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***myosin 7aa*^{-/-} mutant zebrafish show mild photoreceptor degeneration and reduced electroretinographic responses**

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Abstract

Mutations in myosin VIIa (*MYO7A*) cause Usher syndrome 1B (USH1B), a disease characterized by the combination of sensorineural hearing loss and visual impairment termed retinitis pigmentosa (RP). Although the *shaker-1* mouse model of USH1B exists, only minor defects in the retina have been observed during its lifespan. Previous studies of the zebrafish *mariner* mutant, which also carries a mutation in *myo7aa*, revealed balance and hearing defects in the mutants but the retinal phenotype has not been described. We found elevated cell death in the outer nuclear layer (ONL) of *myo7aa*^{-/-} mutants. While *myo7aa*^{-/-} mutants retained visual behaviors in the optokinetic reflex (OKR) assay, electroretinogram (ERG) recordings revealed a significant decrease in both a- and b-wave amplitudes in mutant animals, but not a change in ERG threshold sensitivity. Immunohistochemistry showed mislocalization of rod and blue cone opsins and reduced expression of rod-specific markers in the *myo7aa*^{-/-} ONL, providing further evidence that the photoreceptor degeneration observed represents the initial stages of the RP. Further, constant light exposure resulted in widespread photoreceptor degeneration and the appearance of large holes in the retinal pigment epithelium (RPE). No differences were observed in the retinomotor movements of the photoreceptors or in melanosome migration within the RPE, suggesting that *myo7aa*^{-/-} does not function in these processes in teleosts. These results indicate that the zebrafish *myo7aa*^{-/-} mutant is a useful animal model for the RP seen in humans with USH1B.

Keywords

Myo7a; Zebrafish; Photoreceptor; Ush1B; Cilium; ERG; Usher Syndrome

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1. Introduction

Usher Syndrome is an autosomal recessive disorder characterized by bilateral sensorineural hearing loss and blindness due to retinitis pigmentosa (RP) (Richardson et al., 2011). It is the most common deafness syndrome associated with blindness, with more than 50% of deaf/blind individuals suffering from a type of Usher Syndrome (Boughman et al., 1983; Rosenberg et al., 1997). Three subtypes of Usher Syndrome have been classified based upon the severity of deafness and the onset of photoreceptor degeneration (Davenport and Omenn, 1977), with Usher type I (USH1) being the most severe. Each subtype is genetically heterogeneous and a number of genes and loci have been identified or mapped (Richardson et al., 2011). The gene responsible for Usher 1B (USH1B) was the first Usher syndrome locus to be cloned and its identification was facilitated by the study of the recessive *shaker-1* mutant mouse. The *shaker-1* mutants exhibit hearing loss, head-tossing, and circling behaviors. Following positional cloning strategies, both the *shaker-1* locus and the USH1b gene in humans had mutations in myosin VIIa (*MYO7A*) (Gibson et al., 1995; Weil et al., 1995). More than 120 different mutations in *MYO7A* cause Usher Syndrome (<http://ghr.nlm.nih.gov/gene/MYO7A>).

Myo7a is an unconventional myosin expressed in numerous epithelial cell types, suggesting a role in multiple cellular processes. In both mice and zebrafish, Myo7a is found in the sensory hair cells, specifically at the apical ends of the stereocilia and at the pericuticular necklace that anchors the bases of the stereocilia, both areas rich in F-actin (Hasson et al., 1995). In the mouse retina, Myo7a has been found in the actin-rich microvilli of the apical domain of the RPE cells (Coffin et al., 2007; Hasson et al., 1995) and in the connecting cilia between the inner and outer segments of the photoreceptors (Wolfrum and Schmitt, 2000). More recent evidence indicates that Myo7a localizes to the calyceal processes (Sahly et al., 2012). Calyceal processes are actin-based microvilli projections that encircle the basal end of photoreceptor outer segments in primates, frogs, and teleosts, but are absent in mice (O'Connor and Burnside, 1981; Sahly et al., 2012). Myo7a has also been found in the kidneys, lungs, testis, intestines, and the olfactory epithelium (Wolfrum et al., 1998).

Myo7a is a Mg^{2+} -mediated ATPase motor protein that moves along actin filaments and binds calmodulin in a Ca^{2+} -sensitive manner (Udovichenko et al., 2002). The presence of an actin-based motor protein in ciliated sensory neurons such as the inner ear and photoreceptors has led to hypothesized functional roles ranging from intracellular protein transport (Liu et al., 1999), phagocytosis (Gibbs et al., 2003), endocytosis and membrane recycling (Richardson et al., 1997), and stereocilia assembly and maintenance (Self et al., 1998). In support of these hypotheses, *shaker-1* mice show evidence of defects in aminoglycoside uptake (Richardson et al., 1999), disorganized hair cell bundles (Self et al., 1998), and mislocalization of rhodopsin in the photoreceptor connecting cilium (Liu et al., 1999).

