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Myo15 function is distinct from *Myo6*, *Myo7a* and *pirouette* genes in development of cochlear stereocilia

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The unconventional myosin genes *Myo15*, *Myo6* and *Myo7a* are essential for hearing in both humans and mice. Despite the expression of each gene in multiple organs, mutations result in identifiable phenotypes only in auditory or ocular sensory organs. The *pirouette* (*pi*) mouse also exhibits deafness and an inner ear pathology resembling that of *Myo15* mutant mice and thus may be functionally related to *Myo15*. In order to investigate possible interactions between *Myo15* and *Myo6*, *Myo7a*, and the gene affected in *pirouette*, we crossed *Myo15*$^{sh2/sh2}$ mice to the three other mutant mouse strains. Hearing in doubly heterozygous mice was similar to age-matched singly heterozygous animals, indicating that partial deficiency for both *Myo15* and one of these other deafness genes does not reduce hearing. Viable double mutants were obtained from each cross, indicating that potential overlapping functions between these genes in other organs are not essential for viability. All critical cell types of the cochlear sensory epithelium were present in double mutant mice and cochlear stereocilia exhibited a superimposition of single mutant phenotypes. These data suggest that the function of *Myo15* is distinct from that of *Myo6*, *Myo7a* or *pi* in development and/or maintenance of stereocilia.

INTRODUCTION

Deafness is the most common form of sensory impairment in humans. Approximately 1 in 1000 individuals are born with hearing loss and greater than 50% of this group is of genetic etiology (1). As many as 1 in 300 individuals become deaf or hard of hearing during their lifetime with ~33% due to genetic causes (2,3). The structure and development of the inner ear and the pathology leading to hearing impairment are very similar between mice and humans (4). Thus, the study of mouse genes involved in hearing is valuable for understanding the molecular basis of hearing transduction, and many human forms of deafness have been identified using mouse models. Several forms of hearing loss result from mutations in cytoskeletal proteins including myosins (5).

Myosins are mechanoenzymes defined by their conserved NH_2-terminal head or motor domains which contain actin- and adenosine triphosphate (ATP)-binding sites followed by a variable number of light-chain binding (IQ) motifs in the neck or flexible region, and a variable tail domain. Upon interaction with actin, myosins convert energy from ATP hydrolysis to mechanical force as they pull against or move along actin filaments (6). Myosins are presumed to acquire their specialized functions via their tails, which are tethered to different macromolecular structures that move relative to actin filaments (7). The tails of myosin XV and myosin VIIa share several regions of amino acid identity (8).

Myo15 encodes an unconventional myosin (myosin XV) that is mutated in the shaker-2 (*sh2*) and shaker-2J (*sh2J*) mice, and *DFNB3*, a form of non-syndromic hearing loss in humans

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(8–12). *Myo15* mutant mice are congenitally deaf, and have vestibular defects associated with circling behavior (12). In the inner ear, myosin XV is localized to inner hair cells (IHCs) and outer hair cells (OHCs) of the organ of Corti, at the level of the actin-rich cuticular plate, which anchors the bases of the stereocilia, as well as in the vestibular system of the inner ear (8,11,13). Auditory hair cells of *Myo15* mutant mice have very short stereocilia, although the stereocilia are arrayed in a nearly normal pattern (9), suggesting that *Myo15* may be critical for normal lengthening of the stereocilia.

Myo6 is the unconventional myosin gene affected in Snell's waltzer (*sv*) mice and in two forms of human non-syndromic deafness, *DFNA22* and *DFNB37* (14–16). *Myo6* mutant mice exhibit the typical circling, head tossing, deafness and hyperactivity seen in mice with inner ear defects (17). In the inner ear, myosin VI is localized in the cytoplasm of inner and outer hair cells and within the cuticular plate at the base of the stereocilia, but has not been detected in the stereocilia (14,18). *Myo6* mutations result in fusion of the stereocilia into a giant structure, suggesting that myosin VI acts as an anchor for the stereociliar membrane at the apex of the hair cell (19).

Mutations in the unconventional myosin gene *Myo7a* are responsible for inner ear and vestibular defects in shaker-1 (*sh1*) mice, deafness and blindness in Usher syndrome type 1B (*USH1B*), and two forms of non-syndromic deafness, *DFNB2* and *DFNA11*, in humans (20–25). Within the inner ear, *Myo7a* expression is restricted to IHCs and OHCs, and myosin VIIa protein is found in the apical stereocilia, in the cuticular plate and in the cytoplasm of the hair cells (26). Seven alleles of the *Myo7a* gene are known in mice (27–29). We used the $Myo7a^{4626SB}$ allele, which expresses only 1/250th of wild-type protein levels of myosin VIIa (30). The $Myo7a^{4626SB}$ lesion resides in the head region of the myosin motor domain, and leads to a premature stop mutation that is a presumptive null allele (31). Severe *Myo7a* mutations result in a disorganization of the normal pattern of stereocilia on the surface of the cell. Milder *Myo7a* mutations do not cause visible changes in hair cell morphology, but affected animals are still deaf, suggesting that myosin VIIa performs other functions in the hair cells in addition to ensuring proper organization of the stereocilia (5,32).

