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Report

MYO6, the Human Homologue of the Gene Responsible for Deafness in *Snell's Waltzer* Mice, Is Mutated in Autosomal Dominant Nonsyndromic Hearing Loss

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Mutations in the unconventional *myosin VI* gene, *Myo6*, are associated with deafness and vestibular dysfunction in the *Snell's waltzer* (*sv*) mouse. The corresponding human gene, *MYO6*, is located on chromosome 6q13. We describe the mapping of a new deafness locus, DFNA22, on chromosome 6q13 in a family affected by a nonsyndromic dominant form of deafness (NSAD), and the subsequent identification of a missense mutation in the *MYO6* gene in all members of the family with hearing loss.

Unconventional myosins were among the first family of proteins found to be associated with hearing loss. Mutations in *shaker-1* mutant mice and human Usher syndrome type 1B were identified in the *myosin VIIA* gene (MIM 276903) simultaneously; not long afterwards, forms of both dominant and recessive human hearing loss were found to be associated with *myosin VIIA* mutations (Gibson et al. 1995; Weil et al. 1995; Liu et al. 1997*a*, 1997*b*). Mutations in the unconventional *myosin VI* gene (*Myo6* [MIM 600970]), were found to be associated with deafness and vestibular dysfunction in the *Snell's waltzer* (*sv*) mouse (Avraham et al. 1995). A third unconventional myosin, myosin XV (MIM 602666), is associated with the DFNB3 locus on chromosome 17 and in the shaker-2 mutant mouse (Probst et al. 1998; Wang et al. 1998). Despite the concordance between human and mouse mutations of *myosin VIIA* and *myosin XV* (among the many loci mapped over the years for human nonsyndromic hearing loss), none mapped to the homologous human region of the *sv* locus. This report demonstrates the first identification of a mutation in *myosin VI* associated with human hearing loss.

We included a large Italian kindred affected by NSAD (fig. 1*A*) in a genomewide search. The family is affected by progressive postlingual sensorineural deafness, with onset during childhood (8–10 years old at the onset of symptoms; 6–8 years old at the first audiometric abnormalities). At the age of ~50 years, affected individuals invariably have profound sensorineural deafness. Audiograms showed different degrees of hearing impairment, with sensorineural hearing loss that ranged in severity from moderate to profound (fig. 1*B*). Results of tympanometric testing indicated proper functioning of the tympanic membrane and middle ear (data not shown). Vestibular and/or visual involvement was excluded in all affected individuals. The genomewide search was performed using the ABI PRISM linkage mapping set (Perkin Elmer), characterized by >375 markers that define a human index map with a 10-cM resolution. PCR amplifications, using fluorescence-labeled primers were performed under the conditions recommended by the supplier. An aliquot of each PCR product was tested on an ABI Prism 377 DNA sequencer, and results were processed by GENESCAN software. Allele assignation was performed using GENOTYPER