Whereas Myo7a defects in the inner hair cells explain the auditory and vestibular deficits in *shaker-1* mice, many distinct alleles of *shaker-1* do not show photoreceptor degeneration like USH1b patients, despite the mild mislocalization of rhodopsin (Steel, 1995). The *shaker-1* mice do exhibit some abnormal retinal phenotypes that suggest mechanisms that

lead to blindness. The *shaker-1* ERG a- and b-wave amplitudes were reduced approximately 15–20%, though visual thresholds were unchanged (Libby and Steel, 2001). A recent study compared the rod photoreceptor responses of pigmented *shaker-1* mutants to those placed on an *albino* background (Colella et al., 2013). Whereas pigmented mutants did not show significant differences from their heterozygous siblings, the albino *shaker-1* mutants had age-dependent loss of light sensitivity and slower dark adaptation kinetics after light exposure (Colella et al., 2013). In the RPE, melanosomes failed to extend to the apical processes of the RPE cells in *shaker-1* mice (Gibbs et al., 2004; Liu et al., 1998). Mutants also displayed a slower rate of photoreceptor disk membrane renewal (Liu et al., 1999). The renewal of photoreceptor outer segment disks requires phagocytosis of the distal membranes by the RPE. Given the localization of Myo7a in the apical regions of the RPE (Hasson et al., 1995), it has been suggested that Myo7a plays a role in disk phagocytosis and that defects in this process contributes to the photoreceptor degeneration seen in USH1b patients (Gibbs et al., 2003). Nevertheless, the phenotypes in *shaker-1* mice do not approximate the extent of photoreceptor degeneration seen in Usher Syndrome patients.

Forward genetic screens in zebrafish have identified several mutants affecting hair cell mechanosensation, including several alleles of *mariner*, which has a disrupted *myo7aa* gene (Ernest et al., 2000; Nicolson et al., 1998). Consistent with the *shaker-1* deafness phenotype, *myo7aa*^{-/-} mutants exhibit splayed stereociliary bundles, reduced microphonic potentials, lack of acoustic vibrational sensitivity, and defective endocytosis at the apical surfaces of hair cells (Ernest et al., 2000; Nicolson et al., 1998; Seiler and Nicolson, 1999; Wang and Steyger, 2009). The *myo7aa*^{-/-} mutants are not viable and die between 13–15 days post fertilization (dpf). The mortality likely results from the failure of *myo7aa*^{-/-} mutants to inflate the swim bladder and maintain normal balance while swimming, which inhibits their ability feed effectively. While the effects on inner ear structure and function are characterized, a retinal phenotype of *myo7aa*^{-/-} mutants has not been described.

In the present study, we investigated the histological and physiological phenotypes of the *myo7aa*^{ty220} allele. We report that mild photoreceptor degeneration was observed by 10 dpf and constant light rearing exacerbated photoreceptor degeneration. A reduction in photopic ERG recording suggested defects in outer retinal function. Consistent with the *shaker-1* phenotype, rhodopsin accumulation was seen near the connecting cilium. Retinomotor movements and melanosome migration, however, were normal in *myo7aa*^{-/-} mutants. While the *myo7aa* and *shaker-1* models share many phenotypes, the extent of photoreceptor degeneration we observed indicates that the zebrafish model may be useful model to study USH1b.

2. Materials and methods

2.1 Zebrafish maintenance and husbandry

Five *recessive* alleles of the *myo7aa* mutation were previously identified based upon balance and motor defects (Nicolson et al., 1998). The *myo7aa*^{ty220d} allele results from an A-T nucleotide change, which produces a nonsense mutation (K533X) in the *myo7aa* head domain, and was used for all experiments. Mutants were identified at 5 dpf based on multiple phenotypes, including insensitivity to vibration, the lack of a swim bladder, and

balance defects that result in failure to maintain an upright posture as well as circular looping movements. The *Tg(1.3Xla.Ops:Xla.RhoCT44-GFP)^{a125}* line, hereafter referred to as XOPS:GFP-CT44, was described previously (Perkins et al., 2002). Zebrafish were bred as described by Westerfield (1995). Embryos and adults were raised at 28.5°C on a 14/10 hour light-dark cycle in fish water reconstituted with salts and vitamins. Water containing a 1:1 mix of live paramecium culture (Westerfield, 1995) and fish water was changed daily for embryos and larvae that remained in Petri dishes past 4 dpf. The Harvard University Institutional Animal Care and Use Committee approved all of the protocols.

2.2 Constant light rearing

For constant light treatments, embryos were raised as described by Kainz et al. (2003). Briefly, embryos were placed in constant light starting 2–6 hours post-fertilization (hpf), in transparent Petri dishes sealed with parafilm to prevent evaporation. The dishes were placed on aluminum foil, and illuminated by a 60W incandescent bulb 6 inches above the dishes. A fan was used to dissipate heat generated by the light.

2.3 Light and dark adaptation to assess retinomotor movements

Larvae were dark-adapted by placing fish in complete darkness for two hours prior to fixation and during the first 15 minutes of fixation. Larvae were light-adapted by exposing fish to overhead lights for two hours prior to fixation. All animals were fixed at approximately 5 p.m.

2.4 Optokinetic Response (OKR) assay

A behavioral assay based on the optokinetic reflex (OKR), was performed on 12 light adapted 5 dpf – 10 dpf larvae between the hours of 12 p.m. and 4 p.m. in a darkened room (Brockerhoff et al., 1995). For testing, 2–4 larvae were immobilized in a small Petri dish containing 5% methylcellulose. The Petri dish was positioned in the center of a drum lined with vertical black and white stripes, each 1 cm wide. The drum was illuminated with a tungsten light source at $9.74 \times 10^{-2} \mu\text{W}/\text{cm}^2$ and attenuated by up to 3.5 log units with neutral density filters. The drum was rotated at approximately 8 rpm. During 30 second trials, the direction of drum rotation was changed 4–6 times to elicit a more robust response. Beginning at –3.5 log units, the lowest light level at which a response occurred was determined for each animal. A response was defined as the demonstration of either a smooth pursuit-saccade cycle or eye tracking movements in both the counterclockwise and clockwise directions depending on the rotation of the drum. Larvae that did not show a response at the given light level were retested at 0.5 log unit brighter illumination until the threshold for a response was reached.

