INTRODUCTION

Many inherited retinal diseases are caused by loss of function of a single gene that is expressed in the photoreceptor cells and/or the retinal pigment epithelium (RPE), with progressive retinal degeneration resulting in blindness. These diseases should therefore be preventable by the introduction of a wild-type (WT) version of the mutant gene into the photoreceptor and/or RPE cells, before the onset of retinal degeneration. Proof of this principle has now been demonstrated in clinical trials with patients possessing Leber congenital amaurosis, due to loss of function of the RPE65 gene.1–4

In Usher syndrome, retinal degeneration occurs in conjunction with deafness. In most cases, the deafness is congenital; in Usher syndrome type 1, patients are born profoundly deaf. These patients are thus readily identified when infants, before the onset of retinal degeneration, making gene therapy a viable strategy to prevent their blindness. Here, we have investigated the use of adeno-associated viruses (AAVs) for the delivery of the Usher 1B gene, MYO7A, to retinal cells in cell culture and in MYO7A-null mice. MYO7A cDNA, under control of a smCBA promoter, was packaged in single AAV2 and AAV5 vectors and as two overlapping halves in dual AAV2 vectors. The 7.9-kb smCBA-MYO7A exceeds the capacity of an AAV vector; packaging of such oversized constructs into single AAV vectors may involve fragmentation of the gene. Nevertheless, the AAV2 and AAV5 single vector preparations successfully transduced photoreceptor and retinal pigment epithelium cells, resulting in functional, full-length MYO7A protein and correction of mutant phenotypes, suggesting successful homologous recombination of gene fragments. With discrete, conventional-sized dual AAV2 vectors, full-length MYO7A was detected, but the level of protein expression was variable, and only a minority of cells showed phenotype correction. Our results show that MYO7A therapy with AAV2 or AAV5 single vectors is efficacious; however, the dual AAV2 approach proved to be less effective.

Retinal gene therapy with a large MYO7A cDNA using adeno-associated virus

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Usher 1 patients are born profoundly deaf and then develop retinal degeneration. Thus they are readily identified before the onset of retinal degeneration, making gene therapy a viable strategy to prevent their blindness. Here, we have investigated the use of adeno-associated viruses (AAVs) for the delivery of the Usher 1B gene, MYO7A, to retinal cells in cell culture and in MYO7A-null mice. MYO7A cDNA, under control of a smCBA promoter, was packaged in single AAV2 and AAV5 vectors and as two overlapping halves in dual AAV2 vectors. The 7.9-kb smCBA-MYO7A exceeds the capacity of an AAV vector; packaging of such oversized constructs into single AAV vectors may involve fragmentation of the gene. Nevertheless, the AAV2 and AAV5 single vector preparations successfully transduced photoreceptor and retinal pigment epithelium cells, resulting in functional, full-length MYO7A protein and correction of mutant phenotypes, suggesting successful homologous recombination of gene fragments. With discrete, conventional-sized dual AAV2 vectors, full-length MYO7A was detected, but the level of protein expression was variable, and only a minority of cells showed phenotype correction. Our results show that MYO7A therapy with AAV2 or AAV5 single vectors is efficacious; however, the dual AAV2 approach proved to be less effective.

INTRODUCTION

Many inherited retinal diseases are caused by loss of function of a single gene that is expressed in the photoreceptor cells and/or the retinal pigment epithelium (RPE), with progressive retinal degeneration resulting in blindness. These diseases should therefore be preventable by the introduction of a wild-type (WT) version of the mutant gene into the photoreceptor and/or RPE cells, before the onset of retinal degeneration. Proof of this principle has now been demonstrated in clinical trials with patients possessing Leber congenital amaurosis, due to loss of function of the RPE65 gene.1–4

In Usher syndrome, retinal degeneration occurs in conjunction with deafness. In most cases, the deafness is congenital; in Usher syndrome type 1, patients are born profoundly deaf. These patients are thus readily identified when infants, before the onset of retinal degeneration, making gene therapy a viable strategy to prevent their blindness.

The most common form of Usher syndrome type 1 is Usher 1B, which accounts for at least half of Usher 1 cases. It is caused by mutations in the MYO7A gene.5 Together with light chains, MYO7A forms the actin-based motor, myosin 7a. Myosin 7a has a variety of functions in the photoreceptor and RPE cells.6–11 The recessive inheritance of Usher syndrome, together with MYO7A disease alleles that are likely null, indicate that Usher 1B is primarily due to loss of MYO7A function, for example, see Jacobson et al.12 so that the progressive blindness should therefore be preventable by delivering a WT gene to the photoreceptor and RPE cells before any cell degeneration. A limitation is that MYO7A is a large gene; its cDNA is nearly 7 kb.13,14 This large size is particularly a concern for using adeno-associated virus (AAV) to deliver the gene. AAV has been used successfully in clinical and preclinical retinal gene therapy experiments,1–4,15 but its nominal packaging size was reported to be ~5.3 kb.16 Because of the size of MYO7A, we initially studied the use of a lentiviral vector to deliver MYO7A cDNA to the RPE and photoreceptor cells.17 Following subretinal injection of mice, we observed correction of mutant phenotypes in the RPE and photoreceptor cells. However, the integrating nature of lentiviral vectors poses a concern with respect to the introduction of insertional mutagenesis, as well as different responses among different cells due to cell-to-cell variation in the integration of the gene. By contrast, AAV transduction results in mostly episomal DNA, which has been shown to be stable in terminally differentiated cells, such as photoreceptor and RPE cells.18,19

More recently, we showed that AAV5 was able to mediate the expression of full-length MYO7A protein in RPE cells in primary culture.20 Subsequent studies by three independent groups found that AAV could deliver other large genes, although they showed that the DNA was first fragmented into sizes consistent with canonical AAV packaging (that is, <5 kb), and then the fragmented genes reassemble after delivery to the cell, presumably by recombination, to yield a full-length cDNA.21–23 The ability of different cells to reassemble a given gene is likely to vary. In the present study, we have tested the ability of AAV-MYO7A to transduce RPE and photoreceptor cells in vivo and to correct mutant phenotypes in mice that are null for the Usher 1B orthologue, Myo7a. We demonstrate efficacy not only with AAV5 but also the more commonly used serotype, AAV2.