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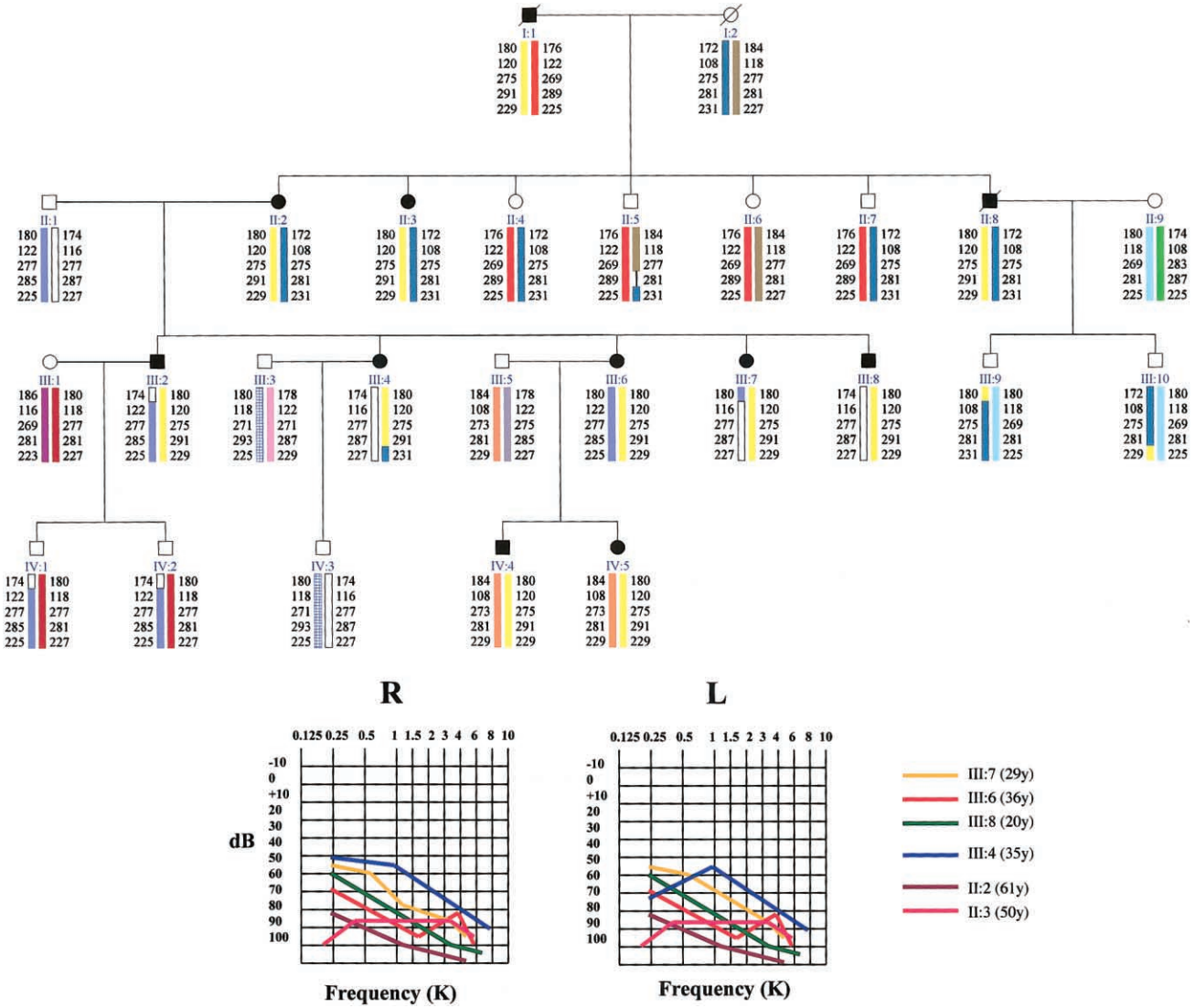

Figure 1 An *MYO6* mutation in affected members of an Italian kindred. *Top*, Pedigree of four generations of the Italian family, showing haplotypes of chromosome 6q13 markers (D6S257, D6S402, D6S681, D6S640, and DS445) and linkage to this region (*yellow*) in deaf individuals. Individuals with hearing loss are indicated by blackened symbols; unaffected individuals are indicated by open symbols. *Bottom*, Pure-tone audiograms, for right and left ears, of five affected family members. Note variability in severity of hearing loss (moderate to profound) among siblings.

software. Statistical analysis was performed on the basis of an autosomal disease with complete penetrance, because all individuals were older than the mean age at onset and because their disease status was well defined by clinical observation and testing. The disease-gene frequency was set to .012, and all marker alleles were considered to be equally frequent. Pairwise linkage analysis was performed using the MLINK program, version 5.1, from the LINKAGE computer software package (Ott 1992). Values for maximum LOD score were calculated with the ILINK program from the same software package. The ~95% confidence limits for the maximum recombination fraction (θ_{max}) at the maximum LOD score (Z_{max}) were calculated by the 1-LOD-down method (Ott 1992). Alleles were down-coded without loss of informativeness, to reduce computing time. Pairwise linkage analysis showed $Z_{max} = 5.11$ with marker D6S460. We further investigated this region with additional markers (D6S257, D6S402, D6S1681, and D6S445), confirming linkage (table 1) and narrowing the candidate region to ~11 cM between markers D6S257 and D6S445. The locus symbol DFNA22 has been assigned to this new NSAD locus.