2.5 Electroretinograms (ERGs)

Isolated whole-eye electroretinograms (ERGs) were obtained using previously published methods (Kainz et al., 2003; Matsui et al., 2006; Wong et al., 2004). The unattenuated stimulus irradiance was $9.5 \times 10^3 \mu\text{W}/\text{cm}^2$ and was attenuated in log-unit steps by neutral-density filters. The isolated eye was bathed in Mangel's Ringers solution throughout the course of a recording session, which lasted between 30–75 minutes. A-waves were isolated

by bathing the eye in 150 μ M L(+)-2-amino-4-phosphonobutyric acid (L-AP4; Tocris, Ellisville, MO) and 15 μ M DL-threo-beta-benzyloxyaspartate (TBOA, Tocris).

2.6 Histology

At appropriate time points, larvae were euthanized with ice-cold fish water and placed in primary fixative consisting of 1% paraformaldehyde, 2.5% glutaraldehyde in 0.06 M phosphate rinse buffer, 3% sucrose, 0.15mM CaCl_2 at pH 7.4 overnight at 4 °C. Larvae were rinsed in 0.06 M phosphate rinse buffer, post-fixed in 1% osmium tetroxide in 0.06 M rinse buffer for 1 hour at 4 °C and rinsed again. The larvae were dehydrated through a graded ethanol series, followed by three washes of 100% propylene oxide. Larvae were infiltrated with Epon/Araldite resin (Polysciences, Warrington, PA) and embedded for transverse sectioning. Serial 1 μ m sections were collected with glass knives, and heat-mounted onto gelatin-coated glass slides. The sections were stained with 1% methylene blue and 1% azure blue in 1% borax and photographed under brightfield illumination.

Cell death was quantified in 10 dpf animals by counting and noting the location of both pyknotic nuclei and acellular holes in 3–4 centrally located transverse sections of the retina. These sections were chosen such that they were far enough apart from each other that there was no overlap in pyknotic cells or acellular holes present between them. Pyknotic nuclei and acellular holes were also counted in the portion of brain tissue included in the chosen sections to distinguish between nonspecific neuronal death and retinal specific apoptosis. RPE hole areas were measured using ImageJ.

2.7 Transmission electron microscopy (TEM)

Larvae were also fixed and embedded as described above for electron microscopy. Transverse sections (60–80 nm) of the central retina were stained with lead citrate and uranyl acetate. Photographs were obtained with a JEOL transmission electron microscope (JEOL USA, Peabody, MA) and the negatives scanned using a UMAX PowerLook 3000 scanner and adjusted using Adobe Photoshop software (v. 7.0).

2.8 TUNEL analysis

For TUNEL analysis, 10 dpf larvae were euthanized in ice cold fish water and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.0 (Sambrook, 1989), overnight at 4 °C. After rinsing in PBS, the larvae were cryoprotected in 35% sucrose in PBS at 4 °C. They were embedded with 10 0% TBS Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) in freezing molds and placed at 20°C until frozen. Cryosections (10 μ m) were mounted onto gelatin-coated glass slides. Apoptotic cells were identified using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay kit according to manufacturer's protocol (Enzo-Boehringer Mannheim, Indianapolis, IN). Slides were also counterstained with DAPI to label DNA. Digital images were collected using a Zeiss Meta 510 confocal microscope (Carl Zeiss Microscopy, Thornhill, NY).

2.9 Immunohistochemistry

Larvae were fixed, frozen, sectioned, and dehydrated as described above for the TUNEL analysis. Sections were then rehydrated in PBST (PBS + 0.1% Tween-20) for 15 minutes

and outlined with a hydrophobic PAP pen. Slides were blocked in 5% normal goat serum, 0.1% DMSO in PBST for 2 hours and incubated with the primary antibody solution overnight at 4°C. After PBST washes, slides were incubated with fluorescently conjugated secondary antibodies, diluted 1:500, and counterstained with Hoescht 33258 or DAPI to label DNA. Digital images were collected with a confocal microscope. The following is a list of primary antibodies used, dilution, and cell types that possessed the antigens: 1D1 (1:500) rods (Fadool, 1999), rhodopsin (1:200) rods (Vihtelic et al., 1999), Zpr1 (1:100) red-green double cones (Larison and Bremiller, 1990), UV opsin (1:200) UV cones (Vihtelic et al., 1999), green opsin (1:200) green cones (Vihtelic et al., 1999).

2.10 In situ hybridization

Two fragments (392 and 445 bp) of the zebrafish *myo7aa* cDNA were amplified by PCR and verified by sequencing. Each fragment differed by at least 30% from *myo7ab* at the nucleotide level. Digoxigenin-labeled riboprobes were synthesized to both fragments and pooled together and in situ hybridization was performed using standard methods (Jowett, 2001).

2.11 Statistics

All statistics and graphs were made using GraphPad Prism 6. Unless otherwise noted, all statistics were done using Unpaired Student's t-test with Welch's correction.