Due to the possible heterogeneous nature of genes delivered by these single AAV vectors, we also tested a dual vector
Cell cultures. Cells derived from Myo7a

Titers of 10^{12}–10^{13} particles per ml were obtained

CBA and activity in mouse retinas as that of the full-length

CBA cDNA encoding the full-length isoform 2 of human MYO7A

proteins that were comparable with that of WT MYO7A

preparation, with two AAV2 vectors, each containing an over-

RESULTS

AAV-MYO7A single vector preparations

AAV vector plasmid was engineered to contain a truncated chimeric CMV/chicken β-actin promoter, smCBA, and the 6.7-kb cDNA encoding the full-length isoform 2 of human MYO7A (Figure 1a). The smCBA promoter exhibits the same tropism and activity in mouse retinas as that of the full-length CBA promoter. Titters of 10^{12}–10^{13} particles per ml were obtained for different lots of AAV2-MYO7A and AAV5-MYO7A. A concentration of 10^{12} particles per ml was regarded as our standard concentration (1×), from which dilutions were made. The experiments were performed with virus obtained from three separate preparations. No differences in expression or phenotype correction, as described below, were observed among the different lots for AAV2-MYO7A or AAV5-MYO7A at a given concentration.

MYO7A expression in cell culture

Transduction of primary cultures of Myo7a-null RPE cells with 1× single AAV2-MYO7A or AAV5-MYO7A resulted in the expression of a polypeptide that, by western blot analysis, had an apparent mass that was comparable with that of WT MYO7A protein and was present at similar levels to that found in primary cultures of Myo7a^{+/−} RPE cells (Figure 1b). Likewise, a single band of appropriate size was detected on western blots of HEK293A cells (data not shown). Immunofluorescence of the primary RPE cells showed that the MYO7A protein, resulting from 1× single AAV-MYO7A treatment of MYO7A-null cells, had a subcellular localization pattern that was comparable with that of endogenous MYO7A in control cells, indicating the generation of appropriately targeted protein (Figures 1c–f). ARPE19 cells were also infected with 1× or diluted (1:100) AAV2-MYO7A or AAV5-MYO7A and compared with non-treated cells. An increase in MYO7A immunofluorescence was detected in the treated cells, and the intracellular localization of the label was comparable with that in untreated cells (Supplementary Figures S1a–e).

Localization of MYO7A in vivo

Most retinal MYO7A is found in the RPE, however, the protein is also present in the connecting cilium and pericilium of the photoreceptor cells. A diagram illustrating this distribution and the retinal functions of MYO7A has been published in a recent review. Three weeks following injection of 1× AAV2-MYO7A or AAV5-MYO7A into the subretinal space of Myo7a-null mice, retinal tissue was examined by immunoelectron microscopy (immunoEM) to test for MYO7A expression. Near the site of injection (within 1.4 mm), immunogold label was evident in the photoreceptor cells, where it was localized in the connecting cilium and pericilium, comparable with that in WT retinas (Figures 2a–e). Label was also present throughout the RPE cells, particularly in the apical cell body region (Figures 2f and g), as found in WT retinas. At the periphery of the retina, no significant immunolabel was detected (Supplementary Figure S2).

MYO7A has a similar distribution in both the rod and cone photoreceptor cells. To test whether treatment with AAV-MYO7A also affected cone photoreceptor cells, we determined whether
Figure 2. Expression of MYO7A from single AAV2 and AAV5 vectors in vivo. (a-e) EM images of MYO7A immunogold labeling of the connecting cilium and pericilium from rod photoreceptors in a Myo7a-null retina. (a) Longitudinal section from an untreated Myo7a-null retina (background label only). (b, c) Longitudinal sections from Myo7a-null retinas treated with 1 × AAV2-MYO7A (b) or AAV5-MYO7A (c). Bar = 200 nm. (d, e) Transverse sections of connecting cilia from rod photoreceptors in Myo7a-null retinas treated with 1 × AAV2-MYO7A (d) or AAV5-MYO7A (e). Bar = 100 nm. (f, g) EM images of RPE cells from Myo7a-null retinas treated with 1 × AAV2-MYO7A (f) or AAV5-MYO7A (g). Bar = 2 μm. BM = Bruch’s membrane, AP = apical processes. Areas indicated by rectangles are enlarged in (f′) and (g′), in order to show MYO7A immunogold labeling (indicated by circles). Bar = 500 nm. (h, i) EM image of a longitudinal section of the connecting cilium and pericilium from a rod (h) and a cone (i) photoreceptor in a Myo7a-null retina, treated with 1 × AAV2-MYO7A. The section was double-labeled with MYO7A (12 nm gold) and rod opsin (15 nm gold) antibodies. Rod outer segments were labeled with the opsin antibody, while cones were identified by lack of rod opsin labeling in their outer segments. The sections show just the base of the outer segments. Nearly all the label in the connecting cilium is MYO7A, even in the rod. Bar = 200 nm. (j-l) Bar graphs indicating MYO7A immunogold particle density in the rod photoreceptor cilium and pericilium (j, k) and in the RPE (l, m), following treatment with AAV2-MYO7A (j, l) or AAV5-MYO7A (k, m) of different concentrations. N = 3 animals per condition. Bars indicate s.e.m.
MYO7A was also present in the ciliary region of cone photoreceptors. We performed double immunoEM of treated retinas, using a MYO7A antibody together with an antibody specific for rod opsin. Although there are only a small number of cones with aligned connecting cilia found in each ultrathin section, MYO7A immunogold label was evident in the connecting cilium and periciliary region of these cones, which were identified by lack of rod opsin labeling in their outer segments (in contrast to the surrounding rod outer segments) (Figures 2h and i). Hence, AAV2-MYO7A and AAV5-MYO7A can transduce cone as well as rod photoreceptor cells.