KCNQ5, a member of the KCNQ potassium-channel

Table 1

Pairwise LOD Scores between Deafness and Chromosome 6 Markers

	LOD Score at θ =								
Marker	.0	.01	.05	.10	.20	.30	.40	Z_{max}	θ_{max}
D6S257	$-\infty$	2.74	3.15	3.08	2.56	1.81	.89	3.16	.05
D6S402	5.41	5.33	4.99	4.54	3.57	2.47	1.22	5.41	.00
D6S1681	3.31	3.25	3.04	2.76	2.14	1.45	.67	3.31	.00
D6S460	5.11	5.03	4.71	4.29	3.37	2.33	1.14	5.11	.00
D6S445	$-\infty$	1.34	2.34	2.64	2.37	1.74	.87	2.64	.10

family, which combines with KCNQ3 (MIM 602232) to form functional heteromeric channel proteins, maps to this region. We examined its expression in the cochlea to determine whether it was a potential candidate for deafness. This gene was considered a candidate because of the association of another member of the potassium channel genes, KCNQ4, to dominant deafness (Coucke et al. 1999; Kubisch et al. 1999). Primers were designed flanking 444 bp of mouse KCNQ5 mRNA sequence, and PCR was performed on cDNA derived from mouse cochlea. A product was obtained, indicating positive expression in the cochlea (data not shown). Primers spanning the exon-intron junctions were also designed, and DNA from the Italian family was screened. No mutations were found to segregate with hearing loss in this family (data not shown).

The human *MYO6* gene also maps within this region and was considered an excellent candidate on the basis of its chromosomal location, cochlear expression, and function. The entire *MYO6* coding sequence and all exon-intron boundaries were scanned for mutations by sequencing and by analysis of single-strand conformation polymorphism (Ahituv et al. 2000) (data not shown). We detected a G→A transition in exon 12 at position 1325 of the cDNA sequence (relative to the ATG, designated +1), which replaces a cysteine (TGT) with a tyrosine (TAT) at residue 442 of the protein (C442Y) (fig. 2*A*). This substitution can be analyzed by a mutagenesis primer that creates an *Hpa*I restriction site (fig. 2*B*). All affected members of this family carried the C442Y mutation, although it was never found in 400 normal chromosomes. The analysis of the coding region and exon-intron boundaries of *MYO6* gene in 50 Spanish and Italian families with NSAD identified several nucleotide variants (not shown) that do not lead to amino acid changes or did not segregate with deafness.

The C442Y mutation affects a cysteine residue in the motor domain that is conserved across other *myosin VI* species, including human, mouse, chicken, pig, striped bass, and sea urchin (fig. 3*A*). In the *Caenorhabditis elegans* and *Drosophila melanogaster myosin VI*, a similar hydrophilic amino acid, serine, resides at this position. Comparison of this residue in a pairwise alignment of 143 myosins revealed no changes to tyrosine. Further analysis of this site, using ConSurf (an algorithmic tool recently developed for the identification of functional regions in proteins by surface-mapping of phylogenetic information) (Armon et al. 2001), reveals that C442Y is located in a position that is conserved at a rate greater than the average among 50 other myosins. Because the primary structures of myosin motor domains are conserved, we were able to use homology-modeling techniques to create a three-dimensional model of myosin VI, using the known structure of myosin II (Rayment et al. 1993) (fig. 3*B*). According to the model shown, the cysteine is partly buried in the protein core, and, if this is the case, replacement of a small amino acid by a bulky one, such as tyrosine, is very likely to destabilize the protein, leading to partial or complete loss of function.

Myosin VI is a member of the superfamily of myosins that hydrolyzes ATP, converting chemical energy to mechanical force in order to move unidirectionally along actin filaments. Myosin VI has the unique feature of being the only motor molecule that is known to move toward the pointed (negative) end of actin filaments (Wells et al. 1999). In the inner ear, myosin VI expression is localized within the inner and outer hair cells of the sensory epithelium and, within these cells, is concen-