The *pirouette (pi)* mutation is another mouse deafness mutant that exhibits profound hearing loss, circling behavior, and is inherited as a recessive trait (33). The responsible gene has not yet been identified, and expression data is not available. This mutant is potentially a model of human *DFNB25*, based on synteny homology between mouse chromosome 5 and human chromosome 4 (34). Stereocilia are very thin in *pi*/*pi* mice (27) and inner hair cells develop an actin-rich pathological structure similar to that found in *Myo15* mutants (35).

All three unconventional myosin genes (*Myo15*, *Myo6* and *Myo7a*) become transcriptionally active at the same time in the developing mouse ear (13,36). Additionally, all three of the myosin genes are expressed in a variety of organs outside the inner ear. Myosin VI and myosin VIIa are expressed in blood, brain, kidney, liver, testis, retina, pituitary and inner ear, while myosin XV expression is more limited, with high levels of expression only in the pituitary and inner ear (8,11,13,14,18,20,21,26,30,37–39). Therefore, these genes may also play important roles in organs outside the inner ear. Retinitis pigmentosa has been reported in humans carrying a subset of *Myo7a* mutations (21). Sensory function, particularly hearing, exhibits an exquisite sensitivity to loss of unconventional myosins.

Individuals with mutations in multiple genes may have more severe phenotypes than expected based on the phenotypes of each mutation alone. This can result from functional overlap of the two genes or from a physical interaction between the two proteins. For example, mice with homozygous null mutations in either *Myf5* or *MyoD* have apparently normal skeletal muscle tissue, but doubly homozygous mutant mice completely lack skeletal muscle. Thus, at least one functional allele of *Myf5* or *MyoD* is necessary for skeletal muscle development (40).

Mutations that disrupt the association of interacting proteins can produce a severe phenotype, even in double heterozygotes. Individuals who are heterozygous for mutations in both the photoreceptor-specific *ROM1* and peripherin/*RDS* genes develop retinitis pigmentosa, while individuals heterozygous for a mutation in only one of the two genes do not (41). This phenomenon is known as digenic inheritance or non-allelic non-complementation. This illustration is particularly relevant, as *ROM1* and *RDS*, like the myosin genes, code for structural proteins and interact to form a complex at the photoreceptor cell membrane that is crucial for normal vision.

Given the similarities in function and pathology among *Myo15*, *Myo6*, *Myo7a* and *pi*, we sought to determine if myosin XV has functional overlap with any of the other three proteins using a classical genetic approach. To do this we crossed $Myo15^{sh2/sh2}$ mice with $Myo6^{sv/sv}$, $Myo7a^{4626SB/4626SB}$ or *pi*/*pi* mice and analyzed their first- and second-generation offspring. Multiple deafness loci may contribute to age-related hearing loss and partial deficiency for *Myo15* and *Myo6*, *Myo7a* or *pi* may be associated with increased risk for age-related hearing loss. Analysis of double heterozygotes and double mutants allowed us to determine whether loss of *Myo15* and one of these other genes produces a more severe phenotype or a superimposition of pathologies, and whether loss of *Myo15* and one of these other genes affects viability or function in other *Myo15*-expressing tissues such as the pituitary. Our data suggest that *Myo15* has a role that is distinct from that of *Myo6*, *Myo7a* or *pi* in development and/or maintenance of sensory hair cells and has no essential overlap with these three genes in other organs.

RESULTS

Hearing is unaffected by partial deficiency of *Myo15* and *Myo6*, *Myo7a* or *pi*

$Myo15^{sh2/sh2}$ mice were crossed with mice homozygous for severe alleles of both Snell's waltzer ($Myo6^{sv/sv}$) and shaker-1 ($Myo7a^{4626SB/4626SB}$) and for *pirouette (pi)*. F_1 doubly heterozygous animals (+/−, +/−) from all three crosses appeared completely normal, did not circle and showed a normal Preyer reflex to noise. Double heterozygotes and single heterozygotes for each mutation from each cross were subjected to auditory brainstem response (ABR) testing at 2, 4 and 6 months of age. Testing was designed to determine if heterozygosity for two recessive deafness genes was a risk factor for age-related hearing loss.

In each of the crosses, risk for age-related hearing loss in doubly heterozygous animals was not significantly greater than animals heterozygous for only one of the genes. *Myo15*, *Myo7a* doubly heterozygous animals showed no age-related hearing loss at any frequency tested, demonstrating that partial deficiency of both these myosin genes poses no hearing loss risk (Fig. 1B). *Myo15*, *Myo6* doubly heterozygous animals showed age-related hearing loss at 10 and 20 kHz frequencies (Fig. 1A), and *Myo15*, *pi* doubly heterozygous animals showed age-related hearing loss at 20 kHz (Fig. 1C). However, differences between the three crosses may be due to modifying genes in the genetic backgrounds because this observed hearing loss was also seen in animals heterozygous for only one of the genes (Fig. 1A–C). While potential genetic background effects make it difficult to detect subtle effects on hearing, double heterozygosity for *Myo15* and *Myo6* or *pi* was not a strong risk factor for age-related hearing loss, and double heterozygosity for *Myo15* and *Myo7a* posed no risk at all.

