P < 0.05 as the criterion for statistical significance. Mean values are quoted ± SD in the text and figures.

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Conflict of Interest statement. None declared.

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