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Whirler Mutant Hair Cells Have Less Severe Pathology than Shaker 2 or Double Mutants

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ABSTRACT

MYOSIN XV is a motor protein that interacts with the PDZ domain-containing protein WHIRLIN and transports WHIRLIN to the tips of the stereocilia. Shaker 2 (sh2) mice have a mutation in the motor domain of MYOSIN XV and exhibit congenital deafness and circling behavior, probably because of abnormally short stereocilia. Whirler (wi) mice have a similar phenotype caused by a deletion in the third PDZ domain of WHIRLIN. We compared the morphology of $Whrn^{wi/wi}$ and $Myo15^{sh2/sh2}$ sensory hair cells and found that $Myo15^{sh2/sh2}$ have more frequent pathology at the base of inner hair cells than $Whrn^{wi/wi}$, and shorter outer hair cell stereocilia. Considering the functional and morphologic similarities in the phenotypes caused by mutations in $Myo15$ and $Whrn$, and the physical interaction between their encoded proteins, we used a genetic approach to test for functional overlap. Double heterozygotes ($Myo15^{sh2/+}$, $Whrn^{wi/+}$) have normal hearing and no increase in hearing loss compared to normal littermates. Single and double mutants ($Myo15^{sh2/sh2}$, $Whrn^{wi/wi}$) exhibit abnormal persistence of kinocilia and microvilli, and develop abnormal cytoskeletal architecture. Double mutants are also similar to the single mutants in viability, circling behavior, and lack of a Preyer reflex. The morphology of cochlear hair cell stereocilia in double mutants reflects a dominance of the more severe $Myo15^{sh2/sh2}$ phenotype over the $Whrn^{wi/wi}$ phenotype. This suggests that MYOSIN XV may interact with other proteins besides WHIRLIN that are important for hair cell maturation.

Keywords: genetics, mouse model, age-related deafness, gene expression, kinocilium

INTRODUCTION

The mammalian inner ear has specialized hair cells that detect sound and movement of the head. The stereocilia on the apical surface of these cells constitute a mechanosensitive organelle with precisely organized actin-filled projections (Moran et al. 1981). These projections develop from microvilli on the hair cell surface. Cochlear hair cells also have a kinocilium, which is a transient organelle that regresses around postnatal day 8 (P8) in mice, and eventually disappears at P12, whereas it is a permanent organelle of the vestibular hair cells (Kikuchi and Hilding 1965; Kimura 1966). Alterations in morphogenesis of the stereocilia can result in hearing loss, balance defects, or both. This maturation involves patterning, increased girth, elongation, and cohesion. MYOSIN XV and the PDZ domain-containing protein WHIRLIN are components of the molecular complex that underlies the elongation process (Delprat et al. 2005; Belyantseva et al. 2005; Kikkawa et al. 2005). Dysfunction of either MYOSIN XV or WHIRLIN causes deafness in humans and mice (Probst et al. 1998; Liang et al. 1999; Mburu et al. 2003). Two recessive deaf mouse mutants, shaker 2 ($Myo15^{sh2/sh2}$) and

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whirler ($Whrn^{wi/wi}$), exhibit abnormally short stereocilia (Probst et al. 1998; Holme et al. 2002), although no direct comparisons of the length of stereocilia have been reported. MYOSIN XV is normally found at the tips of the stereocilia, from embryonic day 18.5 (E18.5) through adulthood, indicating that MYOSIN XV might be important for maintenance of hair cell viability. Although WHIRLIN is also normally localized to the tips of the stereocilia, its expression is dynamic during growth of the sterocilia, exhibiting early appearance of the protein product in inner hair cells (IHCs) that fades out at P11, and later appearance in the outer hair cells (OHC) at P14. This pattern is consistent with the earlier maturation of IHC stereocilia relative to those of OHC (Kikkawa et al. 2005). The colocalization of MYOSIN XV and WHIRLIN in the tips of the stereocilia, and the functional interaction between the MYOSIN XV tail and the PDZ domains of WHIRLIN, suggest that MYOSIN XV is a vehicle for transporting and localizing WHIRLIN to the tips of the stereocilia (Delprat et al. 2005; Belyantseva et al. 2005; Kikkawa et al. 2005).

Previous studies have shown evidence for digenic inheritance of hearing deficiency. Mutations in cadherin 23 ($Cdh23$) and protocadherin 15 ($Pcdh15$) mutations are autosomal recessive (Zheng et al. 2005). Humans and mice that are heterozygous for mutations in both of these genes exhibit deafness, whereas single heterozygotes lack this pathology. Intriguingly, $Cdh23$ and $Pcdh15$, like $Myo15$ and $Whrn$, play essential roles in maintaining the hair cell bundle of stereocilia. Examples of digenic diseases have also been described for retinitis pigmentosa, glaucoma, and diabetes (Goldberg and Molday 1996; Vincent et al. 2002; Savage et al. 2002). Many of these cases of digenic inheritance involve proteins that interact or function in the same pathway (Rudnicki et al. 1993; Goldberg and Molday 1996). Given the functional and morphologic similarities of the cochlear phenotypes caused by mutations in $Myo15$ and $Whrn$ genes, we tested for a potential digenic interaction (or functional overlap) between $Myo15$ and $Whrn$ using a classical genetic approach.