Double mutants of each cross are viable

In all three crosses, double mutant homozygotes (−/−, −/−) were obtained at weaning among the F_2 generation (Tables 1–3), indicating that double mutants were viable. All double mutants circled and had no Preyer reflex. *Myo15*, *Myo7a* and *Myo15*, *pi* double mutants displayed no apparent phenotype besides inner ear pathology. *Myo15*, *Myo6* double mutants had a slight reduction in body size. This was also observed in *Myo6* mutant animals and was judged to be an unreported *Myo6* mutant phenotype, as opposed to an interactive effect between the *Myo15* and *Myo6* mutations.

The *Myo15*, *pi* double mutants, however, were significantly under-represented in the F_2 generation. The chances of such a small number of double mutants (5/238) occurring by chance is only 1% ($P = 0.01$; Table 3). Significant under-representation was observed in the first cohort of 100 F_2 animals bred from the *Myo15*, *pi* cross, and confirmed in a second cohort of over 100 animals. No other genotypes were under-represented in this cross. Some genotypes were under- or over-represented in the crosses of *Myo15* with *Myo6* and *Myo7a* (Tables 1 and 2), but none were significant at the 1% level. The under-representation of double mutant *Myo15*, *pi* mice may be attributable to a rare chance occurrence or may indicate some degree of embryonic or neonatal lethality that is as yet unexplained.

Pituitaries and other organs from all three double mutants are normal

The only organ with high levels of *Myo15* expression besides the sensory epithelia of the inner ear is the pituitary gland (8). Expression of *Myo6* and *Myo7a* was detected and *Myo15* expression was confirmed in two different pituitary preparations by reverse transcriptase-polymerase chain reaction (RT–PCR; Fig. 2). Thus, there is the possibility of a functional overlap of *Myo15* with *Myo6* or *Myo7a* in the pituitary. Pituitary abnormalities (i.e. a corticotrope deficiency) might explain the under-representation of *Myo15*, *pi* double mutants.

Analysis of pituitaries of all three double mutants by immunocytochemistry revealed no anterior pituitary cell deficiencies and there was no evidence of cellular hypertrophy or hyperplasia to suggest diminished capacity for hormone production (data not shown). Unstressed corticosterone levels assayed by radioimmunoassay (RIA) and adrenal morphology also both appeared normal for all double mutants (data not shown).

Because of the wide expression patterns of *Myo6* and *Myo7a* and the expression of *Myo15* in neuroendocrine cells of several organs, double mutants were autopsied at approximately one month of age to search for subtle phenotypes. In all cases, the kidneys, liver, spleen, intestines, lungs, heart, eyes, brain and tongue appeared grossly normal.

Table 1. F_2 distribution of genotypes in 189 progeny of *Myo15*, *Myo6* cross

Genotype		Expected ratio	Number observed	Number expected	Chi-square *P*-value
Myo15	*Myo6*				
+/+	+/+	1/16	7	12	0.16
+/+	+/−	2/16	23	24	0.90
+/+	−/−	1/16	7	12	0.16
+/−	+/+	2/16	35	24	0.02*
+/−	+/−	4/16	45	47	0.74
+/−	−/−	2/16	25	24	0.78
−/−	+/+	1/16	15	12	0.35
−/−	+/−	2/16	24	24	0.94
−/−	−/−	1/16	8	12	0.27

*Significant difference.

Table 2. F_2 distribution of genotypes in 182 progeny of *Myo15*, *Myo7a* cross

Genotype		Expected ratio	Number observed	Number expected	Chi-square *P*-value
Myo15	*Myo7a*				
+/+	+/+	1/16	15	11	0.28
+/+	+/−	2/16	22	23	0.88
+/+	−/−	1/16	11	11	0.91
+/−	+/+	2/16	33	23	0.03*
+/−	+/−	4/16	49	45	0.60
+/−	−/−	2/16	12	23	0.02*
−/−	+/+	1/16	8	11	0.32
−/−	+/−	2/16	19	23	0.43
−/−	−/−	1/16	13	11	0.63

*Significant difference.

Table 3. F_2 distribution of genotypes in 238 progeny of *Myo15*, *pi* cross

Genotype		Expected ratio	Number observed	Number expected	Chi-square *P*-value
Myo15	*pi*				
+/+	+/+	1/16	17	15	0.58
+/+	+/−	2/16	36	30	0.25
+/+	−/−	1/16	16	15	0.77
+/−	+/+	2/16	30	30	0.96
+/−	+/−	4/16	59	59	0.95
+/−	−/−	2/16	36	30	0.25
−/−	+/+	1/16	12	15	0.46
−/−	+/−	2/16	27	30	0.61
−/−	−/−	1/16	5	15	0.01*

*Significant difference.

