Myosin VI is required for the proper maturation and function of inner hair cell ribbon synapses

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The ribbon synapses of auditory inner hair cells (IHCs) undergo morphological and electrophysiological transitions during cochlear development. Here we report that myosin VI (Myo6), an actin-based motor protein involved in genetic forms of deafness, is necessary for some of these changes to occur. By using post-embedding immunogold electron microscopy, we showed that Myo6 is present at the IHC synaptic active zone. In Snell’s waltzer mutant mice, which lack Myo6, IHC ionic currents and ribbon synapse maturation proceeded normally until at least post-natal day 6. In adult mutant mice, however, the IHCs displayed immature potassium currents and still fired action potentials, as normally only observed in immature IHCs. In addition, the number of ribbons per IHC was reduced by 30%, and 30% of the remaining ribbons were morphologically immature. Ca²⁺-dependent exocytosis probed by capacitance measurement was markedly reduced despite normal Ca²⁺ currents and the large proportion of morphologically mature synapses, which suggests additional defects, such as loose Ca²⁺-exocytosis coupling or inefficient vesicular supply. Finally, we provide evidence that Myo6 and otoferlin, a putative Ca²⁺ sensor of synaptic exocytosis also involved in a genetic form of deafness, interact at the IHC ribbon synapse, and we suggest that this interaction is involved in the recycling of synaptic vesicles. Our findings thus uncover essential roles for Myo6 at the IHC ribbon synapse, in addition to that proposed in membrane turnover and anchoring at the apical surface of the hair cells.

INTRODUCTION

Hearing relies primarily on the ability of cochlear inner hair cells (IHCs) to encode sound information with an extreme sensitivity and a high temporal precision (1). Sound-evoked mechanical stimuli are transduced by the mechanoelectrical transduction machinery of the IHC hair bundles into graded membrane potential variations that drive neurotransmitter release. This release can be maintained at high rate in response to sustained stimulation (2). Each mature IHC has 10 to 20 synaptic active zones that are characterized by the presence of an electron-dense structure of submicron diameter, the synaptic ribbon, to which synaptic vesicles are tethered, and which faces the dendrite of a bipolar auditory neuron (3,4).

During the first 2 post-natal weeks in the mouse, the IHCs undergo morphological and electrophysiological changes. The magnitudes of both IHC Ca²⁺ currents and Ca²⁺-dependent exocytosis increase until post-natal day 6 (P6) (5,6). The Ca²⁺ currents then decline progressively during IHC maturation, reaching their mature steady-state level by P12, but leaving the size of Ca²⁺-induced exocytosis almost unaffected. In parallel, the number of ribbons per IHC decreases, and the ribbon shape evolves from spherical to plate-like (3,7). Concomitant with the onset of hearing (P12), the IHCs also acquire fast membrane electrical properties, enabling phase locking of the auditory nerve firing pattern.
RESULTS

Myo6 is present at the IHC ribbon synapse

Two rabbit immune sera were raised against the His-tagged C-terminal part of murine Myo6 (amino acids 1047–1253), and the specific antibodies were affinity-purified against Myo6 globular tail produced as a glutathione S-transferase (GST) fusion protein (GST-Myo6-gt, amino acids 981–1253). The specificity of these antibodies was established by the immunoreactivity of the inner ear sensory epithelia observed in the wild-type mice, but absent in Myo6<sup>sv/sv</sup> mice. The labeling was also absent when the primary antibody was omitted or pre-absorbed on the antigen (Supplementary Material, Fig. S1c, f, g and h). In the inner ear of wild-type mice, Myo6 immunoreactivity was restricted to the sensory cells of the cochlea and vestibule, in agreement with previous reports (14,17) (Fig. 1A, and Supplementary Material, Fig. S1c and f). Immunolabeling experiments on whole-mount preparations of the organ of Corti analyzed by confocal microscopy at P6, P15 and P21, that is before and after the onset of hearing, showed that Myo6 is present throughout the hair cell soma (Fig. 1B). A strong labeling was observed in the two IHC regions known for their intense vesicular trafficking, namely the pericellular necklace (23) and the basolateral region, where the afferent boutons of the primary auditory neurons are located (Fig. 1B) (24). We then carried out post-embedding immunogold electron microscopy experiments on sections of organs of Corti from P10 and P15 mice. In the IHCs, the gold particles were not only associated with the cuticular plate and the peri-cuticular necklace as reported (17,20), but were also seen in the synaptic region of the cells (Fig. 1C–F). In all the ribbon-containing sections examined (10 sections from 3 mice), most of the gold particles (n = 73) were located at the edge of the active zone (48% of the gold particles), whereas 16% of the particles were nearby or associated with the ribbon, and 36% were seen within the cytoplasm. Notably, many of the gold particles seen at the active zone (31%) were associated with tubular features that have been considered to be endocytic structures (Fig. 1F) (25), thus suggesting that Myo6 is involved in synaptic vesicle recycling. The presence of the protein throughout the IHC soma, just like other key synaptic proteins including SNAP25, syntaxin1, otocerin and VGLUT2 (26–28), may reflect the intense synaptic vesicular trafficking of the IHCs.