Results

3.1 Zebrafish *myo7aa*^{ty220d} mutants exhibit elevated photoreceptor cell death

All experiments were done on the *ty220d* allele of *myo7aa* (*mariner*), which introduces a nonsense mutation at amino acid 533 of the zebrafish protein. This mutation (K533X) is located in the head region before the actin-binding domain. Of the five known *myo7aa* alleles, this mutation results in the shortest truncated protein and is one of three nonsense alleles that produce the most severe splaying of sensory hair cell bundles (Ernest et al., 2000). Mutants were identified at 5 dpf primarily by a balance defect phenotype, which results in failure to maintain an upright posture and induces circular looping movements. Both wild-type and heterozygous siblings were phenotypically wild-type and are hereafter referred to as wild-types.

At 5 dpf, the *myo7aa* gene was expressed in the eye, ear, brain, and in neuromasts of the lateral line (Fig. 1A, B). These results are consistent with the widespread expression of mouse *Myo7a* throughout the brain and retina at postnatal day 7 (Magdaleno et al., 2006). Histological sections of retinas from 5 dpf larvae revealed expression in the inner and outer nuclear layers. A pronounced band of expression was observed at the most basal level of the outer nuclear layer, where rod nuclei are located (Figs. 1C, D), although cone expression cannot be ruled out.

Retinal histology of *myo7aa*^{-/-} mutants showed normal cellular organization at both 5 and 10 dpf when observed by light microscopy. At 5 dpf, the *myo7aa*^{-/-} retinas were morphologically indistinguishable from wild-type retinas and no signs of cell death were

observed in the outer nuclear layer or other cellular layers (data not shown). Histological sections of retinas from 10 dpf wild-type and *myo7aa*^{-/-} mutants also showed normal retinal organization (Fig. 2A, B). While we occasionally observed single pyknotic nuclei in semi-thin sections of wild type retinas, a significant number of acellular holes in the ONL, particularly ventrally, were present in 10 dpf *myo7aa*^{-/-} retinas (Fig. 2C, D). Cell death was quantified by combining the number of pyknotic nuclei and acellular holes found in 10 dpf wild type and *myo7aa*^{-/-} retinas (Fig. 2E). Quantification of cell death revealed a 3-fold increase in *myo7aa*^{-/-} retinas (3.4 ± 0.3 ; $n = 16$) compared to wild-type retinas (0.9 ± 0.3 , $n = 11$; $p < 0.001$). There was no significant difference in the number of pyknotic nuclei and acellular holes in the brain tissue present in each section used for retinal cell counts (data not shown). This result indicated that the cell death observed in the retina was not a consequence of nonspecific neuronal degeneration due to poor health of the *myo7aa*^{-/-} mutants. Degeneration was limited to the ONL, and most of the cell death was located in the ventral patch, where there is a high concentration of rod photoreceptors. Non-degenerating photoreceptors at this age extended their outer segments as normal into the RPE in mutant animals.

To confirm that cell death occurred via apoptosis in the *myo7aa*^{-/-} retina, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis was performed at 10 dpf. TUNEL-positive cells were analyzed by confocal microscopy and quantified in single confocal optical sections. An increase in total cell death was observed in *myo7aa*^{-/-} retinas relative to wild type retinas (1.2 vs. 0.4 cells/section; $p < 0.0003$; $n = 6$ or more retinas). These results generally agree with a previous study, which found that fewer than 3 cells per 40 μm -thick retinal section undergo apoptosis in 9 dpf zebrafish (Biehlmaier et al., 2001). In contrast to our finding that degeneration was preferentially restricted to the ventral retina, the number of TUNEL positive cells in the ventral and dorsal ONL of *myo7aa*^{-/-} mutants was very similar (Fig. 3C). These data, along with the acellular holes in the 10 dpf *myo7aa*^{-/-} retina as observed by semi-thin sections, indicate that cell death is first detectable before the 10 dpf time point in *myo7aa*^{-/-} mutants, somewhere in the range of 6–8 dpf.

2.10 Impaired visual function in *myo7aa*^{-/-} larvae

To determine whether the *myo7aa*^{-/-} mutant exhibits physiological defects in the outer retina, we compared electroretinogram responses (ERGs) of dark-adapted 5 and 10 dpf wild-type and mutant larvae to 800–1000 msec full-field light stimuli of increasing intensities, beginning at intensities below threshold. Light evokes a corneal negative potential, the a-wave, generated by photoreceptor hyperpolarization (Dowling, 2012). The a-wave is immediately followed by a positive b-wave, which is generated largely from the second-order ON-bipolar cells postsynaptic to photoreceptors (Dowling, 2012). At the end of the light stimulus, the OFF-bipolar cells generate a positive d-wave, which can be observed following long light impulses. Compared to mice, the larval zebrafish a-wave is largely hidden by the b-wave (Brockerhoff et al., 1995). Representative ERGs from individual wild type and *myo7aa*^{-/-} animals are shown (Fig. 4A).