MATERIALS AND METHODS

Mice

All mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All procedures were approved by the University of Michigan Committee on Use and Care of Animals. In general, the $Whrn^{wi/wi}$ females were bred to $Myo15^{sh2/sh2}$ males, and F1 progeny mice from this cross were intercrossed (F1×F1), and F2 progeny were examined. In some cases double heterozygotes (F1) were replaced with animals mutant at one locus and heterozygous at the other to enhance the frequency of the double mutant genotype above 1/16 predicted for a double heterozygote intercross. At least three animals of each genotype were analyzed at each age (P0–P1, P5–P6, P10, P12, P17, P21, P30, P35, P49, and P180). P0 is designated as the day of birth.

Assessment of hearing by auditory brainstem response

Auditory brainstem responses (ABRs) were conducted as previously described (Beyer et al. 2000). Three to four animals in each category were tested at three frequencies: 4, 10, and 20 kHz.

Apical hair cell morphology assessed by scanning electron microscopy

For scanning electron microscopy (SEM) analysis, animals were killed and temporal bones were removed and fixed with 2% glutaraldehyde in 0.15 M of cacodylate buffer. Cochleae were processed using the OTOTO method, which involves immersing the tissues alternately in thiocarbohydrazide and osmium tetroxide (Osborne and Comis 1991). They were critical point dried and mounted on stubs using colloidal silver paste. Samples were examined with an Amray 1000B SEM. At least three animals of each genotype and age (P17, P21, and p35) were examined.

Phalloidin epifluorescence

For phalloidin analysis, animals were killed and the temporal bones removed. The temporal bones were immersed in 4% paraformaldehyde in phosphate buffer (0.15 M, pH 7.35). Under stereoscopic magnification, the round and oval windows were opened, and the bone from the apical tip of the cochleae was removed to allow fixative to flow throughout the tissue. Two hours later, the otic capsule was trimmed to reveal the modiolus and the organ of Corti. The entire apical turn and several fragments of the basal turn were separated from the modiolus and from the lateral wall tissues. Samples were permeabilized in Triton X-100 (0.3%, 5 min) and incubated with phalloidin 488 (Molecular Probes, Eugene, OR, USA) 1:200 in phosphate-buffered saline for 30 min. After thorough rinsing, the tissues were mounted on glass slides with CrystalMount (Biomeda, Foster City, CA, USA). Tissues were analyzed and photographed on a Leica DMRB epifluorescence microscope using ×40 oil and ×100 oil objectives and a standard fluorescein isothiocyanate (FITC) filter.

Confocal

Confocal microscopy was used to study the differences in stereocilia length between genotypes. Whole mounts of the midcochlear turn were stained with phalloidin as described, then analyzed by confocal microscopy with a Zeiss laser scanning microscope LSM-510. To obtain measurements of stereocilia, Z-series images of 0.5 μm were taken from the apical portion of the sensory epithelium, spanning the entire stereociliar length.

Genotyping

All F1 and F2 animals were genotyped by polymerase chain reaction (PCR). Genotyping for the $Myo15^{sh2}$ and $Whrn^{wi}$ mutations was as previously described (Probst et al. 1998; Holme et al. 2002). Additional primers were designed to distinguish between the $Whrn$ wild type (+/+) and the heterozygote animals, which are 5′-CCGGCATCCACGCCACTGTCCTC-3′ and 5′-AGCCGGCTCCCAGGTGACCCTGA-3′. These primers do not amplify the wild-type allele, but they do amplify the heterozygote allele as a ~2-kb band. PCR parameters are 92°C for 2 min; 4 cycles at 94°C for 5 s, 72°C for 2 min; 4 cycles at 94°C for 5 s, 70°C for 2 min; and 39 cycles at 94°C 5 s, 68°C for 2 min.

Statistical analysis of stereocilium length

For IHCs, stereocilium length was measured in 6–13 cells per animal, 3 animals per genotype. On each cell, 3–10 stereocilia in the outer (tallest) row were measured (±0.08 μm) to establish the range of stereocilium lengths for that cell. In 85% of cells, the observed range of lengths was less than 0.16 μm, which was less than one fifth of the smallest observed midpoint values. The midpoint values were then used to compute the mean stereocilium length for the animal. The animal means were analyzed by ANOVA to test whether stereocilium length differed among genotypes. Subsequently, genotype means were computed from animal means, order by magnitude, and t tests were performed on successively large differences between adjacent pairs to identify which genotypes differ in mean stereocilium length, with appropriate Bonferroni adjustment of the criterion for statistical significance to maintain a table-wide $\alpha=0.05$. Genotype means are reported with the standard error of the mean $(SD/\sqrt{N})$ because this quantity reflects the criterion used in the t test to determine whether the means are different.

For OHCs, 11–16 cells per animal were measured (±0.125 μm). In two third of the cells, the range was less than 0.125 μm, or about one fourth of the smallest midpoint values. Differences between genotypes were evaluated by the same procedures used for IHCs.