Calcium-dependent exocytosis is impaired in mature IHCs lacking Myo6

To investigate a possible role of Myo6 in the synaptic exocytotic process, we studied IHC Ca<sup>2+</sup>-dependent exocytosis in Myo6<sup>sv/sv</sup> mutant mice that lack Myo6 expression. We monitored voltage-gated Ca<sup>2+</sup> currents and membrane capacitance changes (ΔC<sub>m</sub>) in P6–P7 (immature) and P26–P31 (mature) cochlear IHCs (Fig. 2A–C). At both stages, the Ca<sup>2+</sup> currents (Fig. 2A and B, middle panels) were not significantly different between Myo6<sup>sv/sv</sup> mice and their heterozygous littermates. Current–voltage curve of peak I<sub>Ca</sub> were fitted with the equation:

\[
I_{\text{Ca}} = \frac{g_{\text{max}}(V - V_{\text{rev}})}{1 + \exp((V_{1/2} - V)/S)}
\]

where V is the membrane potential, V<sub>rev</sub> the reversal potential, g<sub>max</sub> the maximum chord conductance and V<sub>1/2</sub> the membrane potential at which the conductance is half activated, and the slope factor (S) describes the voltage sensitivity of activation. Fits for P6–P7 Myo6<sup>+/sv</sup> IHCs gave values of g<sub>max</sub> = 8.8 nS, V<sub>1/2</sub> = −29 mV and S = 6.3 mV, whereas for P6–P7 Myo6<sup>sv/sv</sup> IHCs g<sub>max</sub> = 8.7 nS, V<sub>rev</sub> = 36 mV, V<sub>1/2</sub> = −28 mV and S = 6.1 mV (Fig. 2A, middle panel). Fits for P26–P31 Myo6<sup>+/sv</sup> IHCs yielded g<sub>max</sub> = 7.8 nS, V<sub>rev</sub> = 18 mV, V<sub>1/2</sub> = −33 mV, S = 9.9 mV, and for P26–P31 Myo6<sup>sv/sv</sup> IHCs g<sub>max</sub> = 6.2 nS, V<sub>rev</sub> = 15 mV, V<sub>1/2</sub> = −36 mV, S = 8.9 mV (Fig. 2B, middle panel). Reversal potentials in the mature IHCs were about 20 mV less depolarized than in immature IHCs, most likely due to incomplete block of residual outward currents through the K<sup>+</sup> channels (6). In P6–P7 Myo6<sup>sv/sv</sup> IHCs, peak maximum inward I<sub>Ca</sub> was −389 ± 30 pA, n = 4 (versus Myo6<sup>+/sv</sup> −396 ± 25 pA, n = 5), whereas in P26–P31 Myo6<sup>sv/sv</sup> IHCs maximum peak I<sub>Ca</sub> was −184 ± 15 pA, n = 4 (versus Myo6<sup>+/sv</sup> −232 ± 15 pA, n = 5; P < 0.05 at both stages). The Ca<sup>2+</sup> currents of Myo6<sup>+/sv</sup> and Myo6<sup>sv/sv</sup> IHCs declined significantly with age (P < 0.001 for both genotypes), indicating that the normal developmental downregulation of Ca<sup>2+</sup> influx did occur in the IHCs from adult mutant mice (5,6). The Ca<sup>2+</sup>-induced exocytosis of IHCs from mature homozygous mutant mice, however, was markedly reduced compared with the heterozygotes (Fig. 2B, right panel): the maximum ΔC<sub>m</sub> was 11.3 ± 4.1 fF, n = 4, for Myo6<sup>sv/sv</sup>, versus 27.0 ± 2.8 fF, n = 5, for Myo6<sup>+/sv</sup> (P < 0.02). Such a difference was not found in P6–P7 immature homozygous mutants.
(maximum $\Delta C_m = 51.8 \pm 8.0$ fF, $n = 4$, for $\text{Myo6}^{+/sv}$, versus $59.3 \pm 13.1$ fF, $n = 5$, for $\text{Myo6}^{+}/sv$; $P > 0.05$) (Fig. 2A, right panel). Next, we compared synaptic transfer functions (6) that were obtained by plotting $\Delta C_m$ against peak $I_{Ca}$ for 100 ms voltage steps over a range of membrane potentials from $-81$ mV up to $-11$ mV (Fig. 2C). Fitted lines are according to the power function:

$$\Delta C_m \propto I_{Ca}^N,$$

where the power $N$ is a measure for the Ca$^{2+}$-dependence of exocytosis. The synaptic transfer functions at P6–P7 and P26–P31 show that mature $\text{Myo6}^{+/sv}$ IHCs increased their Ca$^{2+}$-efficiency ($\Delta C_m/I_{Ca}$) over the range of their smaller Ca$^{2+}$ currents and decreased their Ca$^{2+}$-dependence of exocytosis ($N = 2.0$ at P6–P7 and $N = 0.93$ at P26–P31), just as in normal development. The P26–P31 $\text{Myo6}^{+/sv}$ IHCs had an even larger developmental reduction in Ca$^{2+}$ dependence ($N = 2.1$ at P6–P7 and $N = 0.51$ at P26–P31, Fig. 2C). Ca$^{2+}$-
efficiency at the maximum inward $I_{\text{Ca}}$ (6), which occurred near a membrane potential of $-21$ mV, in mature $\text{Myo6}^{\text{sv/sv}}$ IHCs (0.066 fF/pA) was about half that of IHCs of their heterozygous littermates (0.119 fF/pA). For smaller depolarizations, the effect became progressively less pronounced due to the reduced $\text{Ca}^{2+}$-dependence, but it was maintained for $\text{Ca}^{2+}$-currents $<20$ pA, so it would affect neurotransmitter release for physiologically relevant receptor potentials, spanning a membrane potential range of about $-60$ to $-20$ mV (Fig. 2B and C) (29). Taken together, these findings suggest that Myo6 is essential for efficient synaptic vesicle exocytosis in IHCs of the mature cochlea.