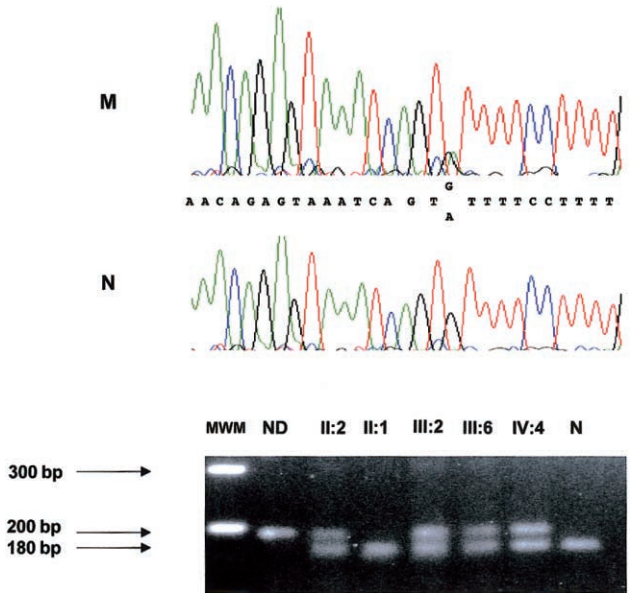

Figure 2 *Top*, Affected members were heterozygous for a G→A transition in exon 12 at position 1325 of the *MYO6* cDNA. *Bottom*, Restriction enzyme analysis with *Hpa*I mutagenesis primers (*Idir*A forward primer 5′-TTAGGTGCACTCTGTGGCAT-3′ and 9* (corresponding to fragment 9 of the gene) reverse mutagenesis primer 5′-GGATGATGTTTCAAAAGGTTAA-3′) identifies the G→A mutation in DFNA22 affected individuals. M = mutant; N = normal; ND = not digested.

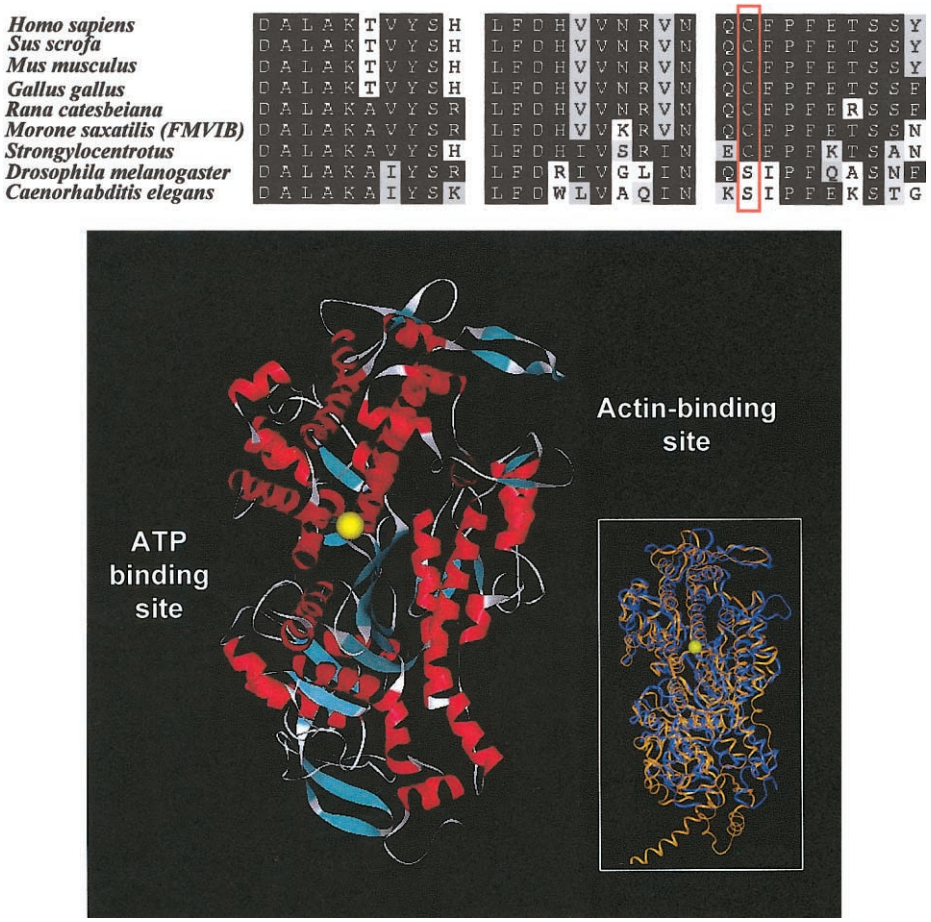

Figure 3 The C442Y missense mutation. *Top,* Alignment of a portion of *myosin VI* from various species. *Bottom,* A ribbon representation of a three-dimensional model of the myosin VI motor domain. *Inset,* the model of myosin VI (*blue*) and the experimentally determined structure of chicken pectoralis myosin II (*gold*), which was used as template. Also shown are C442 (*yellow*), α-helixes (*red*), and β-sheets (*turquoise*). The model was built using the automatic homology modeling facility, Swiss Model, and the three-dimensional structures of several myosins, including the *Dictyostelium discoideum* myosin S1 and chicken pectoralis myosin II motor domain. The figure was created using INSIGHT and WebLabViewerLite, version 3.10 (MSI).