RESULTS

Hearing is unaffected by partial deficiency of $Myo15$ and $Whrn$

Mice carrying $Myo15^{sh2}$ and $Whrn^{wi}$ mutant alleles were crossed to generate double heterozygotes ($Myo15^{sh2/+}$, $Whrn^{wi/+}$). These progeny were intercrossed to generate double mutants, single mutants, and littermate controls. F1 double heterozygote animals appeared normal, did not circle, and showed a normal Preyer reflex. Double heterozygotes and single heterozygotes were subjected to ABR testing at 2 and 6 months of age. Testing was designed to determine if heterozygosity for both of these two recessive deafness genes is a risk factor for initial or age-related hearing loss. At 2 months of age, doubly heterozygous $Myo15^{sh2/+}$, $Whrn^{wi/+}$ mice had ABR thresholds indistinguishable to those of single heterozygote control mice (Fig. 1). At 6 months of age, $Myo15$, $Whrn$ double heterozygotes exhibited shifts in ABR thresholds of about 22 and 10 dB at 4 and 20 kHz frequencies, respectively. Age-related hearing loss in doubly heterozygous mice was not greater than in animals heterozygous for mutation in only one of the genes. Morphological analyses with SEM supported the physiological results of ABR studies that indicated no difference between double heterozygotes and normal mice.
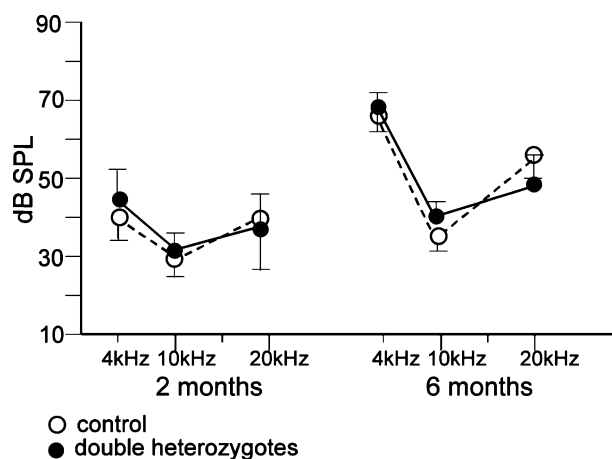

FIG. 1. $Myo15^{sh2}$ and $Whrn^{wi}$ double heterozygotes hear as well as single heterozygotes. ABR tests were performed on 2- and 6-month-old doubly heterozygous mice (filled circles) and single heterozygote controls (open circles) at frequencies of 4, 10, and 20 kHz. The sound pressure level thresholds, measured in decibels (dB), are indistinguishable between double heterozygotes and controls.

The inner ear morphology of double mutants reflects dominance of $Myo15^{sh2/sh2}$ phenotype over the $Whrn^{wi/wi}$ phenotype

Doubly homozygous pups ($Myo15^{sh2/sh2}$, $Whrn^{wi/wi}$) were viable and recovered at expected frequencies. Double mutants, however, circled and had no Preyer reflex. $Myo15$ and $Whrn$ are both expressed in the endocrine glands (Karolyi et al. 2003; Liang et al. 1999). No obvious associated defects were observed for the corresponding double mutants in the function of these organs.

The inner ears of double heterozygotes, single and double homozygotes littermates were examined by SEM and light microscopy at P21 (Fig. 2). SEM of the midturn of the cochlea revealed normal OHC morphology at the apical surface of double heterozygotes, and the presence of all defined cell types of the inner ear sensory epithelium in double mutant mice. OHC degeneration is apparent in $Myo15^{sh2/sh2}$ single mutant and in double mutant mice by P21 in the basal and midturns of the cochlea, whereas degeneration in $Whrn^{wi/wi}$ single mutants is restricted to the basal cochlear turn. Similar results were obtained in younger and older mice (P17 and P35 data not shown).