To directly compare the light responses of wild-type and *myo7aa*^{-/-} mutants, the ERG a-wave and b-wave amplitudes were normalized to the wild-type response at the highest intensity. As the bipolar cell activity depends largely on photoreceptor responses to light, we first used the normalized b-wave amplitudes to assess outer retina function. Animals were exposed to dim light stimuli of increasing intensities until a response was observed. At 5 dpf and 10 dpf (Fig. 4B, top row), most wild type and some of the *myo7aa*^{-/-} animals both responded to the same low intensity flash of light (log I = -3), but the b-wave amplitude elicited from *myo7aa*^{-/-} mutants was significantly reduced at -1 and 0 intensities (Fig. 4B; p<0.05, n = 6–12). The *myo7aa*^{-/-} mutants also showed reduced d-wave amplitudes at the -2 and -1 log intensity levels at both time points (data not shown). Interestingly, wild type and *myo7aa*^{-/-} mutants had similar response thresholds. To test whether the decreased outer retina activity was in part mediated by a decrease in photoreceptor function, the a-wave was isolated by superfusing zebrafish eyes with Ringers containing 150 μ M LAP-4 and 15 μ M TBOA (Matsui et al., 2006; Wong et al., 2004). L-AP4 is a group III metabotropic glutamate receptor agonist, which blocks the light response of rod-driven ON bipolar cells (Kay et al., 2004). TBOA is an inhibitor of glutamate transporters and blocks the responses of cone On-bipolar cells in fish (Grant and Dowling, 1995; Wong et al., 2004). The b-wave is completely abolished when isolated larval zebrafish eyes were treated with L-AP4 and the EAAT inhibitor TBOA (Kainz et al., 2003; Shimamoto et al., 1998; Wong et al., 2004). The remaining response consisted of a sustained, negative-going potential and the positive d-wave. At both 5 dpf and 10 dpf, the normalized a-wave amplitude was reduced in *myo7aa*^{-/-} mutants when compared to wild type larvae at the -1 and 0 intensities (Fig. 4B; p<0.05, n = 6). These data suggest that the responsiveness (i.e. gain) of mutant photoreceptors is decreased at brighter light intensities.

To determine if the reduced photoreceptor responses of the *myo7aa*^{-/-} mutants affected visual behavior, we tested dark-adapted wild-type and *myo7aa*^{-/-} mutants for an optokinetic reflex (OKR) at increasing light intensities over several days (Brockerhoff et al., 1995). Fish were tested daily from 5 dpf, when the *myo7aa*^{-/-} mutants can first be reliably isolated, to 10 dpf. With daily feedings and water changes, the circulation and heart rate of *myo7aa*^{-/-} mutants remained healthy through 10 dpf. Without supplemental feedings, the mutation is lethal by 12–13 dpf (Nicolson et al., 1998). While both wild-type and *myo7aa*^{-/-} animals displayed an OKR on all days tested, a brighter light was required to elicit a response from *myo7aa*^{-/-} larvae (Fig. 4C). Typically, the *myo7aa*^{-/-} OKR response threshold was higher by approximately 0.5 log units. Though the difference was subtle and not statistically significant, the trend was consistent throughout. We conclude that the *myo7aa* mutation does not cause complete loss of visual function at these early developmental time points, though some visual impairment is present.

3.3 Photoreceptor markers reveal differences in *myo7aa*^{-/-} retinas

Mislocalization of opsin can lead to photoreceptor cell death, and defects in opsin transport have been observed in the *shaker-1* mice (Liu et al., 1999). To investigate if the cell death seen in *myo7aa*^{-/-} mutants was a result of abnormal protein transport, we stained cryosections of 8 dpf retinas with a number of rod and cone-specific antibodies. We also bred *myo7aa*^{-/-} mutants to the *Tg(xops:GFP-CT44)* transgenic line, which uses the

Xenopus opsin promoter to express a fusion of GFP to the C-terminal 44 amino acids of *Xenopus* rhodopsin (Perkins et al., 2002). The GFP-CT44 fusion protein localizes exclusively to the rod outer segments and serves as an effective marker of normal protein trafficking at all stages of development. The onset of *Tg(xops:GFP-CT44)* transgene expression is slightly delayed, however, and not all rod photoreceptors express the GFP-CT44 transgene during larval stages (Perkins et al., 2002). Rod opsin distribution was assayed by staining cryosections of GFP-CT44 expressing cells with a polyclonal antibody against zebrafish rhodopsin (Fig. 5A, B). We observed a reduction in both the number of GFP-CT44 expressing cells and in the number of cells positively stained for rhodopsin in the *myo7aa*^{-/-} mutants (Fig. 5A, B). The retina consistently showed reduced ventral and dorsal rhodopsin labeling, and little to no labeling in the ONL of the central retina (Fig. 5B). Although this may reflect rod photoreceptor death, we cannot rule out the alternative possibility that this reflects a subtle delay in rod maturation.

During normal development, rhodopsin can be found in the plasma membrane and synaptic terminals before being restricted exclusively to the outer segment (Fig. 5A). This staining pattern can conceal differences in protein localization caused by abnormal trafficking. The monoclonal antibody 1D1 stains rod photoreceptors in a pattern very similar to rhodopsin but does not stain rod cell bodies or terminals in larvae like the opsin antibody (Fadool, 1999). In wild-type retinas, almost all 1D1 localization (Fig. 5C, red) was restricted to the rod outer segments and co-localized with the GFP-CT44 protein. In *myo7aa*^{-/-} retinas, most of the 1D1 staining and GFP-CT44 fluorescence was observed in the outer segment but more accumulation was also seen in small aggregates at the base of the outer segment, in or near the connecting cilium (arrows in Fig. 5D). This result is reminiscent of the opsin mislocalization seen in the immunoelectron microscopy from *shaker-1* mice (Liu et al., 1999). In both instances, the majority of rod opsin was found in the outer segment but a noticeable mislocalization is seen in *myo7aa*^{-/-} mutants.