**IHC ribbon synapse maturation is delayed in $\text{Myo6}^{\text{sv/sv}}$ mice**

To investigate whether an abnormal structural development could contribute to the observed exocytotic defect, we first carried out quantitative analyses of the IHC ribbons in mutant and wild-type mice at P6, P15 and P21. In the apical part of the cochlea where exocytosis was studied, the ribbons were visualized by using anti-CtBP2/ribeye antibodies that label the ribbons and the cell nucleus (26). The ribbons were quantified after 3D reconstruction of the IHC immunolabeling analyzed by confocal microscopy (Fig. 3A). At P6, the number of ribbons harbored by the IHCs of $\text{Myo6}^{+/+}$ and

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*Figure 2.* $I_{\text{Ca}}$ and $\Delta C_m$ in IHCs of immature and mature cochleas from Snell’s waltzer heterozygous and homozygous mutant mice. (A and B, left panels) Examples of currents (middle traces) and $\Delta C_m$ recordings (lower traces) in immature (A) or mature (B) $\text{Myo6}^{+/+}$ and $\text{Myo6}^{\text{sv/sv}}$ IHCs at the holding potential ($-81$ or $-91$ mV, respectively) and in response to a 100 ms voltage step to $-21$ mV. Top traces show voltage protocols, in which the sinusoid used to track $C_m$ appears as a thick line. (Right panels) Averaged $I_{\text{Ca}}$–$V$ curves for five $\text{Myo6}^{+/+}$ and four $\text{Myo6}^{\text{sv/sv}}$ P6–P7 IHCs (A) and for five $\text{Myo6}^{+/+}$ and four $\text{Myo6}^{\text{sv/sv}}$ P26–P31 IHCs (B), with fitted lines according to Eq. (1). (Right panels) Averaged $\Delta C_m$–$V$ curves for corresponding cells in middle panels. (C) Synaptic transfer functions, relating $\Delta C_m$ and peak $I_{\text{Ca}}$ recorded at different membrane potentials from the holding potential up to the maximum amplitude for $I_{\text{Ca}}$. Dotted lines according to Eq. (2) fit P6–P7 (open symbols), continuous lines P26–P31 (filled symbols) IHCs.
**Myo6**sv/sv mice was similar (27.3 ± 0.8, n = 31, versus 24.7 ± 0.7, n = 29, respectively; P > 0.05). Likewise, we did not observe a significant difference in the number of ribbons per IHC between the two genotypes at P15 (10.9 ± 0.5, n = 71, versus 12.3 ± 0.3, n = 65, respectively; P > 0.05). This result shows that the normal developmental downregulation of the number of ribbons (3) occurred also in the mutant mice. At P21, however, a moderate but statistically significant reduction in the number of ribbons was found in the IHCs of **Myo6**sv/sv mice (13.4 ± 0.3 ribbons per IHC, n = 70 from 3 **Myo6**+/+ cochleas, versus 8.9 ± 0.3 ribbons per IHC, n = 91 from 3 **Myo6**sv/sv cochleas; P < 0.05) (Fig. 3A). Ultrastructural analysis showed that at P6, the IHC synapses of **Myo6**+/+ and **Myo6**sv/sv mice were indistinguishable (Fig. 3B and C). The same analysis carried out at P21 confirmed our immunofluorescence results, showing that the number of ribbon synapses in **Myo6**sv/sv IHCs was lower than that in the wild-type mice (8 ± 0.7 per IHC of **Myo6**+/+, n = 10; versus 4.5 ± 0.4 per IHC of **Myo6**sv/sv, n = 21; P < 0.05). Notably, 70% of the ribbons encountered in P21 **Myo6**sv/sv IHCs (n = 57) exhibited a mature shape and were decorated with synaptic vesicles (Fig. 3H). However, 30% of the ribbons still had an immature shape, indicating a delay in the ribbon maturation process. The immature phenotype of the ribbons in **Myo6**sv/sv...

![Figure 3. Impaired IHC ribbon synapse morphological maturation in the **Myo6**sv/sv mice.](https://academic.oup.com/hmg/article-abstract/18/23/4615/666836)
IHCs was even more pronounced at P15, at which stage most of the synapses had round-shaped ribbons anchored to the active zone through two tubular rosettes (Fig. 3E). In P15 wild-type mice, 46 of 51 ribbons examined (91%) had a plate-like shape characteristic of mature synapses (Fig. 3D), whereas in the mutant mice, such mature ribbons were rarely encountered (11 out 83 ribbons examined in 20 IHCs from 4 cochleae, i.e., 13%). Nevertheless, the immature ribbons (87%) had matured beyond the P6–P8 stage, as their shape was comparable with that of wild-type P10–P12 ribbons (3). Notably, many P15 Myo6sv/sv IHCs were still contacted by efferent nerve fibers, an immature feature that was not observed in the wild-type IHCs of the same stage (Fig. 3F and G). These results show that Myo6 is not necessary for the formation of the IHC ribbon synapse, but is required for its proper maturation and maintenance. This is consistent with what has been reported in neuronal synapses. In cultured hippocampal neurons derived from Myo6sv/sv mice, synapses seemed to develop normally, but were also 21% fewer than in cultures from wild-type mice (30,31). Notably, the loss is even greater when Myo6 was acutely disrupted (30,31).

**IHCs of adult Myo6sv/sv mice exhibit immature electrophysiological membrane properties**

At the onset of hearing (P12), the mature IHCs display the \( I_{K,f} \) current, also called BK current, which enables the cells to switch from spiking pacemakers to high-frequency signal transducers (8,32,33).