trated at the base of the stereocilia that contain the negative ends of actin filaments (Avraham et al. 1997; Hasson et al. 1997). Myosin VI plays a key role in cell motility and shape-change events (reviewed by Cramer 2000), including the invagination of the plasma membrane that is observed during pseudocleavage in *D. melanogaster* embryo blastoderms (Mermall and Miller 1995), during sperm individualization in *D. melanogaster* (Hicks et al. 1999) and *C. elegans* (Kelleher et al. 2000), and during stereocilial development in mice (Self et al. 1999). One *Snell's waltzer* allele (*sv*) contains an intragenic deletion of the *Myo6* gene (Avraham et al. 1995). In this mutant, most stereocilial bundles have a normal appearance at birth, although signs of disorganization of the bundles are already present. At 1 d after birth, early signs of fusion, beginning at the base of the stereocilia, are observed. This fusion progresses along the length of the stereocilia so that, by the age of 20 d, giant stereocilia are seen, along with degeneration of hair cells. The fusion appears to be specific to the lack of myosin VI, because no stereocilial fusion is observed in other deaf mouse mutants, including those with mutations in unconventional myosins (Gibson et al. 1995; Probst et al. 1998). Myosin VI expression is reduced (to 11%–16% of levels found in the inner ear of wild-type mice) in a second *Snell's waltzer* allele, se^{sv}, leading to deafness (Avraham et al. 1995).

The C442Y missense mutation in the motor domain may compromise myosin VI function in a dominant-negative manner and/or cause haploinsufficiency. We speculate that the C422Y mutation might reduce availability of myosin VI in the terminally differentiated hair cells, leading to stereocilial fusion in the human inner ear and subsequently impairing proper auditory function

in a progressive manner. Construction of the appropriate mouse model will help to address this hypothesis.

Our results provide the first evidence of a role for *myosin VI* in human hearing loss, and we have several reasons for proposing C442Y as the causative mutation. First, *myosin VI* is a strong candidate gene, located within the region defined by DFNA22, found to be mutated. Second, other unconventional myosin mutations (VIIA and XV) are associated with different forms of both mouse and human hearing loss. Third, this missense mutation cosegregates with hearing loss in the Italian family and is not present in normal chromosomes. Fourth, a cysteine or amino acid with a similar hydrophilic nature is present in all myosin VI proteins at this position. Despite the effort that has been made to identify an *MYO6* gene mutation in the hearing-impaired population (Ahituv et al. 2000), this is the first report that relates *MYO6* and the corresponding mouse model to a form of human deafness, emphasizing the successful use of mouse models in predicting human disease.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank (for *Myosin VI* [*MYO6*; U90236])

Hereditary Hearing Loss, http://dnalab-www.uia.ac.be/dnalab/hhh (for genetic information on myosins)

Myosin Motor Domain Sequence Alignment, http://www.mrc-lmb.cam.ac.uk/myosin/trees/txalign.html (for structural data)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov./Omim (for *myosin VIIA* gene [MIM 276903], *myosin VI* gene [MIM 600970], *myosin XV* [MIM 602666], and KCNQ3 [MIM 602232])

Swiss Model, http://www.expasy.ch/swissmod/SWISS-MODEL.html (for protein modeling)

References

Ahituv N, Sobe T, Robertson NG, Morton CC, Taggart RT, Avraham KB (2000) Genomic structure of the human unconventional myosin VI gene. Gene 261:269–275

Armon A, Graur D, Ben-Tal N (2001) ConSurf: an algorithmic tool for the identification of functional regions in proteins by surface mapping of phylogenetic information. J Mol Biol 307:447–463

Avraham KB, Hasson T, Steel KP, Kingsley DM, Russell LB, Mooseker MS, Copeland NG, Jenkins NA (1995) The mouse Snell's waltzer deafness gene encodes an unconventional myosin required for the structural integrity of inner ear hair cells. Nat Genet 11:369–375