The $Myo15$ mutant cochlea showed extremely short stereocilia on both IHCs and OHCs and persistence of transient microvilli on the apical cell surface (Fig. 2a–d), as reported previously (Probst et al. 1998; Anderson et al. 2000). Some of the OHC stereocilia in $Myo15$ mutants are arranged in a rounded "U" shape instead of the normal "V" or "W" shape seen in the OHC stereocilia of the double heterozygotes and other normal mice. Many pathological landmarks seen by SEM analysis in the OHCs of $Myo15$ and $Whrn$ single mutants are also evident in the double mutants, such as excess microvilli, U-shaped organization of stereocilia, and shorter than normal stereocilia. However, the $Whrn$ mutant OHC stereocilia seemed intermediate in length to normal and $Myo15$ mutants. In addition, the length of
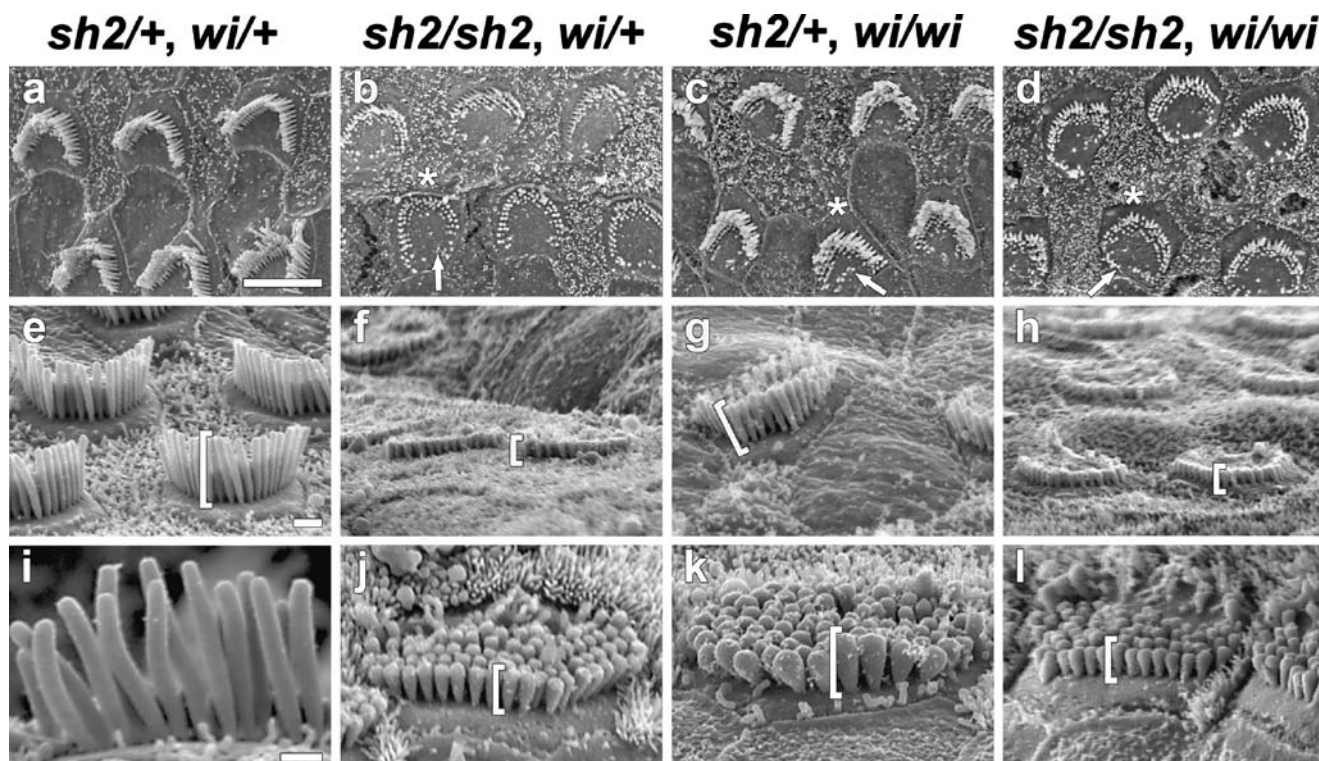

FIG. 2. Double mutants and *sh2* mutants have shorter stereocilia than *wi* mutants. SEM was used to visualize the apical surface of cochlear hair cells in the mid turn region in mice at age P21. OHC stereocilia were analyzed in cochlear whole mounts from double heterozygotes (**a**), both single mutants, $Myo15^{sh2/sh2}$ (**b**) and $Whrn^{wi/wi}$ (**c**), and $Myo15^{sh2/sh2}$, $Whrn^{wi/wi}$ double mutants (**d**). The stereocilia on OHCs of double heterozygotes are indistinguishable from those of wild-type mice. OHC stereocilia in the $Myo15$ mutant are extremely short with abnormally persistent microvilli (arrows). Some stereocilia are arranged in a U shape (asterisk) instead of the normal V shape. The OHC stereocilia of $Whrn$ mutants are shorter than normal, and there are persistent microvilli. Some cells have stereocilia arranged in an abnormal U shape. $Myo15$, $Whrn$ double mutants have an OHC stereocilia phenotype that looks like the $Myo15$ single mutant. Scale bars: **a–d** 5 μm, **e–h** 1 μm, **i–l** 1 μm. The length of the OHC stereocilia of double heterozygotes (**e**) is longer than single mutants of $Myo15$ (**f**) or $Myo15$, $Whrn$ double mutants (**h**), but subtly longer than $Whrn$ single mutants (**g**). Scale bars 1 μm. The lengths of the IHC stereocilia are obviously normal in double heterozygotes and obviously longer (**i**) than in single mutants for $Myo15$ (**j**) or $Whrn$ (**k**), or for double mutants (**l**), which were extremely short. Scale bars 1 μm.