Dose-dependent MYO7A expression in photoreceptor and RPE cells
To determine the levels of MYO7A expression following treatment with different concentrations of AAV2-MYO7A and AAV5-MYO7A (1 ×, 1:10 or 1:100 dilutions), we quantified MYO7A immunogold labeling in EM images, taken within 1.4 mm of the injection site. Reliable detection of MYO7A in the photoreceptor cells, where its distribution is limited to the connecting cilium and pericilium, requires the higher resolution provided by EM. Immunogold particle density was measured in images of the photoreceptor connecting cilium and pericilium, shown in complete longitudinal section (from the basal bodies to the base of the outer segment) and in images showing the RPE cells in apical to basal section. Particle density was expressed as particles per length of cilium for the photoreceptor cells (each connecting cilium is ~1.2 µm long) and as particles per area for the RPE cells (the entire area between the apical and basal surfaces was included). Particle density is dependent on exposure of epitopes on the surface of the section and, as such, provides a relative linear measure of antigen density under the conditions used here (that is, grids were etched and labeled in an identical manner, and the labeling was not so dense as to be affected by steric hindrance).

Treatment with 1 × AAV2-MYO7A or AAV5-MYO7A resulted in 2.5–2.7 times the density of immunolabel in the photoreceptor cilium, compared with that found in WT retinas, while the 1:10 and 1:100 dilutions resulted in a density of immunolabel that was more comparable with WT levels (Figures 2 and I; Supplementary Figure S3). Quantification of immunogold label in the RPE showed that injection of AAV2-MYO7A resulted in 2.7 times more label than in WT, with the 1:10 and 1:100 dilutions showing no significant difference (Figure 2k). By contrast, the level of MYO7A immunolabel in the RPE of retinas injected with AAV5-MYO7A varied in relation to virus titer, with the full-dose virus effecting 2.2-fold more MYO7A than that found in WT RPE, the 1:10 dilution effecting WT levels, and the 1:100 dilution resulting, on average, ~60% of WT levels (Figure 2m).

These counts of labeling density indicate that 1 × AAV-MYO7A resulted in more than double the normal level of MYO7A expression in both the photoreceptor and RPE cells. The distribution of MYO7A was not affected by this overexpression in the photoreceptor cells. In the RPE cells, the overall distribution of MYO7A was comparable with WT, with a higher concentration in the apical cell body region. However, with 1 × AAV2-MYO7A or 1 × AAV5-MYO7A, the proportion of MYO7A that was associated with melanosomes was only just over half that in WT RPE: in WT RPE, we found that 42% of the MYO7A immunogold particles were located near the membrane of melanosomes; in retinas treated with 1 × AAV2-MYO7A, we found 23%; and in retinas treated with 1 × AAV2-MYO7A, we found 24%. This difference is possibly because the proteins that link MYO7A to the melanosomes, MYRIP and RAB27A, may have remained near WT levels and thus limited the absolute amount of MYO7A that could associate with the melanosomes.

Despite the overexpression of MYO7A, no pathology was evident in retinas, up to 3 months after injection of 1 × (or 1:10) AAV2-MYO7A. However, two out of six retinas injected with 10^13 particles per ml of AAAs-MYO7A (that is, 10 ×) showed evidence of photoreceptor cell loss across the retina after 3 weeks (AAV2-MYO7A was not tested at this titer) (Supplementary Figure S4).

Correction of melanosome localization in the RPE
In Myo7a-mutant mice, melanosomes are absent from the apical processes of the RPE cells. This mutant phenotype is evident at all neonatal ages and is due to loss of actin-based transport of the melanosomes by the myosin 7a motor. Three weeks following injection of 1 × AAV2-MYO7A or AAV5-MYO7A into the subretinal space of Myo7a-null mice, melanosomes were observed to have a normal distribution in all RPE cells near the site of injection (within 1.4 mm; n = 10 each for AAV2-MYO7A and AAV5-MYO7A) (Figures 3a–c). Well away from the injection site, a mixture of corrected and uncorrected RPE cells was evident, while, at the periphery of the retina, the cells all exhibited the Myo7a-mutant phenotype, indicating lack of correction in this region (Figures 3d–f). The correction of melanosomes was still evident in retinas that were fixed 3 months after injection (Supplementary Figure S5). Correction was also observed in all the eyes injected with 1:10 dilution AAV2-MYO7A (n = 6) or AAV5-MYO7A (n = 6), as well as in all the eyes injected with 1:100 dilution AAV2-MYO7A (n = 6) or AAV5-MYO7A (n = 6), although with the 1:100 dilution some of the RPE cells near the site of injection were not corrected.

Correction of opsin distribution
Myo7a-mutant mice have an abnormal accumulation of opsin in the connecting cilia of the photoreceptor cells, a phenotype that is evident by immunoEM with opsin antibodies. This mutant phenotype suggests that myosin 7a functions in the vectorial delivery of opsin to the outer segment. Quantification of immunogold opsin labeling in the connecting cilium demonstrated that this phenotype was corrected with 1 × AAV2-MYO7A or AAV5-MYO7A (Figure 3g; Supplementary Figure S6). This analysis also showed phenotype correction with 1:100 dilutions, although the data indicated that a full WT phenotype was not achieved (Figure 3g), despite WT levels of MYO7A (Figures 2 and I), suggesting that some of the MYO7A may not be fully functional.

AAV2-MYO7A dual vector preparations
The preceding results demonstrate that a single AAV vector is capable of delivering functional MYO7A to the RPE and photoreceptor cells in vivo. Because the size of smCBA-MYO7A is ~2 kb larger than the nominal carrying capacity for an AAV, this transduction might involve undefined fragmentation of the smCBA-MYO7A cDNA, followed by reassembly of plus and minus cDNA strands after delivery to the cell, as shown for other large genes. To evaluate whether two AAV vectors containing defined, overlapping fragments of MYO7A cDNA (1365 bases) were also capable of mediating full-length MYO7A expression, we developed an AAV2-based dual vector system (Figure 4a). Two separate lots of the AAV2-MYO7A(dual vector) were prepared, each containing equal concentrations of AAV2-smCBA-MYO7A(5′-half) and AAV2-MYO7A(3′-half). The titer of the first lot contained 2.5 × 10^12 particles per ml of each vector, and the second lot contained 4 × 10^12 particles per ml.