Avraham KB, Hasson T, Sobe T, Balsara B, Testa JR, Skvorak AB, Morton CC, Copeland NG, Jenkins NA (1997) Characterization of unconventional MYO6, the human homologue of the gene responsible for deafness in Snell's waltzer mice. Hum Mol Genet 6:1225–1231

Coucke PJ, Van Hauwe P, Kelley PM, Kunst H, Schatteman I, Van Velzen D, Meyers J, Ensink RJ, Verstreken M, Declau F, Marres H, Kastury K, Bhasin S, McGuirt WT, Smith RJ, Cremers CW, Van de Heyning P, Willems PJ, Smith SD, Van Camp G (1999) Mutations in the KCNQ4 gene are responsible for autosomal dominant deafness in four DFNA2 families. Hum Mol Genet 8:1321–1328

Cramer LP (2000) Myosin VI: roles for a minus end–directed actin motor in cells. J Cell Biol 150:F121–F126

Gibson, F. Walsh J, Mburu P, Varela A, Brown KA, Antonio M, Beisel KW, Steel KP, Brown SD (1995) A type VII myosin encoded by the mouse deafness gene shaker-1. Nature 374: 62–64

Hasson, T, Gillespie PG, Garcia JA, MacDonald RB, Zhao Y, Yee AG, Mooseker MS, Corey DP (1997) Unconventional myosins in inner-ear sensory epithelia. J Cell Biol 137: 1287–1307

Hicks JL, Deng WM, Rogat AD, Miller KG, Bownes M (1999) Class VI unconventional myosin is required for spermatogenesis in *Drosophila*. Mol Biol Cell 10:4341–4353

Kelleher JF, Mandell MA, Moulder G, Hill KL, L'Hernault SW, Barstead R, Titus MA (2000) Myosin VI is required for asymmetric segregation of cellular components during *C. elegans* spermatogenesis. Curr Biol 10:1489–1496

Kubisch C, Schroeder BC, Friedrich T, Lutjohann B, El-Amraoui A, Marlin S, Petit C, Jentsch TJ (1999) KCNQ4, a novel potassium channel expressed in sensory outer hair cells, is mutated in dominant deafness. Cell 96:437–446

Liu X-Z, Walsh J, Mburu P, Kendrick-Jones J, Cope MJ, Steel KP, Brown SD (1997) Mutations in the myosin VIIA gene cause non-syndromic recessive deafness. Nat Genet 16: 188–190

Liu X-Z, Walsh J, Tamagawa Y, Kitamura K, Nishizawa M, Steel KP, Brown SD (1997) Autosomal dominant non-syndromic deafness caused by a mutation in the myosin VIIA gene. Nat Genet 17:268–269

Mermall V, Miller KG (1995) The 95F unconventional myosin is required for proper organization of the *Drosophila* syncytial blastoderm. J Cell Biol 129:1575–1588

Ott J (1992) Analysis of human linkage. The Johns Hopkins University Press, Baltimore

Probst FJ, Fridell RA, Raphael Y, Saunders TL, Wang A, Liang Y, Morell RJ, Touchman JW, Lyons RH, Noben-Trauth K, Friedman TB, Camper SA (1998) Correction of deafness in shaker-2 mice by an unconventional myosin in a BAC transgene. Science 280:1444–1447

Rayment I, Holden HM, Whittaker M, Yohn CB, Lorenz M,

Holmes KC, Milligan RA (1993) Structure of the actin-myosin complex and its implications for muscle contraction. Science 261:50–58

Self T, Sobe T, Copeland NG, Jenkins NA, Avraham KB, Steel KPS (1999) Role of myosin VI in the differentiation of cochlear hair cells. Dev Biol 214:331–341

Wang A, Liang Y, Fridell RA, Probst FJ, Wilcox ER, Touchman JW, Morton CC, Morell RJ, Noben-Trauth K, Camper SA, Friedman TB (1998) Association of unconventional myosin MYO15 mutations with human nonsyndromic deafness DFNB3. Science 280:1447–1451

Weil D, Blanchard S, Kaplan J, Guilford P, Gibson F, Walsh J, Mburu P, et al (1995) Defective myosin VIIA gene responsible for Usher syndrome type 1B. Nature 374:60–61

Wells AL, Lin AW, Chen LQ, Safer D, Cain SM, Hasson T, Carragher BO, Milligan RA Sweeney HL (1999) Myosin VI is an actin-based motor that moves backwards. Nature 401: 505–508