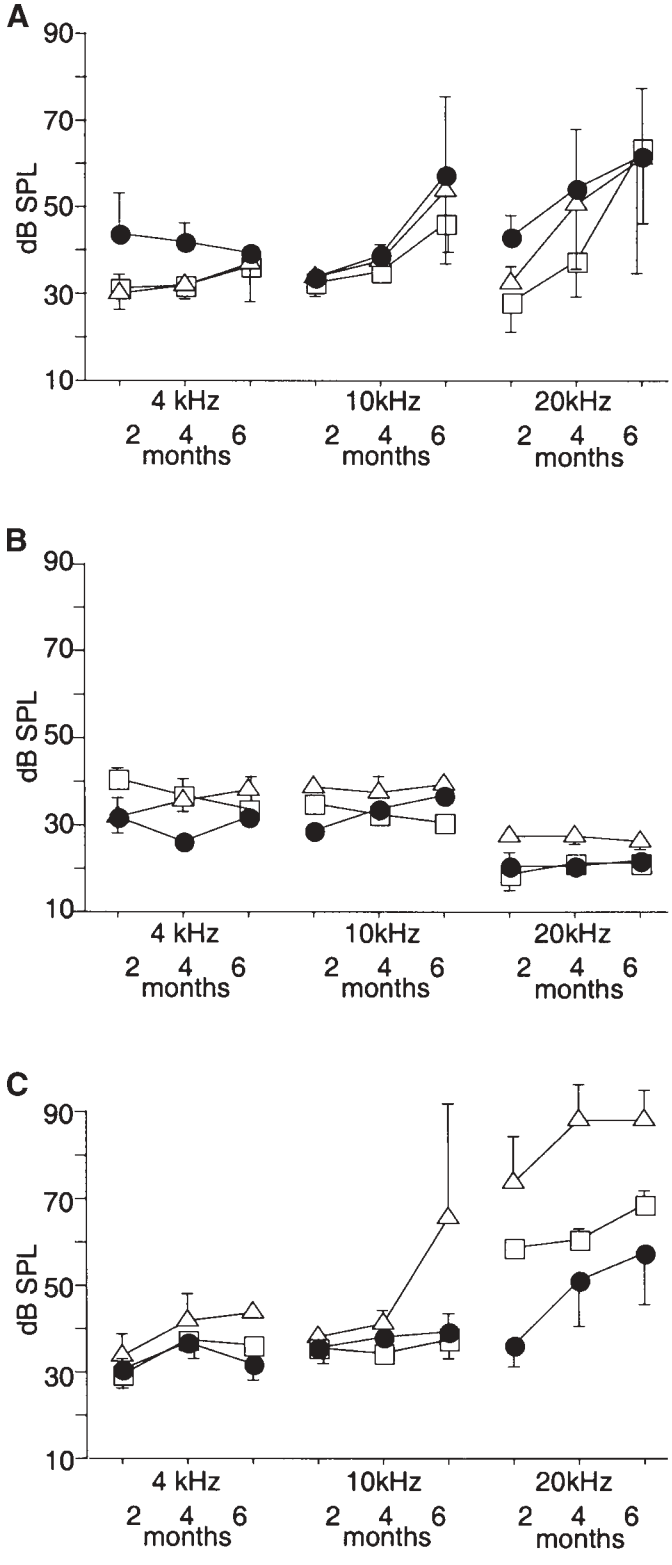

Figure 1. ABR results for heterozygous mice from all three crosses. (**A**) *Myo15*, *Myo6* age-related hearing loss in doubly heterozygous and singly heterozygous animals at frequencies of 4, 10 and 20 kHz. (**B**) *Myo15*, *Myo7a* age-related hearing loss in doubly heterozygous and singly heterozygous animals. (**C**) *Myo15*, *pi* age-related hearing loss in doubly heterozygous and singly heterozygous animals. Symbols: solid circles, double heterozygotes; open squares, *Myo15* single heterozygotes; open triangles; *Myo6* or *Myo7a* or *pi* single heterozygotes.

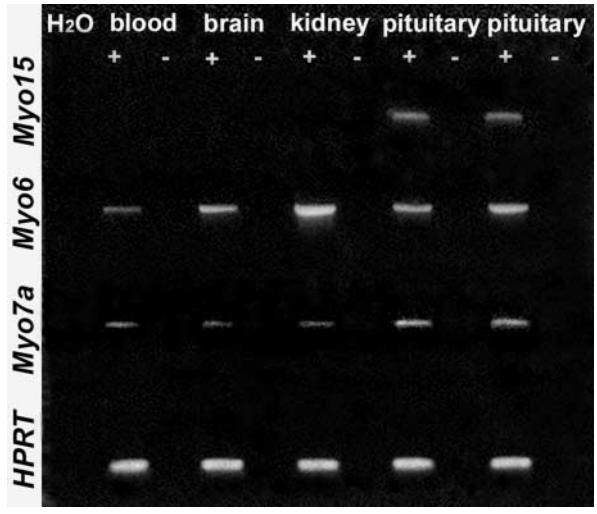

Figure 2. RT–PCR shows *Myo15*, *Myo6* and *Myo7a* in multiple tissues. *Myo15*, *Myo6*, and *Myo7a* are all expressed in pituitary. *Myo6* and *Myo7a* expression is confirmed also in blood, brain and kidney. Two separate pituitary cDNA preparations were tested. *HPRT* is a control for RNA quality and amount and for cDNA quality. (+) With reverse transcriptase; (−) without reverse transcriptase.