The monoclonal antibody zpr1 recognizes Arrestin-3 on the surface of the red-green double cones (Ile et al., 2010; Larison and Bremiller, 1990). In both wild type and *myo7aa*^{-/-} retinas, zpr1 labeled the entire cell bodies of the red-green double cones, and robust staining was observed across the entire ONL (Fig. 5E, F). Furthermore, no noticeable difference was seen when labeling with antibodies against green opsin (Fig. 5G, H) or UV opsin (Fig. 5I, J). Interestingly, the blue cone opsin was partially mislocalized in *myo7aa*^{-/-} retinas and found in the cone inner segment as well as the outer segment (arrows in Fig. 5L).

3.4 Light rearing increased cell death and caused RPE abnormalities in the *myo7aa*^{-/-} retina

As some mouse models of degeneration have shown that increased light exposure to the photoreceptors affects the onset and rate of degeneration (Chen et al., 1999; LaVail et al., 1999; Naash et al., 1996; Sanyal and Hawkins, 1986), we asked if increased light exposure would exacerbate photoreceptor damage in the *myo7aa*^{-/-} mutants. To increase light exposure to photoreceptors during the limited life span of *myo7aa*^{-/-} mutants, larvae were raised under constant light conditions (LL) beginning as blastula stages (2–4 hours post-fertilization). The retinas of LL wild-type animals had an elevated number of pyknotic

nuclei and acellular holes when compared to normally raised wild-type animals (Compare Fig. 6G and Fig. 6E; 3.2 ± 0.5 vs. 0.9 ± 0.3 ; $n = 9$ retinas), indicating a small level of light damage. Several changes were observed in the LL *myo7aa*^{-/-} animals at 10 dpf when compared to LL wild-type retinas (Fig. 6A–F). The LL *myo7aa*^{-/-} animals showed significantly more pyknotic cells and acellular holes in the ONL (Fig. 5G; wt = 3.2 ± 0.5 , *myo7aa*^{-/-} = 7 ± 1.4 , $p < 0.05$; $n = 9$ retinas). All LL *myo7aa*^{-/-} retinas contained large holes in the RPE and a number of smaller holes, which were more numerous than what was observed in LL wild-type retinas (Fig. 6H; wt = 2.4 ± 1.1 , *myo7aa*^{-/-} = 10.4 ± 1.1 , $p < 0.0001$; $n = 10$ retinas). These holes were typically concentrated in either the ventral or dorsal area in a given retina, although on rare occasions, holes were seen both dorsally and ventrally in the same retina. The largest RPE holes were never present in wild-type retinas, although smaller holes were occasionally observed. Furthermore, the RPE holes in *myo7aa*^{-/-} mutants were more than three-fold larger in area, regardless of the total number of holes (Fig. 6I, wt = $9.7 \pm 1.9 \mu\text{m}^2$, *myo7aa*^{-/-} = $35.2 \pm 7.0 \mu\text{m}^2$) These results indicate that light promotes photoreceptor and RPE degeneration in the *myo7aa*^{-/-} mutants.

3.5 Histology of light- and dark-adapted retinas showed normal retinal motor movements

From mouse studies, organelle transport represents one of the few known *in vivo* roles for Myo7a. Studies of the *shaker-1* mouse mutant revealed that Myo7a regulated melanosome distribution in the RPE (Gibbs et al., 2004; Liu et al., 1998) and that phagosome clearance from the apical RPE is considerably slowed (Gibbs et al., 2003). In teleosts, including adult zebrafish, changes in light exposure result in significant morphological changes in both the photoreceptors and RPE known as retinomotor movements. In the light-adapted state, pigment granules extend into the apical RPE processes between photoreceptor outer segments. Meanwhile, rod myoids elongate and cone myoids contract. In the dark-adapted state, the opposite occurs: pigment granules retract to the basal part of the RPE, while rod and cone myoids contract and elongate, respectively (Levinson and Burnside, 1981; Menger et al., 2005). In larval zebrafish, the development of rod and cone myoid movements occurs after 28 dpf but the migration of RPE melanosomes into the apical processes can be observed as early as 5 dpf (Hodel et al., 2006). To determine if pigment migration required Myo7aa, we examined 5 dpf wild-type and *myo7aa*^{-/-} larvae following light-adaptation or dark-adaptation (Fig. 7). The RPE melanosomes were retracted in both dark-adapted wild-type and *myo7aa*^{-/-} larvae (Fig. 7A, B). Also, light-adapted wild-type and *myo7aa*^{-/-} larvae showed melanosome extension into the RPE apical processes (Fig. 7C, D). Similar results were observed at 10 dpf (data not shown). This was especially visible in the ventral patch because of the high concentration of rod photoreceptors with long outer segments in this region. Thus, Myo7aa does not appear to regulate melanosome migration in zebrafish.

3 Discussion

Our data show that the loss of *myo7aa* in zebrafish results in a number of phenotypes characteristic of the photoreceptor degeneration observed in USH1B patients. Previous reports established that *myo7aa*-deficient zebrafish exhibited auditory and balance defects like those seen in humans, yet the retinal phenotype was not investigated (Nicolson et al., 1998). In *myo7aa*^{-/-} mutants, mild photoreceptor degeneration occurs within 10 dpf, and

light rearing can exacerbate photoreceptor degeneration. Rod, but not cone, specific markers were also reduced. Interestingly, rod photoreceptors contribute little to overall visual function in zebrafish before 2 weeks of age (Bilotta et al., 2001; Moyano et al., 2013), and yet significant reductions in the ERG a- and b-wave responses were observed in *myo7aa*^{-/-} mutants, indicating loss of cone photoreceptor function.