stereocilia on IHC of wild-type mice appeared longer than stereocilia in *Myo15* or *Whrn* single mutants. To quantify this perception, SEM specimens were photographed at the midturn of the cochlea and stereocilia were measured (Fig. 2e–l). Specimens were oriented so that stereocilia were as close to parallel with the screen as possible and the entire vertical length of the stereocilia could be visualized. Significant differences among genotypes in mean stereocilium length were found for both IHCs (F=493.1, df=3.8, p<0.001) and OHCs (F=424.9, df=3.8, p<0.001). As Figure 3 shows, mean stereocilium length of IHCs was longest in the wild type (4.18±0.13, n=3) and much shorter in $Whrn^{wi/wi}$ (1.19±0.02, n=3) and $Myo15^{sh2/sh2}$ (0.88± 0.02, n=3). The mean of $Myo15^{sh2/sh2}$, $Whrn^{wi/wi}$ (0.85±0.06, n=3) was nearly identical to $Myo15^{sh2/sh2}$, and not significantly different from it (p=0.695). The

larger differences between $Myo15^{sh2/sh2}$ and $Whrn^{wi/wi}$ and between $Whrn^{wi/wi}$ and wild type are both significant (p<0.001). Mean stereocilium length of OHCs was also longest in the wild type (2.08±0.06, n=3) and shorter in $Myo15^{sh2/sh2}$ (0.51±0.02, n=3) than in $Whrn^{wi/wi}$ (1.36±0.02, n=3). The mean was slightly greater in $Myo15^{sh2/sh2}$, $Whrn^{wi/wi}$ (0.55±0.04, n=3) than in $Myo15^{sh2/sh2}$, but the difference was not significant (p=0.353). The larger differences between wild type and $Whrn^{wi/wi}$ (p=0.007) and between $Whrn^{wi/wi}$ and $Myo15^{sh2/sh2}$, $Whrn^{wi/wi}$ (p<0.001) were significant.

The stereocilia of animals of each genotype was also examined by confocal microscopy. Wild type and double heterozygotes have similar stereocilia length (Fig. 4a, b). The Z-series images confirm differences in stereocilia length between double heterozygotes, single mutants, and double mutants, consistent with quantitative analysis of SEM images.

The kinocilium normally disappears by P12 (Kikuchi and Hilding 1965; Kimura 1966). However, we observed persistence of kinocilia in IHCs at P16–P17 in both single mutants and double mutants, relative to double heterozygotes at the same ages (data not shown). All single mutant littermates exhibited persistent kinocilia in the majority of IHCs of the apical turn (70% of the IHCs), a substantial number of IHCs in the midturn (30% of the cells), and negligible (0.02%) IHCs in the basal turn of the cochlea. At P21, somewhat fewer persistent kinocilia were evident in IHC of mutants (Fig. 5). At this time kinocilia were noted in 60% of the IHC within the apical turn and 20% of IHC in the midturn. At P35 there was even less persistence of the kinocilia, and regression was apparently in progress in some of the remaining kinocilia (data not shown). This phenomenon was less obvious in OHC than IHC. At P16–P17, very few kinocilia were observed in the OHCs of the apical turn of the $Myo15^{sh2/sh2}$ and $Whrn^{wi/wi}$ single mutants or double mutants (0.02% of the cells) (data not shown).

The cytocaud, an actin rich pathological structure, appears in the hair cells of single and double mutants