MYO7A expression with AAV2 dual vectors
Western blot analysis of primary cultures of Myo7a-null RPE cells, infected with AAV2-MYO7A(dual vector) of either lot, showed that the cells expressed a MYO7A-immunolabeled polypeptide of comparable mass with that of WT MYO7A (Figure 4b). However, the overall expression level of MYO7A in the treated Myo7a-null RPE cells was significantly less than that found in primary cultures.
Myo7a-null RPE cells, infected with AAV2-MYO7A(dual vector), showed that a few cells scattered throughout the culture exhibited very high levels of MYO7A, but all other cells contained insignificant levels (Figures 4d–f). The cells overexpressing MYO7A typically had altered morphology, suggesting that the high levels of MYO7A may be toxic. Similarly, immunofluorescence of ARPE19 cells, infected with AAV2-MYO7A(dual vector), resulted in a minority of cells that were labeled intensely with MYO7A antibody, with most of the cells appearing to express only endogenous levels of MYO7A (Figure 4g; Supplementary Figure S1f).

Immunolabeling of retinas, prepared 3 weeks after subretinal injection with AAV2-MYO7A(dual vector) of either lot, also showed only a few RPE cells and photoreceptor cells with clear MYO7A expression, although significant overexpression was not evident in this in vivo experiment. Immunogold particle counts from images of ultrathin sections were used to quantify the level of MYO7A expression in Myo7a-null retinas that were treated with the second lot of AAV2-MYO7A(dual vector). Within 1.4 mm of the injection site, MYO7A immunolabeling of the connecting cilium and pericilium of the photoreceptor cells was a mean of 48% of that in WT retinas: 2.8 particles per μm (n = 3 retinas) compared with 6.5 particles per μm for WT (n = 3 retinas). The mean label density in apical–basal sections of the RPE was 35% of that in WT retinas: 11 particles per 100 μm² compared with 31 particles per 100 μm² for WT. However, it was clear that these lower means were achieved by some cells expressing near normal amounts of MYO7A and the majority expressing very little; over half the cells had fewer than 10 particles per 100 μm² (Figure 4h).

Correction of Myo7a-mutant phenotypes with AAV2 dual vectors

Eyes were analyzed for correction of melanosome localization and ciliary opsin distribution within 1.4 mm of the injection site. With either lot of AAV2-MYO7A(dual vector), some RPE cells (29% for lot 1 treatment (n = 6 retinas), 35% for lot 2 treatment (n = 9 retinas)) were observed to have a normal apical melanosome distribution, but most of the cells in this region retained the Myo7a-mutant phenotype, resulting in a mosaic effect (Figure 5a) that contained a much lower proportion of corrected cells than that observed with a 1:10 dilution of either of the single vectors. The only correction observed in the three eyes injected with a 1:10 dilution of AAV2-MYO7A(dual vector) (first lot) was in 18% of the RPE cells in one of the retinas. With full strength of AAV2-MYO7A(dual vector) (second lot), opsin immunogold density averaged 3.2 ± 0.4 particles per μm of cilium length, which was reduced from untreated retinas (4.2 ± 0.8 particles per μm; P = 0.003), but still greater than WT levels (1.1 ± 0.2 particles per μm), suggesting that most cells were not corrected. An example of a corrected cell is shown in Figure 5g.

Using immunoEM, we tested for a correlation between phenotype correction and the expression level of MYO7A, determined by the mean concentration of immunogold particles in an apical–basal section of each RPE cell (Figures 5b–e). From the eyes injected with AAV2-MYO7A(dual vector) (second lot), we found that the corrected RPE cells contained a mean of 108% of the WT level of MYO7A (the minimum level was 82%). RPE cells that were not corrected contained a mean of 26% of the WT level of MYO7A (the maximum level was 92%). Although these data show that higher expression of MYO7A is correlated with phenotype correction (Figure 5f), it also indicates that some of the labeled MYO7A protein is not functional, given that melanosomes are localized normally in mice that are heterozygous for the Myo7a-null allele and have only ~50% of the WT level of MYO7A.

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The efficacy of AAV2- and AAV5-mediated MYO7A delivery to the retinas of Myo7a-mutant mice was tested in a preclinical study aimed at developing a treatment for preventing blindness in Usher 1B. Despite the large size of smCBA-MYO7A relative to the carrying capacity of AAV, single vector preparations of either serotype effected MYO7A expression in RPE and photoreceptor cells and corrected the mutant phenotypes in both the cell types. In a different approach, using two discrete AAV2 vectors, each containing a different half of MYO7A cDNA, with a central overlapping region, MYO7A expression was detected, but only in a minority of cells, both in culture and in vivo. Likewise, the correction of mutant phenotypes was sporadic. These findings demonstrate the therapeutic potential of using a single AAV vector to deliver MYO7A, but they indicate limitations with an overlapping dual AAV vector approach.

**Figure 4.** Expression of MYO7A from the overlapping AAV2-MYO7A dual vectors. (a) Diagram of the the overlapping AAV2-MYO7A dual vectors. The overlapping region contains 1365 bases. (b) Western blot of proteins from primary RPE cultures derived from Myo7a-null mice and not infected (lane 1) or infected with AAV2-MYO7A(dual) (lane 2); and primary RPE cultures derived from Myo7a+/– mice (lane 3). All lanes were immunolabeled with anti-MYO7A and anti-actin. (c) Western blot of primary cultures derived from Myo7a-null mice and not infected (lane 1) or infected with AAV2-MYO7A(dual) (lane 2) or with AAV5-MYO7A single vector (1 ×) (lane 3). Lanes were immunolabeled with anti-MYO7A and anti-actin. Densitometry of the actin labeling showed that lane 2 was loaded with threefold more protein than lane 3; the MYO7A-to-actin ratio is sevenfold greater in lane 3 compared with lane 2 in this blot. (d–g) Immunofluorescence of cultured RPE cells transduced with AAV2-MYO7A(dual), (d–f) Primary RPE cultures derived from Myo7a-null mice and (g) ARPE19 cells. Bar = 10 μm. (h) Bar graph indicating the distribution of MYO7A immunogold particle density among RPE cells from retinas of Myo7a-null mice, injected with AAV2-MYO7A(dual). N = 3 animals.