The presence of evoked and spontaneous spiking in the absence of Myo6 was studied by recording voltage responses to current injection in three IHCs from three P5–P6 Myo6sv/sv mice. Spontaneous and evoked spiking activity was recorded in all three cells (Supplementary Material, Fig. S2), indicating that at least up to P6, the IHC basolateral currents develop normally in Myo6sv/sv mice, which is consistent with our morphological observations. Surprisingly, we found that IHCs from adult Myo6sv/sv mice also fired action potentials (Fig. 4A right), just like immature IHCs (8). This contrasts with the normal graded voltage responses recorded in the mature Myo6+/-sv IHCs (Fig. 4A left). The resting potentials of mature Myo6sv/sv IHCs were hyperpolarized compared with those of their heterozygous littermates (−75.2 ± 2.2 mV, \( n = 6 \), versus −62.7 ± 2.2 mV, \( n = 5 \); \( P < 0.01 \)). This suggested that mutant and heterozygote IHCs have differences in membrane currents, which we studied under voltage clamp. Myo6sv/sv IHCs lacked \( I_{K,f} \) recorded in IHCs of mature heterozygous littermates (\( I_{K,f} \) measured 3 ms following a voltage step to −25 mV: Myo6+/-sv 2.27 ± 0.37 nA, \( n = 6 \), versus Myo6sv/sv −0.10 ± 0.02 nA, \( n = 6 \); \( P < 0.0001 \)) (Fig. 4B). In addition, the delayed rectifier potassium current \( I_{K,a} \) (8,33) recorded in P21–P31 Myo6sv/sv IHCs was smaller than that recorded in IHCs from heterozygotes (\( I_{K,a} \) measured by subtracting \( I_{K,f} \) from the total current at the end of a voltage step to −25 mV: Myo6+/-sv 1.74 ± 0.14 nA, \( n = 5 \), versus Myo6+/-sv 2.82 ± 0.31 nA, \( n = 5 \); \( P < 0.01 \)) (Fig. 4C). Notably, Myo6sv/sv IHCs also lacked the deactivating inward \( K^+ \) current \( I_{K,n} \), characterized by a very negative activation range, which appears in mature wild-type IHCs (34,35) and was found in Myo6+/-sv mice. The amplitude of the \( I_{K,n} \) current recorded was measured as the difference between the peak inward current and the steady-state current in response to a voltage step to −124 mV from a holding potential of −84 mV (36), and was −94 ± 24 pA (\( n = 3 \)). The inward current in the Myo6sv/sv IHCs was distinctly different from that of the Myo6+/-sv mice. Currents increased rather than deactivated during hyperpolarizing voltage steps in the Myo6sv/sv IHCs (Fig. 4D), thus resembling those of the inward rectifier \( I_{K,f} \) found in immature IHCs. IHCs normally increase in size during development, which is reflected as an increase in resting membrane capacitance (\( C_m \)) (34). This increase failed to occur in Myo6sv/sv IHCs. \( C_m \) was 8.0 ± 0.1 pF in immature Myo6+/-sv IHCs (P4–P6, \( n = 17 \)), increasing to 9.9 ± 0.4 pF upon maturation (P25–P31, \( n = 17 \)). In Myo6sv/sv IHCs, \( C_m \) was 7.5 ± 0.2 pF (P4–P7, \( n = 17 \)) early in development, and 7.4 ± 0.3 pF later on (P21–P31, \( n = 15 \)). Mature Myo6+/-sv IHCs had a significantly larger \( C_m \) than the other groups (all \( P < 0.001 \)). In conclusion, although the electrophysiological development of Myo6+/-sv IHCs appeared entirely normal, as investigated in normal CD-1 mice (32,34), the Myo6sv/sv IHCs retained immature electrophysiological properties.