Superimposition of single mutant phenotypes in the inner ears of double mutants

Inner ears of double mutant mice were examined by scanning electron microscopy (SEM) at 3–4 weeks of age. Single mutant and double heterozygote controls were similarly prepared. In all double mutants, SEM of the organ of Corti exhibited an apparent superimposition of the single mutant phenotypes (Figs 3–6). *Myo15* mutants had extremely short stereocilia, and several stereocilia on each hair cell were located outside of the typical bundle location (9) (Fig. 3). *Myo6* mutants typically had one or a few giant stereocilia extending from the surface of the organ of Corti, and the stereocilia often had bulbous regions at or near their tips (19). The *Myo15*, *Myo6* double mutant had features of both the *Myo15* and the *Myo6* mutants, with a few short stereocilia protruding from each cell in addition to the giant stereocilia. The shorter stereocilia tended to be longer than the extremely short ones seen in the *Myo15* mutant, and shorter than the *Myo6* mutant, with fewer blebs at the tips (Fig. 4). The severe $Myo7a^{4626SB}$ allele produced disorganized stereocilia. The stereocilia formed rows with graded heights, but the orientation of these rows was erratic and were often oriented in opposite directions on a single hair cell (5,32). *Myo15*, *Myo7a* double mutants exhibited both phenotypes, short and disorganized stereocilia (Fig. 5). Similarly, *pi*/*pi* mutants had thinner stereocilia than WT. These stereocilia were slightly shorter or longer than wild-type (42). The *Myo15*, *pi* double mutant had uniformly short and thin stereocilia (Fig. 6).

DISCUSSION

In this study, we demonstrate that the function of *Myo15* is distinct from that of *Myo6*, *Myo7a* or *pirouette* in the mouse inner ear. Doubly heterozygous mice from all three crosses exhibited normal startle responses to sound and normal

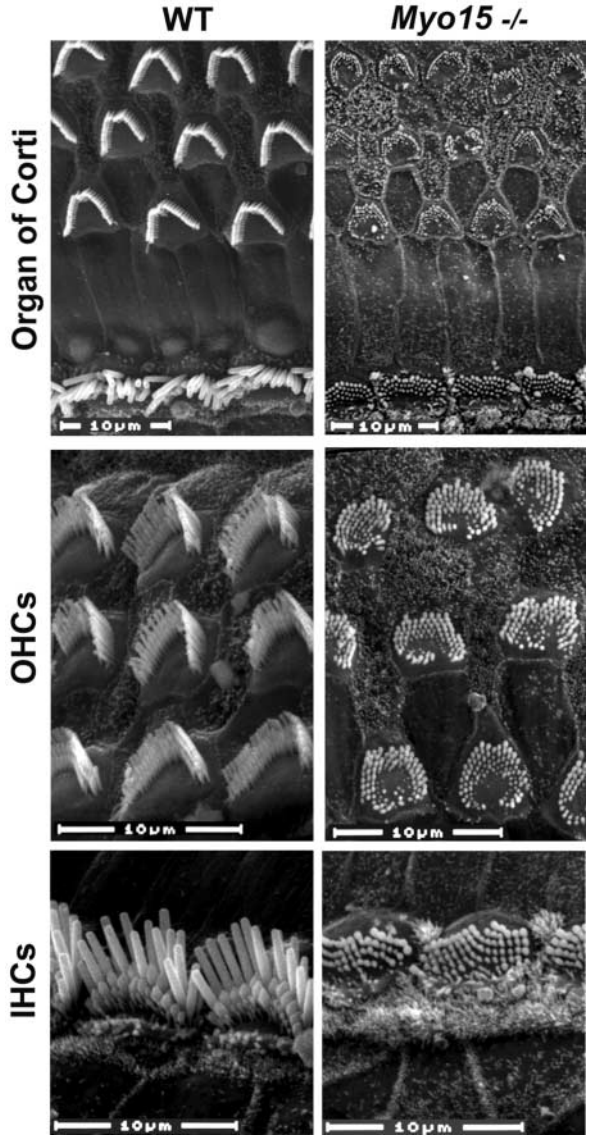

Figure 3. SEM analysis of cochlear hair cells in WT and *Myo15* mutants. Stereocilia in the *Myo15* mutant cochlea are extremely short and some are located away from the typical bundle site.