**DISCUSSION**

The efficacy of AAV2- and AAV5-mediated MYO7A delivery to the retinas of Myo7a-mutant mice was tested in a preclinical study aimed at developing a treatment for preventing blindness in Usher 1B. Despite the large size of smCBA-MYO7A relative to the carrying capacity of AAV, single vector preparations of either serotype effected MYO7A expression in RPE and photoreceptor cells and corrected the mutant phenotypes in both the cell types. In a different approach, using two discrete AAV2 vectors, each containing a different half of MYO7A cDNA, with a central overlapping region, MYO7A expression was detected, but only in a minority of cells, both in culture and in vivo. Likewise, the correction of mutant phenotypes was sporadic. These findings demonstrate the therapeutic potential of using a single AAV vector to deliver MYO7A, but they indicate limitations with an overlapping dual AAV vector approach.

MYO7A delivery by AAV2 and AAV5 single vectors

Demonstration that AAV vectors mediate safe and effective therapy in clinical trials has been limited to the delivery of small genes or reduced versions of larger ones. Recombinant DNA can only be efficiently packaged into AAV if the total DNA is less than about 5.3 kb. Capsids containing DNA > 5.3 kb exhibit a progressively reduced transduction efficiency until reaching the physical encapsidation limit of ~6 kb. The strategy of creating ‘mini’ proteins, reduced to minimally essential domains, has been used in an attempt to make gene therapy with AAV tractable for large genes, such as dystrophin, although, limitations of this approach have been reported recently. However, the mini gene approach is of little use for large molecular motor genes, such as MYO7A. MYO7A is composed of essential motor, neck and tail domains, which provide the mechanoenzymatic properties, a lever for the step-wise motion and cargo-binding
sites, respectively. The essential nature of each domain is clear from structure–function studies of myosins.\textsuperscript{34–36} Moreover, MYO7A mutations throughout the gene have been shown to cause Usher 1B, demonstrating that a perturbation of any major domain is pathogenic, for example, see Jacobson \textit{et al.}\textsuperscript{12} (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=MYO7A).

Previously, we showed that a single AAV5 vector was able to deliver \textit{MYO7A} and \textit{ABCA4} (8.9 kb) cDNA, resulting in full-length protein expression in cell culture.\textsuperscript{20} In vivo studies with \textit{Abca4}-mutant mice indicated \textit{ABCA4} expression and a reduction in the abnormally high levels of lipofuscin in the RPE that results from \textit{ABCA4} dysfunction. More recently, it has been shown that full-length, large gene reconstruction results from recombination between fragmented plus and minus cDNA strands packaged into separate capsids.\textsuperscript{21–23} Using single-molecule sequencing, Kapranov \textit{et al.}\textsuperscript{37} found that the accommodation of large genomes by AAV involves encapsidation of only the 3' inverted terminal repeat and cleavage at the 5' end, resulting in a heterogeneous population of fragmented genomes. These fragments thus require a recombination event between appropriate overlapping genome fragments to reconstitute a full-length cDNA in cells containing multiple viral copies.

Our results show that transduction of photoreceptor and RPE cells with either the AAV2 or AAV5 single vector results in the
expression of MYO7A of the correct molecular mass. If fragmenta-
tion of the gene is occurring during packaging into the vectors, 
these results indicate that the fragments recombine successfully 
within the target cells. The extent of recombination may be 
dependent on the recombinogenic nature of the transgene itself; 
MYO7A, in the present study, seems to recombine well from 
the fragments, whereas dystrophin, for example, recombines relatively 
poorly.22

The level of expression of MYO7A observed in vivo was related 
to the titer of the vector delivered, with the exception of MYO7A 
levels in the RPE following treatment with AAV2-MYO7A; these 
levels were similar across a 100-fold dilution range. Even with 
this exception, the relative expression between the photoreceptor and 
RPE cells did not vary substantially from the ratio found in WT 
retinas, despite the use of a non-native promoter in the vector, the 
large difference in MYO7A levels between the photoreceptor and 
RPE cells and the use of two different AAV serotypes.

Overexpression of MYO7A has been shown to be toxic in a 
previous study, using lentiviral delivery of MYO7A under a full CMV 
promoter,12 and, in the present study, we observed extensive 
photoreceptor cell death, following subretinal injection of 10^{13} 
vector genome containing particles per ml of AAV5-MYO7A 
(that is, 10 \times). Nevertheless, the present results indicate that 
there is a fairly large tolerance for excessive MYO7A levels. Phe-
notype correlation with no indication of deleterious effects was 
observed over a wide range of vector titers (10^{10}–10^{12} particles 
per ml), resulting in a wide range of MYO7A levels, up to nearly three 
times to that found in the photoreceptor and RPE cells of WT 
retinas. Some overexpression may be necessary in the photo-
receptor cells, where some of the MYO7A was suggested to be not 
fully functional, based on counts of MYO7A immunogold particles, 
and the degree of correction of the opsin distribution.

**MYO7A delivery by AAV2 dual vector**

We also investigated whether a dual vector system composed of 
two vectors containing specific overlapping MYO7A cDNA frag-
ments would also promote therapy. AAV2 dual vectors were 
packaged so that each contained a 3’ or 5’ segment of the cDNA 
that was <5 kb, and each part shared an overlapping region of 
1365 bases. Western blot analysis showed that infected cells were 
able to generate full-length MYO7A from the two parts; however, 
immunolabeling of cells in vitro and in vivo showed that the 
majority of cells failed to make significant amounts of the protein.

Moreover, the RPE cells in culture that did contain MYO7A 
expressed it at excessive levels, and our data correlating MYO7A 
levels were slightly higher than the 1 \times titer used for the single AAV2 
vector, yet the single vector was efficacious, resulting in WT levels 
of MYO7A, even at a 1:100 dilution. The probability of the two 
halves of MYO7A cDNA recombining therefore appears to be very 
low in most of the RPE and photoreceptor cells.