**Myo6 and otoferlin interact in vivo**

By using a yeast two-hybrid assay and molecular and biochemical analyses, we have identified Myo6 as a potential otoferlin-interacting protein. Otoferlin, a six C2-domain transmembrane protein of synaptic vesicles (Fig. 5A), is responsible, when mutated, for a recessive form of deafness (DFNB9) and has been proposed to be a major \( Ca^{2+} \) sensor at IHC ribbon synapses (26). The otoferlin N-terminal fragment, containing the first three C2 domains (amino acids 1–761), was used as a bait to screen a library constructed from P3–P5 mouse inner-ear sensory epithelia (Fig. 5A) (37). The Myo6 globular tail (Fig. 5A), a domain regarded as a cargo-binding region (22), was among the prey identified. The direct interaction between otoferlin and Myo6 was confirmed by in vivo binding assays. In addition to Myo6 full length (Myo6-f), two different fragments of Myo6, its globular tail (Myo6-gt amino acids 981–1253) and the protein without its globular tail (Myo6-\( \Delta \)gt amino acids 1–981) (Fig. 5A), produced in vitro and labeled with \( 35S \)-methionine, were incubated with the N-terminal fragment of otoferlin produced as a GST fusion protein. Myo6 full-length and its globular tail domain did bind to the otoferlin fragment, whereas Myo6 deleted from its globular tail domain did not (Fig. 5B). We then assessed the co-localization of otoferlin and Myo6 in IHCs. Whole-mount preparations of the organ of Corti from P21 mice labeled for otoferlin and Myo6 were analyzed by confocal microscopy. Myo6 and otoferlin labelings largely overlapped in the IHCs: in particular, many co-labeled varicose processes overlapped in the IHCs: in particular, many co-labeled varicose processes were visible in the basolateral region of these cells (Fig. 5C). We next carried out double-immunogold electron microscopy on sections of the organ of Corti, which showed the presence of the two proteins at the IHC active zones, but with slightly different distributions (Fig. 5D). Indeed, Myo6 was preferentially detected at the edge of the active zone (Fig. 1C–F), whereas otoferlin was mainly localized around the ribbon, as described previously (Fig. 5D) (26). In some
Figure 4. Defective electrophysiological maturation in Myo6\textsuperscript{sv/sv} IHCs. (A) Voltage responses to current injection from a P25 Myo6\textsuperscript{+/sv} IHC [resting membrane capacitance (C\textsubscript{m}): 11.4 pF, R\textsubscript{s}: 2.1 M\Omega, resting potential (V\textsubscript{m}): −63 mV] and a P28 Myo6\textsuperscript{sv/sv} IHC (C\textsubscript{m}: 8.1 pF, R\textsubscript{s}: 5.2 M\Omega, V\textsubscript{m}: −67 mV). The voltage responses of the Myo6\textsuperscript{+/sv} IHC show graded increases in response to increasing current injection (left panel). The Myo6\textsuperscript{sv/sv} IHC responses to current injection are markedly different (right panel). Current injection of 100 pA results in fluctuations in the membrane potential, resembling spiking activity seen in IHCs of neonatal mice. Injection of larger currents (1000 pA) results in large depolarizations and is characterized by a single peak followed by a decline to a steady level where the cell remains until current injection ceases. (B) Representative current traces elicited from a series of depolarizing voltage steps from a −84 mV holding potential in 10 mV increments in a P25 Myo6\textsuperscript{+/sv} IHC (C\textsubscript{m}: 13.5 pF, R\textsubscript{s}: 0.86 M\Omega, zero-current potential (V\textsubscript{z}): −70 mV, G\textsubscript{leak}: 4.2 nS) and a P21 Myo6\textsuperscript{sv/sv} IHC (C\textsubscript{m}: 8.7 pF, R\textsubscript{s}: 2.5 M\Omega, V\textsubscript{z}: −74 mV, G\textsubscript{leak}: 1.3 nS). Fast time scale emphasizes the contribution of the fast potassium current I\textsubscript{K,f}, which is absent in the Myo6\textsuperscript{sv/sv} IHC (B, right panel). (C) Currents from the same cells as panels (B) on a longer time scale to show the contribution of the slow K\textsuperscript{+} current I\textsubscript{K,s}, which remains present in the Myo6\textsuperscript{sv/sv} IHC (C, right panel). (D) Currents elicited from a series of hyperpolarizing voltage steps in 10 mV increments from a holding potential of −84 mV for a P27 Myo6\textsuperscript{+/sv} IHC (C\textsubscript{m}: 11.5 pF, R\textsubscript{s}: 0.74 M\Omega, V\textsubscript{z}: −64 mV) and a P21 Myo6\textsuperscript{sv/sv} IHC (C\textsubscript{m}: 6.9 pF, R\textsubscript{s}: 0.95 M\Omega, V\textsubscript{z}: −65 mV). The deactivating I\textsubscript{K,n} current was not detected in the Myo6\textsuperscript{sv/sv} cell (right panel), which had an inward rectifier I\textsubscript{K1} current instead.
Figure 5. Myo6 and otoferlin co-localize at the IHC synaptic active zone and directly interact. (A) Schematic illustration of otoferlin and Myo6 structures. Upper panel depicts otoferlin with its six predicted C2 domains (C2A–F) and its transmembrane (TM) domain. The bait used in the yeast two-hybrid screening is indicated by a red horizontal line (Otof-Nt). Lower panel represents Myo6 and its alternative splice forms, which may or may not include a large insertion (LI) immediately preceding the globular tail (in blue), and a second, smaller insertion (SI) within the globular tail. The overlapping sequences of the two preys (red bar) identified in the yeast two-hybrid screening map to the globular tail of Myo6, which lacks both inserts. (B) In vitro-translated 35S-methionine-labeled full-length Myo6 (Myo6-fl, 1253 amino acids) (see Materials and Methods), cloned from our mouse cochlear cDNA library, lacks both inserts. (B) In vitro-translated 35S-methionine-labeled full-length Myo6 (Myo6-fl) or its truncated fragments, namely the globular tail (Myo6-gt) and the protein deleted from its globular tail (Myo6-Dgt), were incubated with GST-Otof-Nt fusion protein or GST alone. Myo6-fl and Myo6-gt, but not Myo6-Dgt, directly interact with the N-terminal fragment of otoferlin. (C, left panel) Scheme of an IHC and its basolateral region, where the afferent boutons (in brown) are located. (Middle panel) Transverse section of a P21 IHC after 3D reconstruction from a cochlear whole-mount preparation labeled for otoferlin and Myo6 (in red and green, respectively). Isolated varicosities of the basolateral area of the IHC (arrow heads) are immunoreactive for both antibodies. (Right panel) Close-up view of a section through the basolateral region of three IHCs. Scale bar: 10 μm. (D) Co-localization by immunogold electron microscopy of Myo6 and otoferlin in the mouse IHC. Arrowheads point to juxtaposed 10 and 5 nm gold particles, reflecting otoferlin and Myo6 immunoreactivity, respectively. The Myo6 labeling is especially abundant at the edge of the active zone (double-arrows), whereas otoferlin is more abundant around the ribbon (arrow). Scale bar: 125 nm. (E) Extracts from transfected HEK-293 cells expressing Myo6 alone (1) or with otoferlin (2) were subjected to immunoprecipitation (IP) with the anti-otoferlin antibody. Myo6 is co-immunoprecipitated with otoferlin. (F) Co-immunoprecipitation experiment carried out with protein extracts from mouse cochlear sensory epithelium. The antibody directed against otoferlin immunoprecipitates Myo6 with otoferlin in addition to syntaxin1 and SNAP25, whereas only otoferlin and Myo6 are co-immunoprecipitated by the antibody directed against Myo6. (G) Equal amounts of Myo6-ct (Input) were incubated with Glutathione Sepharose 4B beads alone (R) or pre-incubated with GST, GST-C2A, GST-C2B or GST-C2C in the presence of 2 mM EDTA or of 1 mM free Ca2+ concentration. Red Ponceau-staining (upper panel) was used to assess the levels of GST fusion proteins bound to the resin. Only the C2B domain is able to bind to Myo6-ct (lower panel).
locations, the two types of gold particles were found just a few nanometers apart, including at the edge of the active zone (Fig. 5D), suggesting that the two proteins can physically interact in vivo. This was confirmed by co-immunoprecipitation experiments carried out on protein extracts from transfected human embryonic kidney (HEK)-293 cells producing otoferlin and Myo6 (Fig. 5E), and on protein extracts from inner ears of P15 mice (Fig. 5F). The anti-otoferlin antibody immunoprecipitated not only Myo6, but also syntaxin-1 and SNAP25, two members of the SNARE complex (26). The anti-Myo6 antibody, however, immunoprecipitated only otoferlin. This suggests that the SNARE proteins do not physically interact with Myo6, and that otoferlin interaction with Myo6 is independent of the interaction between otoferlin and the SNARE proteins. To determine which otoferlin domain(s) is involved in the binding to Myo6, the precise boundaries of each of the C2 domains present in the otoferlin N-terminal fragment, namely, C2A-C, were determined. This was achieved by combining programs of secondary elements search (HCA and PSI) and multiple sequence alignment (Dialign and Clustal W), followed by in vitro expression. The C2A, C2B and C2C domains were produced as GST-fusion proteins and purified by affinity chromatography. These proteins were then incubated with Myo6-ct (amino acids 835–1253) or Myo6-gt. Only C2B domain was able to specifically bind to Myo6 carboxy terminal tail (Fig. 5G and data not shown). To determine whether this interaction could be modulated by Ca$^{2+}$ ions, we repeated this test in the presence of 2 mM EDTA or 1 mM free Ca$^{2+}$. Modifying the free Ca$^{2+}$ concentration did not affect the binding of C2B to Myo6 carboxy terminal tail (Fig. 5G). Indeed, C2B sequence lacks most of the residues known to be involved in Ca$^{2+}$-binding in other C2 domains (38,39). It is noteworthy that a different otoferlin–Myo6 interaction has recently been reported, which involves the otoferlin C2D domain (40) that can bind to Ca$^{2+}$ ions (26). The Ca$^{2+}$-dependence of this interaction, however, remains to be determined.