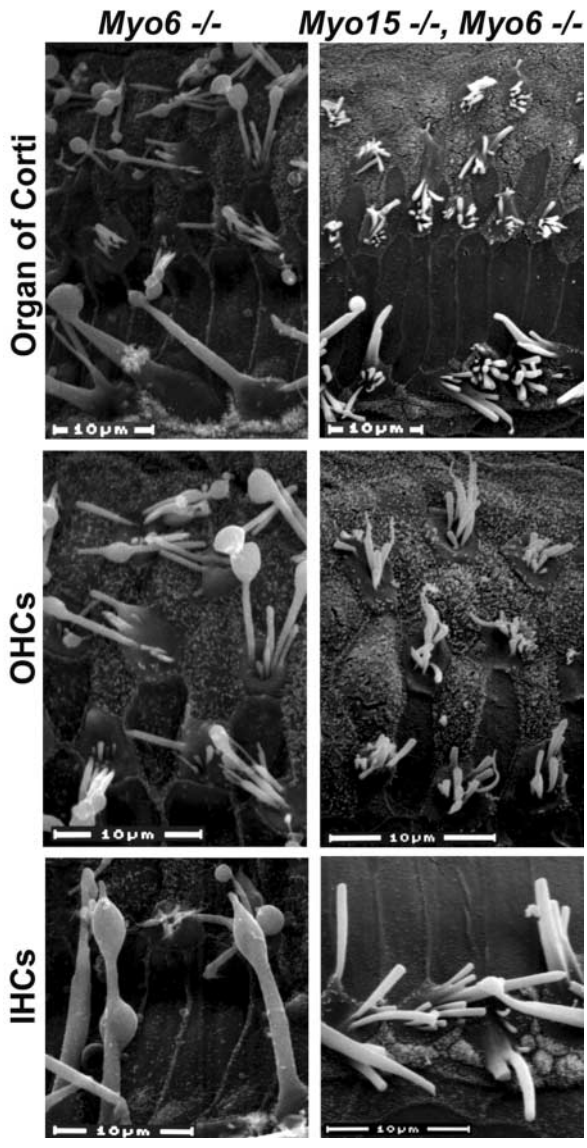

Figure 4. SEM analysis of cochlear hair cells in *Myo6* mutants and *Myo15*, *Myo6* double mutants. Stereocilia in the *Myo6* mutant cochlea coalesce and fuse to form giant stereocilia. In the *Myo15*, *Myo6* double mutant, giant stereocilia are seen. Unfused stereocilia appear shorter than the *Myo6* mutant or the WT animals, but not as short as the *Myo15* mutants.

vestibular function. There was no increased risk for age-related hearing loss in *Myo15*, *Myo7a* double heterozygotes, and no evidence of reduced hearing in double heterozygotes from the *Myo15*, *Myo6* or *Myo15*, *pi* crosses, although some age-related hearing loss was detected. This hearing loss is probably due to different modifying genes in the genetic backgrounds of these crosses as hearing loss was also exhibited by animals heterozygous for single mutations in those cross. Genetic backgrounds of the *Myo15* and *Myo7a* stocks are undefined, the genetic background of *Myo6* is primarily C57BL6/J and the genetic background of the *pi* strain is C57BL6/J. Thus, considerable numbers of unidentified modifying genes may be segregating in all of these crosses. Although early studies reported age-related hearing loss in mice heterozygous for both waltzer (*v*) and a mild allele of shaker-1 ($Myo7a^{sh1}$) (27,43), a recent thorough study showed no correlation between the loss of Preyer reflex response and double heterozygosity for waltzer and the severe allele of shaker-1 ($Myo7a^{4626SB}$) (44). A genetic background effect, rather than genetic interaction between *v* and *Myo7a* was probably responsible for the initial observations.

The double mutants of *Myo15* and *Myo6*, *Myo7a* or *pi* revealed no overt phenotype other than profound deafness and vestibular dysfunction. *Myo15* has been hypothesized to play a role in hormone secretion based on its expression in the endocrine cells of many organs (37). We detected expression of all three myosins, *Myo15*, *Myo6* and *Myo7a*, in the mouse pituitary. However, examination of pituitaries of the surviving double mutants revealed no obvious pituitary deficiency indicating that the role of these myosins in the pituitary is