Can the dual vector design be improved so that it could be used 
for Usher 1B retinal gene therapy? Different overlapping regions of 
the gene may offer more reliable homologous recombination, 
although the choice of regions is quite limited for MYO7A, as 
neither half can be made much larger without exceeding the 
vector packaging limit. Previous studies have compared transduc-
tion with a single vector and dual overlapping vectors, and 
showed that transduction is typically less efficient with the dual 
vector, although they have only tested genes that are <5 kb.38,39 

An alternative is to use dual vectors in which the two segments of 
the CDNA are designed to trans-splice together. An earlier report 
showed an order of magnitude improvement with this design 
compared with homologous recombination via a simple overlapp-
ing region, although it still fell short of transduction with a single vector.40 Combining trans-splicing with recombination of 
an overlapping region has yielded more promising results41 and 
could be applied to MYO7A. However, when such hybrid 
(trans-splicing + overlapping) vectors were directly compared with 
an overlapping only dual vector system in vivo, the overlapping 
vector proved to be most efficient.24 An alternative approach for 
increasing the efficiency of a dual vector system may be the use of a different AAV serotype. The 
decision to test AAV2 in the present study was based on the 
current clinical use of this serotype.2,4,15 Other serotypes, such as AAV5 and AAV8, have been reported to transduce photoreceptor 
and RPE cells with greater efficiency,42,43 although, in the present 
study, the AAV5-MYO7A single vector was not found to transduce 
these cells with any greater efficiency than the AAV2-MYO7A 
single vector (Figure 2). AAV vectors containing single or multiple 
tyrosine to phenylalanine (Y-F) mutations on their capsid surface 
have been found to increase transduction efficiency and expres-
sion kinetics relative to unmodified vectors, possibly due to 
the ability of the virus to avoid proteosomal degradation.44 The 
increased capacity of an AAV8(Y-F) mutant to restore function and 
prevent photoreceptor degeneration has been shown in two other mouse models of 
retinal disease.45,46 However, in the case of MYO7A therapy, an 
approach that increases the general level of expression of MYO7A protein might also be 
resulted in pathalogy of the higher expressing cells, as already indicated by 
the cultured RPE cells that were treated with the dual vector in the 
present study.

Potential treatment for Usher 1B

The mouse model we have used in the present study contains a 
null mutation for Myo7a.74 Like other Myo7a-mutant mice, and 
indeed most other Usher 1 mouse models, their retinas do not 
degenerate.30,47 Nevertheless, the mouse represents a useful 
model for testing the efficacy of retinal gene therapy for Usher 1B. 
First, MYO7A has been demonstrated to function in the motility of 
melanosomes of human RPE cells, as it does in mouse RPE cells.48 Second, although null MYO7A alleles seem to have less severe 
retinal disease among Usher 1B patients, the primary effect of 
MYO7A mutations is loss of function of the mechanoenzyme.12 Therefore, testing the correction of mutant phenotypes in a 
Myo7a-null mouse, as an indicator of appropriate expression of 
functional MYO7A in the retina, is a valid preclinical approach for 
identifying a treatment to prevent blindness in Ush1B patients.

In the present study, we have shown that single AAV2 or AAV5 
vector delivery of MYO7A to the photoreceptor and RPE cells 
provides effective therapy. Although this approach likely includes 
the production of random fragments of MYO7A, these fragments 
successfully recombined to express functional MYO7A, resulting 
in correction of mutant phenotypes. By contrast, recombination of 
two defined overlapping fragments of MYO7A, delivered by dual 
AAV2 vectors, was found to be inconsistent, with only sporadic 
phenotype correction observed.

**MATERIALS AND METHODS**

**Animals**

Shaker1 mice carrying the 46265b allele, an effective null mutation,7,47 were used on the C57BL6 genetic background and maintained 
and genotyped as described.7,8 They were maintained on a 12-h light/12-h dark cycle, with exposure to 10–50 lux of fluorescent lighting during 
the light phase, and were treated according to NIH and UCLA animal 
care guidelines. Homozygous mutants were distinguished from the
heterozygous controls by their hyperactivity, head-tossing and circling behavior\textsuperscript{13,14} and/or by a PCR/restriction digest assay.

Construction of AAV vectors

Single vector platform. AAV vector plasmid, containing the truncated chimeric CMV/chicken $\beta$-actin promoter (smCB\textsuperscript{A})\textsuperscript{15} and MYO7A cDNA, was constructed by removing the full MYO7A cDNA from pEGFP-C2 by EagI and Sal I digest and ligating into pTR-smCB\textsuperscript{A}-GFP (green fluorescent protein) digested with Nco I and Sal I (to remove GFP). The MYO7A cDNA (6.7 kb) corresponds to isoform 2 of human MYO7A and was the same as that used previously,\textsuperscript{13,14} which was based on the sequence published by Chen et al.\textsuperscript{13} MYO7A isoform 2 is 114-kb shorter than isoform 1.\textsuperscript{13,14} The MYO7A cDNA and resulting junctions were fully sequenced before packaging. Vector plasmid was packaged and titered in AAV2 and AAV5 according to previously published methods.\textsuperscript{20,21} Titters of $10^{12}$–$10^{13}$ particles per ml were obtained for different batches of AAV2-MYO7A and AAV5-MYO7A.

Dual vector platform. Two separate vector plasmids were constructed. Vector A contains the strong, ubiquitous 'smCB\textsuperscript{A}' promoter and MYO7A cDNA encoding the N-terminal portion. Vector B contains MYO7A cDNA encoding the C-terminal portion and a poly-A signal sequence. Each vector plasmid contains both inverted terminal repeats. Using PCR with full-length cDNA of MYO7A isoform 2 (NCBI # NM_0011271780) as template, the MYO7A cDNA was divided roughly in half with ampicillin encompassing nucleotide positions 1–3644 (Vector A) and 2279–6647 (Vector B) relative to ATG start position 1. The resulting two vector plasmids share 1365 bp of overlapping MYO7A sequence and are 5.0 and 4.9 kb in length, respectively, well within the size limitation of a standard AAV vector. Both vector plasmids were sequence verified and separately packaged by standard AAV production methods.\textsuperscript{22,23} The titers of the first lot contained $2.5 \times 10^{12}$ particles per ml of each vector, and the second lot contained 4 $\times 10^{12}$ particles per ml of each vector.

Viral delivery in vitro

HEK293A cells (Innvitrogen, Carlsbad, CA, USA), grown in DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum and 1X NEAA (non-essential amino acids) and Pen/Strep (Innvitrogen) were plated in six-well plates. The next day cells were incubated at 37 °C and 5% CO\textsubscript{2} with AAV2- and AAV5-MYO7A at a multiplicity of infection of 10,000 viral particles per cell in 500 μl of complete medium, containing 100 μM of calpain inhibitor (Roche, Indianapolis, IN, USA). Two hours later, complete medium was added. The next day the medium was changed, and cells were incubated for an additional 48 h. Alternatively, some cells were transfected with 1 μg of vector pTR-smCB\textsuperscript{A}-MYO7A, complexed with Lipofectamine 2000 (ratio 1:3), according to the manufacturer's instructions (Innvitrogen).