**DISCUSSION**

We found that Myo6 is present at the IHC synaptic active zone and that its absence prevents the IHC ribbon synapse maturation from proceeding normally. We also found that Myo6 defect leads to a markedly reduced synaptic exocytosis and to the lack of several K$^+$ currents in adult IHCs. Finally, we provide evidence that Myo6 and otoferlin, a putative Ca$^{2+}$ sensor of synaptic exocytosis also involved in a genetic form of deafness, DFNB9, interact at the IHC ribbon synapse.

The implication of Myo6 in IHC exocytosis is consistent with several studies showing that this myosin is involved both in endocytic and exocytotic membrane-trafficking pathways (41). This was shown not only in non-neuronal cells, in which Myo6 was found to be required for efficient secretion and for the maintenance of the structure of the Golgi apparatus (42), but also in synaptic vesicle exocytosis at hippocampal synapses (30,31). In hippocampal neurons, both spontaneous and evoked synaptic vesicle exocytosis were reduced by 35% in Myo6$^{xv/xv}$ mice (31,41). The more dramatic reduction of synaptic exocytosis reported here (~60%) may reflect a specific requirement of the IHC ribbon synapse for a high rate of exocytosis and recycling, and/or the inability of mutant IHCs to develop compensatory mechanisms as efficient as hippocampal neurons do in the absence of Myo6. The marked decrease of IHC synaptic exocytosis observed in mature Myo6$^{xv/xv}$ mice could, in principle, result from a failure of IHC synaptic development, a vesicular trafficking defect, an inefficient Ca$^{2+}$-exocytosis coupling, or a combination of these defects.

The finding that the IHCs of adult Myo6$^{xv/xv}$ mice harbor morphologically mature ribbon synapses excludes a major ultrastructural abnormality as the sole cause of the exocytosis defect. Likewise, the moderate reduction of the number of ribbons (30%) is unlikely to fully account for the dramatic decrease of IHC exocytosis, since in the bassoon mutant mice that have over 90% of ribbon loss, IHC exocytosis is largely unaffected (4).

Part of the IHC exocytosis defect of Myo6$^{xv/xv}$ mice could be the outcome of the delayed maturation of the synapse we observed, which ultimately leads to IHCs in which mature and immature ribbon synapses coexist. Such a configuration may hinder IHC transmitter release, as the two types of synapses have different dependences on local Ca$^{2+}$ concentration. It is generally believed that during development, IHC exocytosis switches from a mode of Ca$^{2+}$-dependence in which each release event requires the cooperative action of overlapping Ca$^{2+}$ microdomains, to a nanodomain configuration in which very few channels are required for the release of a single vesicle (6,43,44). It has been suggested that the more efficient coupling between Ca$^{2+}$ entry and vesicle exocytosis in the mature IHCs arises from a developmental tightening between synaptic vesicles and the Ca$^{2+}$ channels, which would compensate the lower Ca$^{2+}$ influx in mature IHCs (44–46). Therefore, the immature ribbon synapses may not function properly in IHCs that display smaller adult-type Ca$^{2+}$ currents suitable for a release machinery operating in a nanodomain configuration.

Insufficient supply of synaptic vesicles due to an endocytosis defect could also contribute to the observed reduction of exocytosis. Indeed, Myo6 has been found to be associated with clathrin-coated pits/vesicles and to be able to modulate endocytosis in the kidney (42), a mechanism that is likely to also take place at glutamatergic synapses during synaptic exocytosis and the recycling of AMPA receptors (30,31). Myo6 is also required for the efficient transportation of nascent endocytotic vesicles from the actin-rich periphery of the retinal pigment epithelium cell to supply the early endosome compartment with endocytotic vesicles (47). In the IHC, endocytosis at the synaptic active zone and at the peri-cuticular necklace has been proposed to be the main source for IHC synaptic vesicle pool replenishment (24,25). The abundance of Myo6 in these two regions, its association with tubular structures, likely endocytotic structures, at the edge of the active zone, and the increasing evidence that the protein is involved in endocytotic membrane-trafficking together suggest that Myo6 is implicated in the transport of vesicles on actin filaments from the IHC apical region to the ribbon synapse and/or in the retrieval of IHC synaptic vesicles after exocytosis (41,48,49). Our results suggest that at the IHC
ribbon synapse, Myo6 could achieve such a task through its binding to the vesicular membrane protein otoferlin. Two findings support this proposal. First, Myo6 and otoferlin are co-localized at the edge of the synaptic active zone, where IHCs endocytosis is suggested to take place (24,25). Second, the amount of otoferlin is apparently reduced in the Myo6sv/sv IHCs (40).