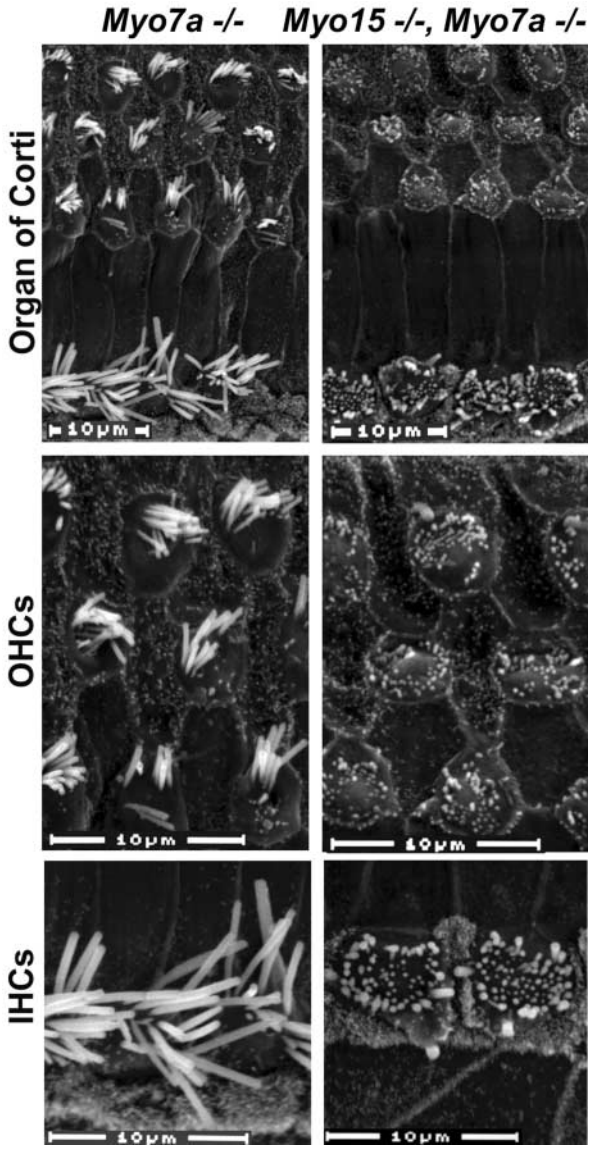

Figure 5. SEM analysis of cochlear hair cells in *Myo7a* mutants and *Myo15*, *Myo7a* double mutants. Stereocilia in the *Myo7a* mutant cochlea are of normal length, but are haphazardly scattered over the surface of the cell. In the *Myo15*, *Myo7a* double mutant, stereocilia are shortened and disorganized.

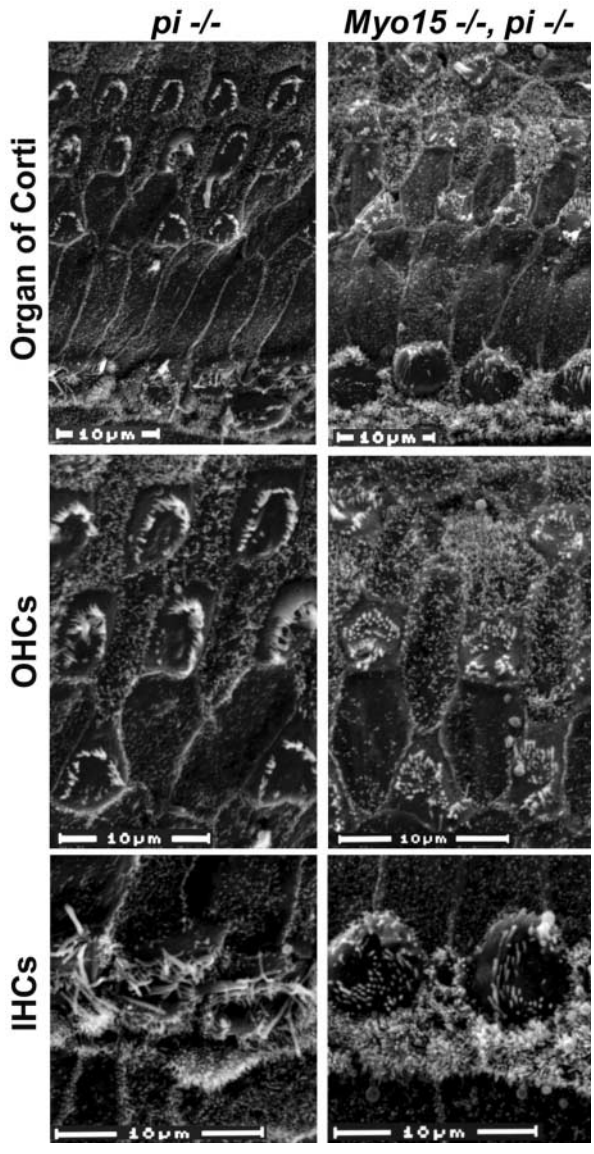

Figure 6. SEM analysis of cochlear hair cells in *pi* mutants and in Myo15, *pi* double mutants. The *pirouette* mutants exhibit thinned stereocilia as compared to WT animals. In the *Myo15*, *pi* double mutant, stereocilia are thin and short.

non-essential or is compensated for by other myosins. Other non-sensory organs examined in double mutants appeared grossly normal, again indicating compensation by other genes.

We observed fewer double mutants from the *Myo15*, *pi* cross than were statistically expected ($P = 0.01$), and found no evidence for pituitary dysfunction as a cause. It is possible that some of the *Myo15*, *pi* double mutants are dying of pituitary dysfunction at birth or in the early neonatal period and that could account for the statistical under-representation of those animals in the F_2 generation. This issue can be explored further after the *pi* gene is identified and its expression pattern is described.

The morphology of cochlear hair cell stereocilia in doubly homozygous mutant animals suggests a superimposition of the phenotypes generated by each of the single homozygotes. Overlapping functions would be expected to generate a more severe phenotype, potentially causing premature loss of hair cells or related structures. Our studies indicate that myosin XV is most likely performing a distinct function in the ear that does not obviously overlap with the functions of myosin VI, myosin VIIa or *pirouette.* The superimposition of phenotypes in the double mutants indicates that *Myo15* and *Myo6*, *Myo7a* and *pi* have unique and specific functions in hair cells. This is surprising given the high degree of homology between the tail regions of myosin 15 and myosin 7a (8).