The results presented here show a number of similarities, but also some critical differences with the retinal phenotypes found in the *shaker-1* mouse. First, both *myo7aa*^{-/-} and *shaker-1* show abnormalities in ERG recordings. While visual thresholds remain unchanged, the amplitudes of the a- and b-waves were reduced at high light intensities in both animal models (Libby and Steel, 2001), this study), suggesting a defect in photoreceptor gain. Second, while most rhodopsin is transported to the outer segments, a small amount of protein accumulates at the base of the connecting cilium, indicating problems with rhodopsin trafficking. This phenotype was also observed in the *shaker-1* mouse (Liu et al., 1999). However, with the exception of blue opsin, none of the cone visual pigments were mislocalized. Why this phenotype was observed only in blue cones, but not with other cone subtypes is unknown. As noted previously (Libby and Steel, 2001), the ERG abnormalities do not reflect greatly reduced opsin concentrations, as this would lead to increased visual thresholds (Dowling, 1960). As Myo7a is located at the connecting cilium of photoreceptors and possesses an ATPase motor activity, it would appear that the function of this protein would be to transport cargo through the outer segment (Liu et al., 1997; Udovichenko et al., 2002). Two pieces of evidence argue against this hypothesis. First, the phenotypes observed in both mouse and fish do not support the notion of Myo7a as the primary motor for rhodopsin transport. Second, and perhaps more significant, a large body of evidence indicates that microtubule-based kinesin motors regulate ciliary trafficking (Insinna et al., 2009; Insinna et al., 2008; Jimeno et al., 2006; Marszalek et al., 2000; Trivedi et al., 2012; Tsujikawa and Malicki, 2004). Thus, our results complement previous work from the *shaker-1* mouse that indicates a requirement for Myo7a in normal photoreceptor function, although the exact function of Myo7a remains elusive.

The cause of retinal pathology in humans with USH1 mutations remains unclear. Myo7a is the only USH1 protein to be expressed in photoreceptors and the RPE (Sahly et al., 2012). The RPE supports the neural retina by providing several critical functions for photoreceptors including 11-cis retinal for visual pigment regeneration, and phagocytosis of spent outer segment material (Strauss, 2005). Further, dysfunction of the RPE leads to photoreceptor death (D'Cruz et al., 2000; Dowling and Sidman, 1962; Mullen and LaVail, 1976). Primary cultures of RPE from *shaker-1* mice degrade photoreceptor outer segments slower than control RPE (Gibbs et al., 2003), and incomplete degradation, and thus clearance, of photoreceptor material by the RPE can lead to retinal dystrophy (Dowling and Sidman, 1962; Okubo et al., 2000). The inability to degrade photoreceptor outer segments could lead to RPE degeneration and subsequent photoreceptor cell death in USH1B patients. Consistent with this hypothesis, we observed holes in the RPE of *myo7aa*^{-/-} retinas following light-damage. Membranes or other lipid material was not observed in these holes, suggesting that the RPE itself was degenerating. However, the presence of Myo7a and other USH1 proteins in the calyceal processes of frogs and primates have raised the intriguing possibility that the

USH1 protein network may function as an adhesion belt necessary for outer segment disk formation and stability (Sahly et al., 2012). Photoreceptors from teleost fishes, including zebrafish, are also surrounded by calyceal processes. Indeed, zebrafish mutants in the USH1 genes *myo7aa* (this study) and *harmonin* (*ush1c*) (Phillips et al., 2011) both show defects in photoreceptor function. Studies of the *harmonin* mutants suggest a primary defect in the Müller glial cells of the retina that caused a subsequent defect in ribbon synapse function (Phillips et al., 2011), while this study indicates a photoreceptor and RPE defect.

Perhaps the most interesting difference between *myo7aa*^{-/-} and the *shaker-1* mouse model is the degree of photoreceptor degeneration in the zebrafish, which is exacerbated by constant light rearing. Constant light causes additional stress on the photoreceptors and RPE by increasing chromophore recycling and causing photic damage to outer segment proteins. Increased exposure to elevated light levels may also increase metabolic stress and oxidative damage. Increasing light exposure, therefore, should reduce the time needed to cause degeneration. Indeed, the mild degeneration observed in *myo7aa*^{-/-} zebrafish was exacerbated by increasing overall light exposure over the course of only 10 days. A recent study found that exposure to moderate light (2500 lux) led to a reduction in photoreceptor number in *shaker-1* mice, although the effects on the RPE were not mentioned (Peng et al., 2011). It was noted, however, that the *Myo7a*^{sh1-11j} allele used in that study were backcrossed nine times to the 129 Sv/J background and carried the L450 RPE65 quantitative trait locus that increases sensitivity to light damage (Danciger et al., 2000; Redmond et al., 2007; Wenzel et al., 2001). The similarities in light-induced photoreceptor degeneration observed in the *myo7aa*^{-/-} and *shaker-1* models warrants further study, although the light-dependent degeneration should be confirmed in different alleles of the *shaker-1* mouse.

One goal of investigating animal models for a human disease is to design and test appropriate clinical therapies. Further characterization of the retinal phenotype of the *myo7aa*^{-/-} mutant, particularly the role of the RPE and calyceal processes, will offer insight into how loss of Myo7a contributes to photoreceptor damage in this mutant. The results obtained from the characterization of the *myo7aa*^{-/-} mutant suggest that it is an excellent model system in which to continue to search for a greater understanding of disease pathology.

