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

I. Jill Karolyi¹, Frank J. Probst¹, Lisa Beyer², Hana Odeh², Gary Dootz², Kelly B. Cha¹, Donna M. Martin¹,³, Karen B. Avraham⁴, David Kohrman², David F. Dolan², Yehoash Raphael² and Sally A. Camper¹,*

¹Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109-0638, USA, ²Department of Otolaryngology, University of Michigan, Ann Arbor, MI 48109-0648, USA, ³Department of Pediatrics, University of Michigan, Ann Arbor, MI 48109-0688, USA and ⁴Department of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

Received June 16, 2003; Revised and Accepted August 28, 2003

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 Myo15sh2/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 NH₂-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 macro-molecular 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.
(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 stereociliary 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 IB (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<sup>4626SB</sup> allele, which expresses only 1/250th of wild-type protein levels of myosin VIIa (30). The Myo7a<sup>-/−</sup> 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<sup>sh1/sh2</sup> mice with Myo6<sup>4626SB</sup>, Myo7a<sup>4626SB/4626SB</sup> 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<sup>sh1/sh2</sup> mice were crossed with mice homozygous for severe alleles of both Snell’s waltzer (Myo6<sup>4626SB</sup>) and shaker-1 (Myo7a<sup>4626SB/4626SB</sup>) and for pirouette (pi). F1 doubly heterozygous animals (+/−, +/−) from all three crosses appeared completely normal, did not circle and showed a normal Preray 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 F2 generation (Tables 1–3), indicating that double mutants were viable. All double mutants died 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 F2 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 F2 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 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. F2 distribution of genotypes in 189 progeny of Myo15, Myo6 cross**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Expected ratio</th>
<th>Number observed</th>
<th>Number expected</th>
<th>Chi-square</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo15</td>
<td>Myo6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>++</td>
<td>++</td>
<td>1/16</td>
<td>7</td>
<td>12</td>
<td>0.16</td>
</tr>
<tr>
<td>++</td>
<td>--</td>
<td>1/16</td>
<td>35</td>
<td>24</td>
<td>0.02**</td>
</tr>
<tr>
<td>++</td>
<td>++</td>
<td>4/16</td>
<td>25</td>
<td>24</td>
<td>0.74</td>
</tr>
<tr>
<td>++</td>
<td>--</td>
<td>1/16</td>
<td>8</td>
<td>12</td>
<td>0.27</td>
</tr>
<tr>
<td>++</td>
<td>++</td>
<td>1/16</td>
<td>12</td>
<td>12</td>
<td>0.90</td>
</tr>
<tr>
<td>++</td>
<td>--</td>
<td>2/16</td>
<td>23</td>
<td>24</td>
<td>0.16</td>
</tr>
<tr>
<td>++</td>
<td>--</td>
<td>1/16</td>
<td>7</td>
<td>12</td>
<td>0.06</td>
</tr>
<tr>
<td>++</td>
<td>++</td>
<td>2/16</td>
<td>35</td>
<td>24</td>
<td>0.02**</td>
</tr>
<tr>
<td>++</td>
<td>--</td>
<td>1/16</td>
<td>15</td>
<td>12</td>
<td>0.35</td>
</tr>
<tr>
<td>++</td>
<td>--</td>
<td>2/16</td>
<td>24</td>
<td>24</td>
<td>0.94</td>
</tr>
<tr>
<td>++</td>
<td>--</td>
<td>1/16</td>
<td>8</td>
<td>12</td>
<td>0.27</td>
</tr>
</tbody>
</table>

*Significant difference.

**Table 2. F2 distribution of genotypes in 182 progeny of Myo15, Myo7a cross**

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

*Significant difference.

**Table 3. F2 distribution of genotypes in 238 progeny of Myo15, pi cross**

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

*Significant difference.
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 Myo7a4626SB 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...
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<sup>162888b</sup>) (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<sup>162888b</sup>) (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
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, Myo7a 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 F2 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

![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.](image)

![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.](image)
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 cell’s apical surface, as the stereocilia are disorganized in the Myo7a null mutant. The pi mutation results in short stereocilia, making it likely that Myo7a may be important for the original organization of stereocilia on the cell’s apical surface, as the stereocilia are disorganized in the Myo15 null mutant. The pi mutation 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 by circumferential addition of actin filaments (49).

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 stereociliary development and organization.

MATERIALS AND METHODS

Mice

All mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), except for Myo7a4626SB/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.

Myo15sh2/sh2 females were bred to Myo6sv/sv, Myo7a4626SB/4626SB and pi/pi males, and progeny F1 mice were fostered with lactating CD-1 female mice, as Myo15sh2/sh2 females often do not nurse their pups (unpublished observation). F1 mice from each cross were then intercrossed (F1 × F1), and F2 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 F2 progeny.

Genotyping

All F2 animals were genotyped by polymerase chain reaction (PCR). Genotyping for the Myo15sh2 mutation was as previously described (9). PCR primers for the Myo6sv mutation are 5′-TGAAAAGATCAACCTGTGGC-3′ and 5′-TGGTTTCTGCATTTATGC-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 Myo6sv DNA (−) yields a ~1.2 kb band. Myo7a4626SB 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 CO2 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.

ACKNOWLEDGEMENTS

We thank Karen Steel for providing mice and for helpful discussions. We thank Audrey Seasholtz for blood corticosterone assays. This work was funded by National Institutes of Health DC03049 (D.K.), DC05188 (D.F.D.), DC02982 (D.F.D.), DC05401 (Y.R.), DC05053 (S.A.C.), the Deafness Research Foundation...
REFERENCES