Primary mouse RPE cells were derived from P14-P16 Myo7a-null animals and cultured in 24-well dishes, as described.\textsuperscript{8,25} After 48 h in culture, cells were transduced with viruses. Cells were incubated in 100 μl of complete medium, containing 40 μM of calpain inhibitor (Roche, Indianapolis, IN, USA). Two hours later, complete medium was added to each well and incubated overnight. The medium was changed the following day, and cells were incubated for an additional 48 h. ARPE19 cells (ATCC) were cultivated in DMEM/F-12 with 10% fetal bovine serum and split into 24-well plates with glass coverslips. Cells were grown to confluency and then transduced in the same way as it were for the primary RPE cells.

MYO7A expression analysis by western blot and immunofluorescence

HEK293A and primary mouse RPE cells that were transduced with AAV-MYO7A were collected 3 days post-transduction. For western blot analysis, cells were collected and lysed in 20 mM TRIS, pH 7.4, 5 mM MgCl\textsubscript{2}, 10 mM NaCl, 1 mM dithiothreitol and 1 μM protease inhibitor cocktail (Sigma, St Louis, MO, USA). Equivalent amounts of total protein were separated on a 7.5% SDS-PAGE (sodium dodecyl sulfate-polyacylamide gel electrophoresis) gel. After transfer, blots were blocked with 5% non-fat milk and probed with mouse anti-MYO7A antibody, generated against residues 927–1203 of human MYO7A (Developmental Studies Hybridoma Bank, Iowa city, IA, USA),\textsuperscript{26} and mouse anti-actin antibody (Sigma) as a loading control.

Immunofluorescence was performed with ARPE19 and mouse RPE primary cells, 3 days after infection. Cells were fixed in 4% formaldehyde, blocked with blocking solution (0.5% bovine serum albumin/0.05% saponin in phosphate-buffered saline), incubated with the mouse anti-MYO7A followed by goat anti-mouse Alexa-568 (Molecular Probes, Invitrogen). Coverslips were mounted with mounting medium containing DAPI (4,6-diamidino-2-phenylindole; Fluorogel II, Electron Microscopy Sciences, Hatfield, PA, USA) and visualized on a Leica confocal system.

Viral delivery in vivo

Mice were anesthetized with 2.0–3.0% isoflurane inhalation. The pupils of the animals were dilated with 1% (w/v) atropine sulfate and 2.5% phenylephrine. A local anesthetic, 0.5% proparacaine hydrochloride, was also administered. A sclerotomy in the temporal limbus was performed with a 27-G needle. A 32-G blunt needle, attached to a microsyringe pump (WPI, Sarasota, FL, USA), was inserted, and 1 μl of viral solution was injected into the ventral subretinal space of P14-P16 animals. Retinal detachment was visualized under a dissecting microscope and registered as indication of a positive subretinal injection.

Light microscopy and immuno-EM of retinas

Eye cups were processed for embedding in either LR White or Epon, and semithin and ultrathin sections were prepared. Semithin sections were stained to toluidine blue and visualized on a Leica confocal system. Ultrathin sections were labeled with purified MYO7A pAb 2.229 and monoclonal anti-opsin (1D4, R. Molday), followed by gold-conjugated secondary antibodies (Electron Microscopy Sciences), as described previously.\textsuperscript{10} Negative control sections processed at the same time included those from Myo7a-null retinas, and, as positive control, WT animals were used.

MYO7A immunogold density was determined on sections of age-matched WT, Myo7a-null retinas and retinas of Myo7a-null animals that had been injected with AAV-MYO7A at P14-16 and dissected 3 weeks later. For quantification of the immunolabel, all the gold particles in a complete section of each RPE cell were counted. The area of each cell's profile was determined using ImageJ software (http://rsbweb.nih.gov/ij/). For background labeling, the concentration of label in sections of untreated Myo7a-null animals was measured. Data are expressed with this background labeling subtracted.

The concentration of MYO7A and opsin immunogold labeling in the connecting cilia of photoreceptor cells was determined by counting gold particles along longitudinal profiles of connecting cilia and measuring the length of each profile.

Analysis and quantifications were performed in a minimum of three different retinas from three different animals. Statistical analysis was performed using one tailed Student's t-test.

CONFLICT OF INTEREST

WWH and the University of Florida have a financial interest in the use of AAV therapies and own equity in a company (AGTC Inc.), which might, in the future, commercialize some aspects of this work.

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REFERENCES

1 Bainbridge JW, Smith AJ, Barker SS, Robbins S, Henderson R, Balaggan K et al. Effect of gene therapy on visual function in Leber's congenital amaurosis. N Engl J Med 2008; 358: 2240–2248.
2 Maguire AM, Simonelli F, Pierce EA, Pugh Jr. EN, Mingozzi F, Bennicelli J et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. N Engl J Med 2008; 358: 2240–2248.
3 Hauswirth WW, Alemas TS, Kaushal S, Cideciyan AV, Schwartz SB, Wang L et al. Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. Hum Gene Ther 2008; 19: 979–990.
4 Cideciyan AV, Aleman TS, Boye SL, Schwartz SB, Kauhal S, Roman AJ et al. Human gene therapy for RPE65 isomeroreceptor deficiency activates the retinal cycle of vision but with slow rod kinetics. Proc Natl Acad Sci USA 2008; 105: 15112–15117.

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

6 Liu X, Ondek B, Williams DS. Mutant myosin VIIA causes defective melanosome distribution in the RPE of shaker-1 mice. Nat Genet 1998; 19: 117–118.

7 Liu X, Udoidechenko IP, Brown SD, Steel KP, Williams DS. Myosin VIIA participates in opsin transport through the photoreceptor cilium. J Neurosci 1999; 19: 6267–6274.

8 Gibbs D, Kitaromo J, Williams DS. Abnormal phagocytosis by retinal pigmented epithelium that lacks myosin VIIa, the Usher syndrome 1B protein. Proc Natl Acad Sci USA 2003; 100: 6481–6486.