In addition to its role at the synapse, we report that Myo6 is necessary for the maturation of the electrophysiological membrane properties of the IHCs. Mature Myo6sv/sv mice lack Ik,f and Ik,n, whereas they exhibit a reduced amplitude of the delayed rectifier potassium current I K, f, I K, n current not only prevents the mature IHCs from firing action potentials, but also lowers their membrane electrical time constant to well under 1 ms (8), enabling phase-locking of transmitter release for sound frequencies up to a few kHz. In the absence of this current in Myo6sv/sv IHCs, the membrane time constant is expected to be abnormally large, which will prevent phase-locking of action potentials in the auditory nerve for sound frequencies above a few tens of Hz. Mature Myo6sv/sv mice are therefore expected to have defects in the precision of spike timing in the auditory nerve, as reported for BK knockout mice which lack Ik,f and Ik,n (50). Somewhat unexpectedly, the immature electrophysiological membrane properties of the IHCs recorded in adult mutant mice did not include the normal Ca2+ currents, suggesting that maturation of K+ and Ca2+ currents in IHCs proceed independently. The absence of adult-type K+ currents in the Myo6sv/sv IHCs could in principle result from a gene expression defect or from a mistargeting of the corresponding channels. The latter possibility is the most likely, since a missorting of the BK channel α-subunit has recently been reported in the Myo6sv/sv IHCs (40).

Finally, it is worthy of note that although otoferlin and Myo6 directly interact, otoferlin and Myo6 mutants display major differences in their phenotypes. The lack of otoferlin does not interfere with IHC ribbon or K+ currents maturation, whereas it leads to an almost completely abolished Ca2+-induced endocytosis, despite normal Ca2+ currents (26). Moreover, this failure of Ca2+-induced endocytosis is already present at P6 (26), whereas in the absence of Myo6, the defect of Ca2+-induced endocytosis is partial and only appears in mature IHCs. This suggests that the retrieval of synaptic vesicles, where otoferlin may act as a cargo adapter, is just part of the variety of cellular functions wherein Myo6 is involved (51).

Together, our results broaden the roles of Myo6 in hair cells and uncover an unexpected and essential role of this protein in the maturation of the IHC electrophysiological membrane properties and in the morphological and functional maturation of the ribbon synapse.

MATERIALS AND METHODS

Animals
Snell’s waltzer mice (Myo6sv/sv), which have an intragenic deletion of Myo6 leading to a null allele (13,14), were bred with C57BL/6J mice. Genotyping at the Myo6 locus was conducted by PCR as described in what follows. The day of birth was denoted P0. All experiments reported were carried out according to INSERM, Institut Pasteur and UK Home Office welfare guidelines.

Genotyping
Genomic DNAs from Myo6sv/sv inter-cross litters were amplified using primers (5’TGGTGAAGAGCTAACCTGTG-3’ and 5’-GCTTCAGGTCGATTTTTATT-3’), flanking the deletion in the Myo6sv mutation allele (14). The reaction mixture contained 200 ng of genomic DNA, LA PCR Buffer II with 2.5 mM MgCl2, 0.4 mM of each dNTP, 500 nm of each primer and 0.05 unit/ml of reaction mixture of TaKaRa LA Taq polymerase (TaKaRa Bio Inc). PCR was performed as follows: 5 min denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C, 2.5 min extension at 72°C and finally an additional 10 min at 72°C. Wild-type mice displayed a PCR product of 2255 bp, whereas Myo6sv/sv mice displayed a 1245 bp fragment, and DNA from Myo6sv/sv mice generated both fragments (Supplementary Material, Fig. S2).

Yeast two-hybrid screening
We used the yeast two-hybrid cDNA library constructed from a P3–P5 inner-ear sensory epithelia previously reported (37). Yeast two-hybrid screenings were carried out as reported (52,53), using otoferlin amino terminal region (Otof-Nt, amino acids 1–761) as bait (Fig. 5A). The interacting ‘prey’ fragments of the positive clones were PCR-amplified and sequenced. The resulting sequences were used to identify the corresponding gene in the GenBank database (NCBI) using a fully automated procedure.

DNA constructs
The Myo6 full-length cDNA (NM_001039546) was cloned by three successive rounds of RACE-PCR/subcloning on a BALB/c mouse cochlear cDNA library. The predicted protein sequence was 1253 amino acid 835–1253, Myo6-gt: 981–1253 and Myo6-ct: amino acid 1–761 as bait (Fig. 5A). The interacting ‘prey’ fragments of the positive clones were PCR-amplified and sequenced. The corresponding gene in the GenBank database (NCBI) using a fully automated procedure.

Protein expression in Escherichia coli
GST-tagged proteins were expressed in Escherichia coli BL21-CodonPlus (DE3)-RIPL (Stratagene). Production was made in 2YT medium (17 g of bacto-tryptone, 10 g bacto-yeast extract and 5 g NaCl per liter) overnight at 14°C, following...
were transfected with otoferlin full length cDNA alone or in combination with Myo6 full length (10 μg total DNA/10 cm dish), using Lipofectamin with Plus reagents (Invitrogen) according to the manufacturer’s instruction. After 36 h, cells were rinsed in cold PBS, harvested and pelleted by centrifugation. Cell extracts were prepared using 1% Triton X-100, 0.5% deoxycholate and 0.2% SDS, 5 mM DTT supplemented with 3 mM ATP/MgCl2 and EDTA-free cocktail of protease inhibitors (Roche). Immunoprecipitation was carried out using the polyclonal antibody directed against Myo6 or the anti-otoferlin monoclonal antibody, pre-incubated with protein G-agarose (Pharmacia) for 1.5 h at room temperature. The resin was pelleted by centrifugation, washed twice with 50 mM Tris–HCl, pH 7.4, and 0.1% Triton X-100, and three times in the same buffer complemented with 150 mM NaCl. Proteins were transferred electrophoretically to nitrocellulose sheets. Blots were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20 and probed with the following antibodies: anti-Myo6 1:500, anti-otoferlin 1:500, anti-SNAP25 1:2500, anti-syntaxin1 1:2500. Horse-radish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (BioRad) and the ECL chemiluminescence system (Amersham) were used for detection.