Epifluorescence examination of phalloidin/FITC-stained whole mounts of the organ of Corti revealed normal organization of cytoskeletal actin in 21-day-old double heterozygote mice (Fig. 6a). The organization at the reticular lamina (the luminal surface of the auditory epithelium) and the cuticular plate of the IHC appeared normal. In *sh2/sh2* animals of a similar age, at a focal plane just beneath the reticular lamina, an abnormally thick actin bundle was evident (Fig. 6b). The bundle extended from each IHC toward the modiolar axis of the cochlea. This path-
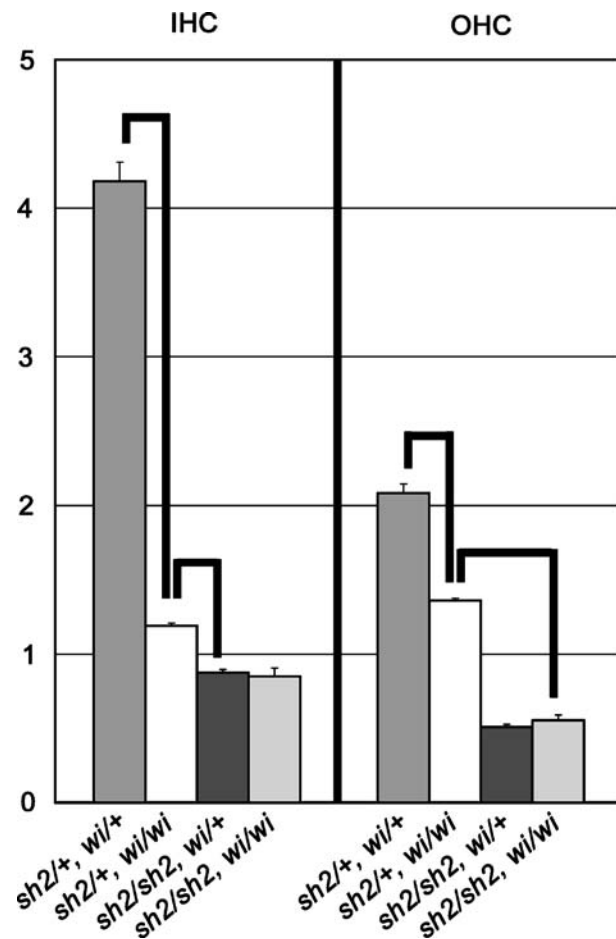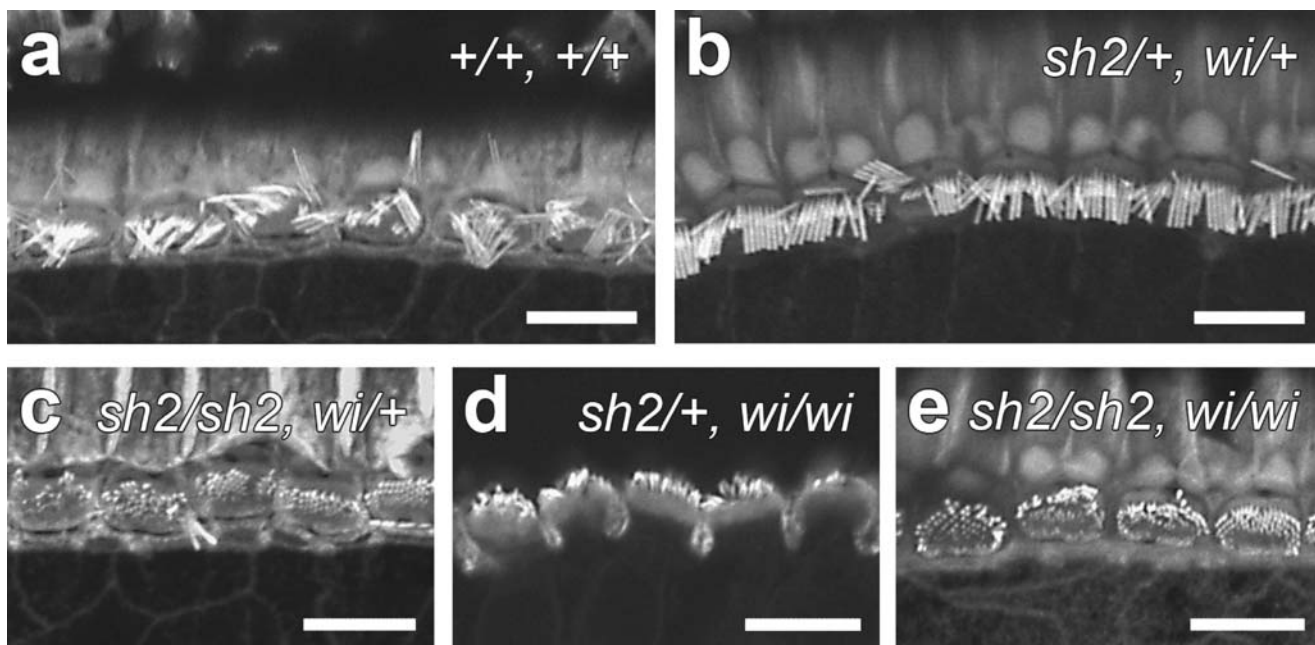

FIG. 3. Quantification of mean stereocilia length confirms differences in stereociliar length. The length of the stereocilia was measured in micrometers from SEM photographs of IHC and OHC for the four genotype classes. Means±SE of the mean of stereocilia length are shown for IHCs and OHCs of each genotype. Brackets indicate consecutive means shown to be significantly different by *t* test, after Bonferroni adjustment for multiple tests.

FIG. 4. Differences in stereocilia lengths are apparent by confocal microscopy of the actin cytoskeleton. Confocal images of phalloidin labeled organ of Corti show that stereocilia of inner hairs cells are long in wild types (**a**) and double heterozygotes (**b**) and distinctly shorter in mutants (**c**–**e**). Scale bars 10 μm.

ological structure has been previously described to contain cytoplasm and organelles, and has been termed a cytocaud (Beyer et al. 2000; Probst et al. 1998). Cytocauds were present at P10 in nearly every IHC of $Myo15^{sh2/sh2}$ mutants. Cytocauds have not previously been reported for $Whrn^{wi/wi}$ mutants. We observed approximately one cytocaud in every 25–30 IHC of $Whrn^{wi/wi}$ mutants examined at P10, which is significantly fewer than in $sh2$ mice (Fig. 6c). Cytocauds are also evident in the IHCs of double mutants, with a frequency similar to $Myo15^{sh2/sh2}$ mutants (Fig. 6d).

Analysis of the vestibular sensory epithelium (ampulla, saccula, and utricule) revealed that the actin core of the cytocaud is apparent in most vestibular hair cells of $Myo15^{sh2/sh2}$ and $Whrn^{wi/wi}$ single mutants and double mutants at birth (Fig. 7). There does not appear to be any difference in frequency of cytocauds in vestibular hair cells of single and double mutants, in contrast to the reduced frequency observed in $Whrn^{wi/wi}$ IHC in the organ of Corti.