Based upon the present study, and upon the individual differences in stereociliar phenotypes of each of the single mutants, each of these genes appears to play a distinct role in cochlear stereociliar maturation. Myosin XV is essential for

normal actin organization within the IHCs of the cochlea, and may function similarly in stereocilia (9,13,45). Myosin VI is thought to play a role in anchoring stereocilia to the cuticular plate. In the absence of functional myosin VI, the stereocilia fuse together during development, leading to the production of giant stereocilia (19). By connecting stereociliary membrane to its actin core, myosin VIIa may contribute to anchoring and causing tension in membrane-bound elements such as transducer channels, tip links and lateral links (18,32,46–48).

These genes may also have different developmental roles. The stereocilia begin as rudimentary protrusions that stud the apical surface of the hair cell. Successive ranks of processes then begin to lengthen at increasing intervals. As longitudinal growth progresses, individual stereocilia also increase in girth by circumferential addition of actin filaments (49). *Myo7a* may be important for the original organization of stereocilia on the cell's apical surface, as the stereocilia are disorganized in the *Myo7a* null mutant. The *pi* mutation results in thin stereocilia suggesting *pi* may be important for increasing girth of the developing stereocilia. Likewise, the *Myo15* null mutation results in short stereocilia, making it likely that *Myo15* is involved in the elongation phase of stereociliary development. *Myo15* may also have a role in maintenance of stereocilia, as recent studies suggest actin replacement in stereocilia occurs every 48 h (50). The *Myo6* gene is proposed to be important in the anchoring of the stereocilia to the cuticular plate (19) and homozygous *Myo6* null mutations results in fusion of stereocilia into giant stereocilia.

In the present study, we observed no stereocilia defects in any of the double heterozygotes, and found that mice of these genotypes exhibited no significant increase in susceptibility to age-related hearing loss. Doubly homozygous mutants exhibited a superimposition of phenotypes in the cochlea, which suggests that *Myo15* has a role distinct from that of *Myo6*, *Myo7a* and *pi* in stereociliar development and organization.

MATERIALS AND METHODS

Mice

All mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), except for $Myo7a^{4626SB/4626SB}$ mice, which were obtained from Karen Steel of the MRC Institute of Hearing Research (Nottingham, UK). All procedures were approved by the University of Michigan Committee on Use and Care of Animals. All experiments were conducted in accordance with the principles and procedures outlined in the National Research Council *Guide for the Care and Use of Laboratory Animals*.

$Myo15^{sh2/sh2}$ females were bred to $Myo6^{sv/sv}$, $Myo7a^{4626SB/4626SB}$ and *pi*/*pi* males, and progeny F_1 mice were fostered with lactating CD-1 female mice, as $Myo15^{sh2/sh2}$ females often do not nurse their pups (unpublished observation). F_1 mice from each cross were then intercrossed ($F_1 \times F_1$), and F_2 progeny were examined. For simplicity, we refer to these progeny as mutants (−/−) or heterozygotes (+/−) rather than repeat the gene names and specific alleles. Double mutants are (−/−, −/−). Double heterozygotes are (+/−, +/−). Single mutants are (−/−) at one allele and either (+/+) or (+/−) at the other allele. All comparisons were made among F_2 progeny.

Genotyping

All F_2 animals were genotyped by polymerase chain reaction (PCR). Genotyping for the $Myo15^{sh2}$ mutation was as previously described (9). PCR primers for the $Myo6^{sv}$ mutation are 5′-TGAAAAGAGTCAACCTGTGGC-3′ and 5′-TGGGTTTCTGCATTTTAATGC-3′, with PCR parameters: 92°C for 2 min; 30 cycles at 92°C for 10 s, 55°C for 30 s, 68°C for 4 min; and 68°C for 10 min. Wild-type DNA (+) yields a ∼2.3 kb band, while $Myo6^{sv}$ DNA (−) yields a ∼1.2 kb band. $Myo7a^{4626SB}$ PCR genotyping was as previously described (47). PCR genotyping of *pirouette* animals was as previously described (35).

Auditory brainstem response

Auditory brainstem responses (ABRs) were conducted as previously described (35). Two or three animals in each category were tested at three frequencies: 4, 10 and 20 kHz.

Reverse transcriptase–polymerase chain reaction

RT–PCR was performed as previously described (51).

Immunocytochemistry

Immunocytochemistry was performed as previously described (52).

Radioimmunoassay

Blood collection and RIA was performed as previously described (53).

Inner ear morphology

Morphological analysis was performed with SEM. Sample preparation and procedures were as previously described (35). Briefly, samples were dehydrated in a graded series of ethanol, critical point dried with CO_2 in a SamDri 790 (Tousimis, Rockville, MD, USA) and mounted on stubs using colloidal silver paste. Samples were analyzed and digitally recorded using a Philips XL30 Field Emission Gun Scanning Electron Microscope (FEI, Hillsboro, OR, USA). Two to four animals of each genotype were examined.

Statistical analysis

All statistical analysis was performed with STATVIEW 4.0 or EXCEL.

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