**Immunohistofluorescence**

Cochlear tissue preparation and immunohistochemistry for light microscopy were carried out as reported previously (26). Cochlear whole-mount preparations were fixed with 4% paraformaldehyde (PFA) in PBS, permeabilized with 0.3% Triton X-100 in PBS containing 20% normal goat serum for 1 h at room temperature and incubated with the polyclonal antibody directed against Myo6 (1:400), otoferlin (1:500) or CtBP2 (Transduction Laboratories, France) (1:100), overnight at 4°C. After three washes in PBS, the cochleas were incubated for 1 h with F(ab)2 fragment of goat anti-rabbit IgG antibody conjugated with Alexa488 fluoroescein (Interchim, France) alone or with goat anti-mouse IgG antibody conjugated with Cy3 fluorophore (Jackson Immunoresearch Laboratories) diluted at 1:500 in PBS. Rhodamine phalloidin (1:2000, Invitrogen) was used to label F-actin. The preparations were then washed three times in PBS and finally mounted in one drop of Fluorsave medium (Biochem Laboratories, France).

The samples were analyzed using a confocal laser scanning microscope, LSM510 Meta (Zeiss, Pasteur Institute, Imageo-ple). The images taken with a step size of 0.1 μm were used to generate 3D reconstructions of the IHCs using Osirix software (Antoine Rosset, Department of Radiology, Geneva University Hospital, Switzerland). The total number of ribbons counted was divided by the number of IHCs analyzed. All comparative images between wild-type and mutant mice were done in the same conditions of preparation, acquisition and analysis.

**Electron microscopy**

Mouse cochleas were fixed as described (26). The organs of Corti were microdissected and processed by the progressive temperature-lowering technique as reported (26). Ultrathin sections (70 nm) were cut with a Leica Ultracut S microtome.
and transferred to formvar-coated single-slot grids. Immunogold-labeling was carried out as described (26). The sections were incubated overnight with the anti-Myo6 polyclonal antibodies alone or in combination with the anti-otoferlin monoclonal antibody, diluted at 1:200 and 1:100, respectively. The sections were washed and then incubated for 2 h with 10 nm gold-conjugated goat anti-mouse and 5 nm gold-conjugated goat anti-rabbit antibodies (1:50, Tebu, France). The sections were then stained with uranyl acetate and lead citrate and examined under a Jeol1200EX electron microscope. Gold particle distribution in the synaptic region was quantified compared with the ribbon considered as the center of the active zone. A gold particle was counted as being at the edge when it was associated with the presynaptic density over 100 nm away from the ribbon. A gold particle was counted as being nearby the ribbon when it was not associated with the membrane but located within 50 nm from it. For morphological analyses, cochleas were perfused with 4% PFA and 2% glutaraldehyde in PBS at pH 7.4 and immersed in the fixative solution for 2 h. They were then post-fixed by overnight incubation in 1% osmium tetroxide at 4°C, dehydrated in graded acetone concentrations and embedded in Spurr’s low-viscosity epoxy resin hardened at 70°C. Ultrathin sections were transferred to formvar-coated single-slot grids, stained with uranyl acetate and lead citrate and examined under a Jeol1200EX electron microscope (Pasteur, Imageopole).

Electrophysiological recordings and capacitance tracking

Voltage- and current-clamp recordings were obtained from apical-coil IHCs, using an Optopatch amplifier (Cairn Research). Voltage-clamp experiments were done at room temperature (20–24°C), current-clamp and capacitance tracking close to body temperature (32–35°C). The extracellular solution contained (in mM): 135 NaCl, 5.8 KCl, 1.3 CaCl2, 0.9 MgCl2, 0.7 Na2HPO4, 2 Na pyruvate, 5.6 glucose, 10 HEPES–NaOH, amino acids and vitamins for Eagle’s MEM. Osmolality was around 308 mOsm/kg and pH 7.5. The pipette filling solution for voltage- and current-clamp experiments was (mM) 131 KCl, 3 MgCl2, 5 Na2ATP, 1 EGTA–KOH, 5 HEPES–KOH (osmolality 282 mOsm/kg, pH 7.3). Currents under voltage clamp were corrected offline for linear leak conductance calculated from 10 mV steps from the holding potential, usually −84 mV, including a −4 mV liquid junction potential correction. Membrane potentials were corrected for voltage drop across the residual series resistance (Rm) of 1.7 ± 0.3 MΩ (n = 12) after compensation of around 80%. Sizes of the K+ currents IC sat and IC s were determined as reported before (8).

Real-time changes in membrane capacitance (∆Cm) were studied using the Optopatch (6), applying a 2.5kHz sine wave of 13 or 18.5 mV amplitude around a holding potential of −81 or −91 mV (including −11 mV correction for the liquid junction potential), to avoid activating significant voltage-dependent membrane currents, as accurate capacitance tracking requires a membrane resistance (Rm) that is relatively constant and high compared with R, (6). For the capacitance-tracking experiments, no R, compensation could be applied. Nevertheless, no offline correction of membrane potentials for R, (5.0 ± 0.1 MΩ, n = 18) was necessary, as maximum voltage errors were in the order of 1 to 2 mV. Rm did not differ significantly between the four groups of IHCs (mature versus immature, heterozygote versus mutant) and averaged 803 ± 108 MΩ (n = 18). The extracellular superfusion solution for capacitance tracking and the associated Ca-current recordings contained 30 mM TEA–Cl (Fluka) to help block the large currents through the K+ channels. Some of the apparent inactivation of the inward Ca2+ currents (Fig. 2A and B, left panels) is likely to be due to residual unblocked currents. We therefore measured Ca2+ current size as that of the initial peak current. In the superfusion solution, amino acids and vitamins were omitted and NaCl was reduced to 115 mM to keep osmolality near 308 mOsm/kg. The intracellular solution was (mM) 140 Cs-glutamate, 3 MgCl2, 5 Na2ATP, 0.3 Na2GTP, 1 EGTA–CsOH, 5 HEPES–CsOH (osmolality around 282 mOsm/kg and pH 7.3).

Electrophysiological recordings used for the data analysis were mostly in response to single presentations of the stimulus protocol, but occasionally averaged from two successive presentations. All quantitative data are presented as mean ± SEM. Student’s t-test or one-way ANOVA followed by the Tukey post-test was used to analyze statistical significance (criterion P < 0.05).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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