DISCUSSION

MYOSIN XV contributes specifically to the differential elongation of the stereocilia by increasing the delivery of essential elements, like WHIRLIN and probably other cargoes, to the tips of the stereocilia. The fact that MYOSIN XV expression is maintained after the

elongation process is completed while WHIRLIN expression wanes suggests that MYOSIN XV might be playing a different role than WHIRLIN in the long-term maintenance of hair cell through its interaction with different proteins. This theory is supported by the presence of additional predicted interaction domains in the tail encoded by $Myo15$ and by the more severe
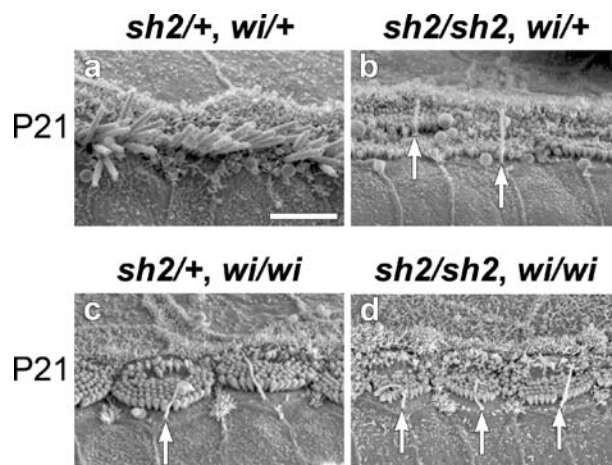

FIG. 5. Kinocilium regression is impaired in Myo15 and Whrn mutants. SEM analysis of IHCs in the apical turn of cochlea revealed abnormalities in apical surface morphology of mutants relative to controls. Cochlear whole mounts from P21 animals revealed abnormal persistence of kinocilia (arrows) in IHCs of single mutants of $Myo15$ (**b**) and $Whrn$ (**c**), and $Myo15$, $Whrn$ double mutants (**d**), relative to double heterozygotes in which the kinocilia have regressed (**a**). Scale bars 5 μm.
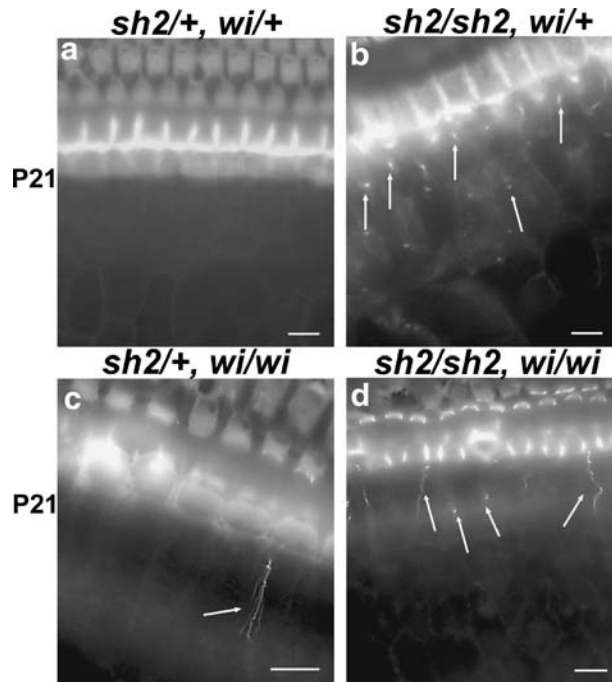
FIG. 6. Cytocaud pathology is more severe and appears earlier in mutants with the shortest stereocilia. Epifluorescence images of phalloidin-labeled whole mounts of the organ of Corti of P21 control mice (**a**), single mutants of *Myo15* (**b**) and *Whrn* (**c**), and *Myo15*, *Whrn* double mutants (**d**) reveal cytoskeletal actin organization abnormalities in mutants relative to controls (arrows). At the focal plane near the base of the cuticular plate, an abnormal actin-positive stain, known as a cytocaud, is detected in the IHCs of single mutants of *Myo15* (**b**) and *Whrn* (**c**), and *Myo15*, *Whrn* double mutants (**d**).

phenotype of shaker 2 (*sh2*) sensory hair cells compared to those of whirler (*wi*) mutants. Both IHC and OHC stereocilia in *Myo15* mutants are shorter than those in *Whrn* mutants, and the frequency of cytocaud pathology is higher in the IHC of *Myo15* mutants than in *Whrn* mutants. The $Myo15^{sh2/sh2}$, $Whrn^{wi/wi}$ double mutants have a phenotype similar to that of *Myo15* single mutant. Taking all of the pathologies together, *Whrn* mutant hair cells are less affected than those of *Myo15* mutants and double mutants. This suggests that MYOSIN XV has a greater effect on IHC and OHC stereocilia elongation than WHIRLIN.

MYOSIN XV is necessary for transportation of WHIRLIN to the tips of the stereocilia, and the two proteins have been shown to interact physically (Belyantseva et al. 2005). Adult double heterozygotes, $Myo15^{sh2/+}$, $Whrn^{wi/+}$, exhibited normal ABR thresholds and normal vestibular function. There was no obvious difference in the susceptibility to age-related hearing loss in double heterozygotes compared to the single heterozygotes. Thus, 50% of the normal level of MYOSIN XV and WHIRLIN is sufficient for

normal hair bundle formation, maintenance, and function. This contrasts with other genes encoding proteins that interact in which decreased dosage of both genes causes significant abnormalities (Zheng et al. 2005; Goldberg and Molday 1996). For example, *Cdh23* and *Pcdh15* double heterozygotes are deaf by 5 months and have abnormal stereocilia, whereas single heterozygotes are unaffected (Zheng et al. 2005).