9 Gibbs D, Azarian SM, Lillo C, Kitaromo J, Klomp AE, Steel KP et al. Role of myosin VIIA and Rab27a in the motility and localization of RPE melanosomes. J Cell Sci 2004; 117: 6473–6483.

10 Lopes VS, Gibbs D, Libby RT, Aleman TS, Welsh DL, Lillo C et al. The Usher 1B protein, MYO7A, is required for normal localization and function of the visual retinoid cycle enzyme, RPE65. Hum Mol Genet 2011; 20: 2560–2570.

11 Williams DS, Lopes VS. The many different cellular functions of MYO7A in the retina. Biochem Soc Trans 2011; 39: 1207–1210.

12 Jacobson SG, Cideciyan AV, Gibbs D, Sumaroka A, Roman AJ, Aleman TS et al. Retinal disease course in Usher syndrome 1B due to MYO7A mutations. Invest Ophthalmol Vis Sci 2011; 52: 7924–7936.

13 Chen ZY, Hasson T, Kelley PM, Schwindt BJ, Schwartz MF, Ramakrishnan M et al. Molecular cloning and domain structure of human myosin-VIIa, the gene product defective in Usher syndrome 1B. Genomics 1996; 36: 440–448.

14 Weil D, Levy G, Sahly I, Levi-Acubas F, Blanchard S, El-Amraoui A et al. Human myosin VIIA responsible for the Usher 1B syndrome: a predicted membrane-associated motor protein expressed in developing sensory epithilia. Proc Natl Acad Sci USA 1996; 93: 3322–3327.

15 Bowles DE, McPhee SW, Li C, Gray SJ, Samulski JJ, Camp AS et al. Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. Mol Ther 2012; 20: 443–455.

16 Griefer JC, Samulski RJ. Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. Hum Gene Ther 2012; 23: 46–55.

17 Halbert CL, Allen JM, Miller AD. Efficient mouse airway transduction following recombination between AAV vectors carrying parts of a larger gene. Nat Biotechnol 2002; 20: 697–703.

18 Acland GM, Aguirre GD, Bennett J, Aleman TS, Cideciyan AV, Bennicelli J et al. Next generation of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. Hum Gene Ther 2002; 13: 258–269.

19 Acland GM, Aguirre GD, Bennett J, Aleman TS, Cideciyan AV, Bennicelli J et al. Next generation of adeno-associated virus serotypes: optimized AAV vectors for expression in the retina. Invest Ophthalmol Vis Sci 2007; 48: 377–385.

20 Hasson T, Welch DL, Lillo C, Chen M, Walker ML, Knight PJ et al. A novel allele of myosin VIIA reveals a critical function for the C-terminal FERM domain for melanosome transport in retinal pigment epithelial cells. J Neurosci 2009; 29: 15810–15818.

21 Yang Y, Babbolot TG, Sithinathananth V, Chen M, Walker ML, Knight PJ et al. A FERM domain autoregulates Drosophila myosin 7a activity. Proc Natl Acad Sci USA 2009; 106: 4189–4194.

22 Wu L, Pan L, Wei Z, Zhang M. Structure of MyTH4-FERM domains in myosin VIIA tail bound to cargo. Science 2011; 331: 757–760.

23 Kapranov P, Chen L, Dederich D, Dong B, He J, Steinmann KE et al. Native molecular state of adeno-associated viral vectors revealed by single-molecule sequencing. Hum Gene Ther 2012; 23: 117–127.

24 Auricchio A, Kobinger G, Anand V, Hildinger M, O'Connor E, Maguire AM et al. Exchange of surface proteins impacts on viral vector cellular specificity and transduction characteristics: the retina as a model. Hum Mol Genet 2001; 10: 3075–3081.

25 Allocca M, Manfredi A, Iodice C, Di Vicino U, Auricchio A. AAV-mediated gene replacement, either alone or in combination with physical and pharmacological agents, results in partial and transient protection from photoreceptor degeneration associated with betaPDE deficiency. Invest Ophthalmol Vis Sci 2011; 52: 5713–5719.

26 Zhong L, Li B, Mah CS, Govindasamy L, Agbandje-McKenna M, Cooper M et al. Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. Proc Natl Acad Sci USA 2008; 105: 7827–7832.

27 Boye SL, Conlon T, Erker G, Rylas R, Neeley A, Cossette T et al. Long-term preservation of cone photoreceptors and restoration of cone function by gene therapy in the guanylate cyclase-1 knockout (GC1KO) mouse. Invest Ophthalmol Vis Sci 2011; 52: 7098–7108.

28 Pang JJ, Dai X, Boye SE, Baron I, Boye SL, Mao S et al. Long-term retinal function and structure rescue using capsid mutant AAV8 vector in the rd10 mouse, a model of recessive retinitis pigmentosa. Mol Ther 2011; 19: 234–242.

29 Hasson T, Walsh J, Cable J, Mooseker MS, Brown SDM, Steel KP. Effects of shaker-1 mutations on myosin-VIIa protein and mRNA expression. Cell Motil Cytoskeleton 1997; 37: 127–138.

30 Gibbs D, Diemer T, Khanobadee K, Hu J, Bok D, Williams DS. Function of MYO7A in the human RPE and the validity of the shaker1 mice as a model for Usher syndrome 1B. Invest Ophthalmol Vis Sci 2010; 51: 1130–1135.

31 Gibson F, Walsh J, Mburu P, Varela A, Brown KA, Antonio M et al. Type VII myosin encoded by mouse deafness gene shaker-1. Nature 1995; 374: 62–64.

32 Zolotukhin S, Potter M, Zolotukhin I, Sakai Y, Loiler S, Frazites Jr J et al. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 2002; 28: 158–167.

33 Jacobson SG, Acland GM, Aguirre GD, Aleman TS, Schwartz SB, Cideciyan AV et al. Safety of recombinant adeno-associated virus type 2-RPE65 vector delivered by ocular subretinal injection. Mol Ther 2006; 13: 1074–1084.

34 Gibbs D, Williams DS. Isolation and culture of primary mouse retinal pigmented epithelial cells. Adv Exp Med Biol 2003; 533: 347–352.

35 Soni LE, Warren CM, Bucci C, Orten DJ, Hasson T. The unconventional myosin-VIIa associates with lysosomes. Cell Motil Cytoskeleton 2005; 62: 13–26.