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Highlights

- Zebrafish *myosin 7aa*^{-/-} mutants exhibit elevated photoreceptor death
- The ERG a- and b-waves are reduced in zebrafish *myosin 7aa*^{-/-} mutants
- Light exposure leads to severe photoreceptor and RPE degeneration
- Loss of *myosin 7aa*^{-/-} does not affect melanosome migration or retinomotor movements

Figure 1. Expression of *myo7aa* in neural tissues in 5 dpf zebrafish

(A, B) Whole-mount in situ hybridization of 5 dpf larvae shows expression in eyes, ear, brain and in neuromasts (arrows). (C, D) Expression in the retina is seen in inner nuclear layer (INL) and outer nuclear layer (ONL). Scale bar = 500 μ m (A), 250 μ m (B), 100 μ m (C), 20 μ m (D).

Figure 2. Outer retina cell loss occurs in *myo7aa*^{-/-} mutants

(A and B) Transverse histological sections of retinas from 10 dpf wild-type and *myo7aa*^{-/-} larvae revealed no significant changes in gross retinal morphology. (C and D) Higher magnification of the boxed areas in A and B revealed occasional pyknotic cells and acellular holes (arrows) in the ventral outer retina of *myo7aa*^{-/-} mutants. (E) Quantification of pyknotic nuclei and acellular holes showed a significant increase in *myo7aa*^{-/-} retinas (*p<0.001; Unpaired Student's *t*-test). Error bars = SEM. Scale bar = 50μm (A and B) and 10 μm (C and D).

Figure 3. Increased TUNEL staining in *myo7aa*^{-/-} retinas (A and B) TUNEL staining (green) on cryosections of 10 dpf zebrafish retinas revealed apoptotic cells in the outer nuclear layer (arrows). Sections were counterstained with DAPI (pseudocolored red). (C) Quantification of TUNEL-positive cells showed a significant increase in *myo7aa*^{-/-} retinas (*p<0.004, **p<0.0003; Unpaired Student's t-test with Welch's correction). Error bars = SEM. Scale bar = 50 μ m (A and B)

Figure 4. *myo7aa*^{-/-} mutants show reduced visual function

(A) Representative ERG recordings from wild-type (left) and *myo7aa*^{-/-} mutants (right) under constant background illumination following a 1000 millisecond light stimuli at increasing light intensities over a range of 4 log units. (B) Normalized ERG b-wave (top) and a-wave amplitudes (bottom) to increasing light stimuli. Both the b-wave and a-wave amplitudes were reduced in the *myo7aa*^{-/-} mutants, although thresholds were unchanged (*p<0.05, Student's *t*-test; *n* = 6–10 larvae per condition). (C) Dark adapted optokinetic response (OKR) in 5–10 dpf wild-type and *myo7aa*^{-/-} larvae (*n* = 12 larvae; *p*>0.05). During a 30-second trial, the light intensity required to generate a positive OKR response was determined (see Materials and Methods for criteria). The unattenuated light stimulus (*log I* = 0) was $9.74 \times 10^{-2} \mu\text{W}/\text{cm}^2$. In all cases, error bars = SEM.

Figure 5. Photoreceptor outer segment proteins show abnormal localization in *myo7aa*^{-/-} mutants

Transverse cryosections of 8 dpf retinas were stained with markers for rod and cone photoreceptors. (A and B) Immunoreactivity from polyclonal antibodies against rhodopsin (red) and fluorescence from the XOPS:GFP-CT44 transgene (green) label rod photoreceptor outer segments. (C and D) Immunoreactivity from a monoclonal antibody against rhodopsin (1D1, red) and fluorescence from XOPS::GFP-CT44 transgene (green). Rhodopsin mislocalization is observed near the connecting cilium (arrows). (E and F) Zpr-1 immunoreactivity labels red-green double cones (red). Polyclonal antibodies against green opsin (G and H), UV opsin (I and J), and blue opsin (K and L) labeled cone outer segments. Partial mislocalization of blue opsin was seen near the connecting cilium in *myo7aa*^{-/-} mutants (L, arrows). All sections were counterstained with DAPI to label nuclei (blue). Scale bar = 80 μ m (A, B, E, F) and 20 μ m (C, D, G–L).

Figure 6. Constant light exposure causes photoreceptor damage and death in *myo7aa*^{-/-} mutants Transverse histological sections of retinas from 10 dpf wild-type (A and B) and *myo7aa*^{-/-} larvae (C–F) reveal outer retina damage in *myo7aa*^{-/-} retinas following constant light exposure. (B, D, F) Higher magnifications of boxed areas show regions of cell death or degeneration in both the photoreceptor layer and in the RPE (arrows). (G) Quantification of pyknotic nuclei and acellular holes showed a significant increase in light-damaged *myo7aa*^{-/-} retinas. (H) Quantification of the number of RPE holes per retina section following light damage. (I) Quantification of the average area of holes in the RPE of light-damage retinas. Results were compared using an Unpaired Student's t-test with Welch's correction. *p<0.05, **p<0.001, ***p<0.0001 Error bars = SEM. Scale bar = 50 μ m (A, C, E) and 10 μ m (B, D, F).

Figure 7. Melanosome migration occurs normally in *myo7aa*^{-/-} mutant RPE cells

(A and B) Transverse histological sections of retinas from 5 dpf wild-type and *myo7aa*^{-/-} larvae following two hours of dark adaptation (DA) or (C and D) light adaptation (LA). The dashed line indicates the base of the outer segments. Note the distribution of melanosomes into the RPE apical processes adjacent to the base of photoreceptor outer segments in light adapted retinas and the gap between the melanosomes and the base of the outer segments in dark adapted retinas. Scale bar = 10 μm.