The kinocilia and microvilli in hair cells of the organ of Corti persist longer than normal in the *Myo15* and *Whrn* single and double mutants. The persistence of these structures may result from roles for the *Myo15* and *Whrn* genes in general cellular maturation. Consistent with this idea, the kinocilia persist more in the IHCs than the OHCs of the mutants, and the apical pole of the IHCs normally reaches adult-like morphology more slowly than that of the OHCs (Anniko 1983). Differences in kinocilium development might contribute to the rounded U-shaped pattern of stereocilia in the mutants, instead of the normal V or W shape. In contrast to MYOSIN XV, MYOSIN VII, which is known to interact with WHIRLIN, is expressed in the kinocilium, but additional studies will be necessary to determine whether WHIRLIN isoforms localize there as well
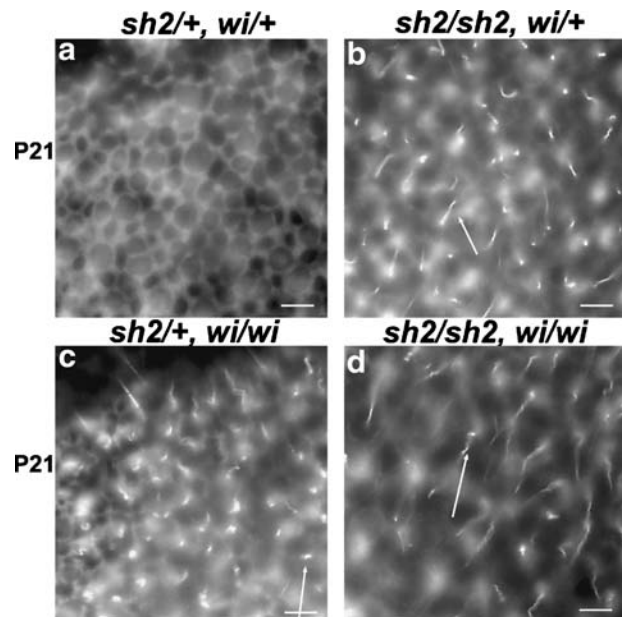

FIG. 7. Vestibular hair cells of *Myo15* and *Whrn* single and double mutants exhibit cytocaud pathology at birth. Epifluorescence images of whole mounts of the ampulla of P21 control mice (**a**), single mutants of *Myo15* (**b**) and *Whrn* (**c**), and *Myo15*, *Whrn* double mutants (**d**) labeled with phalloidin. At the focal plane just beneath the cuticular plate, an actin-positive stain is detected in most of the vestibular hair cells of *Myo15* (**b**) and *Whrn* (**c**) single mutants and *Myo15*, *Whrn* (**d**) double mutants (arrows), but not in control mice.

(Delprat et al. 2005; Wolfrum et al. 1998). This potential interaction raises the possibility of cross talk between the different motors and cargoes during the development of the inner ear hair cells.

Some, but not all, deaf animal models exhibit pathological actin-rich cytoplasmic extensions from the basal surface of IHC and/or vestibular hair cells, known as cytocauds (Sobin et al. 1982; Sobin and Flock 1983; Beyer et al. 2000; Sobin and Flock 1981; Ernstson et al. 1969). We describe for the first time the presence of cytocauds in *Whrn* mutants. The frequency of these cytocauds, however, is lower than that seen in *Myo15* mutants or double mutants. The basis for the pathology is unknown, but it is intriguing that cytocaud-containing cells are predominantly innervated by afferent neurons and have higher rates of actin turnover and higher expression level of MYOSIN XV than the unaffected OHCs (Schneider et al. 2002, Rzadzinska et al. 2004; Sekerková et al. 2006). By analogy to actin assembly in yeast, the cytocaud may result from inability to nucleate and orient actin monomers in *Whrn* and *Myo15* mutants (Evangelista et al. 2003; Kamasaki et al. 2005; Schneider et al. 2002).

We did not detect any genetic interaction between the *Myo15* and *Whrn* genes in double heterozygotes and double mutants. In an extreme case, overlapping functions could result in a much more severe phenotype in the double mutant, potentially causing premature loss of hair cells or related structures, or even effects in other tissues. Despite coexpression of *Whrn* and *Myo15* in endocrine tissues, no endocrine phenotypes were manifested. Intriguingly, there is no evidence for novel phenotypes arising from double heterozygotes or double homozygotes of *Myo15* mutants and mutants in other motor myosin genes, including $Myo7a^{4626SB/4626SB}$ or $Myo6^{sv/sv}$, or in the unrelated gene, $Grxcr1^{pi/pi}$ (Karolyi et al. 2003). In each of those cases the stereocilia of double mutants exhibited additivity of the single mutant phenotype. The reason why so many different molecular motors are required is not clear, nor is the full panoply of proteins that interact with each motor well defined. The roles of MYOSIN XV and WHIRLIN in development and regulation of normal cytoskeletal morphology may be advanced through the identification of additional protein partners.

ACKNOWLEDGMENTS

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