

**USING ZEBRAFISH MODELS OF USHER SYNDROME
TYPE 2A TO INVESTIGATE
RETINAL CELL FUNCTION AND SURVIVAL**

by

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Monte Westerfield

Usher syndrome is the main cause of hereditary deaf-blindness. Patients diagnosed with Usher syndrome experience hearing loss and progressive blindness due to photoreceptor degeneration over several decades. The most common form of Usher syndrome, type 2A, is caused by mutations in the *USH2A* gene, which encodes the large protein Usherin. In this study, I characterized three different mutations affecting distinct regions of the zebrafish *ush2a* gene. In these mutant backgrounds, I studied the co-localization of other proteins known to interact with Usherin to test whether the loss of the normal Usherin protein disrupted localization of these other proteins. In humans, photoreceptor cell death takes place over many years, causing slow, progressive vision loss. The *USH2A* mouse model of Usher syndrome has a mild retinal phenotype compared to the degree of vision loss that human patients experience, perhaps due in part to their shorter lifespan. Zebrafish, similarly, have a much shorter lifespan than humans, so we devised a system that would challenge the retina with increased levels of light in an effort to accelerate the rate of photoreceptor damage. These studies have established a functional zebrafish model of *USH2A* induced Usher syndrome, which will provide researchers with a valuable tool for investigating treating the functional role of Usherin in vision and hearing, as well as providing a model for which to test new treatment options.

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INTRODUCTION

I completed my thesis in the Westerfield lab, which uses zebrafish as a model for studying Usher syndrome. Usher syndrome is the most common cause of hereditary deaf blindness and has no cure (van Wijk et al. 2004). It is estimated that somewhere between 1/17,000 to 1/25,000 people are affected worldwide (Boughman et al. 1983; Rosenberg et al. 1997), but a more recent study by Kimberling et al. (2011) suggests that this number is more like 1/6,000. Usher syndrome is a hereditary disorder, which means that it is passed genetically from parent to child. To put this in perspective, the incidence of cystic fibrosis is about 1/3,500 in the U.S and the incidence of Huntingtons disease is somewhere between 1/14,000 to 1/20,000 (“Monogenic diseases”). Usher syndrome is recessive, so the child must inherit a copy of the mutated gene from each parent. Children who have one normal copy of an Usher syndrome gene and one mutated copy will have normal hearing and vision, but can potentially pass on the condition to their progeny (“Usher Syndrome” 2014). Currently, there are eleven known genes that can cause Usher syndrome when mutated. Usher syndrome is classified into three subtypes: type 1, 2, and 3, and each has slightly different manifestations of the main symptoms of hearing and vision loss (Millán et al. 2011).

My project focused on the gene implicated in Usher syndrome type 2A (*USH2A*), which encodes the large multidomain protein Usherin. Patients diagnosed with *USH2A* have congenital hearing impairment that ranges from moderate to severe and experience progressive vision loss beginning in the second decade of life (“Usher Syndrome” 2014). This progressive vision loss, called retinitis pigmentosa (RP), is due to degeneration of the photoreceptor cells in the retina, which causes night blindness and tunnel vision

(Keats and Lentz 2013). Mutations in the *USH2A* gene are responsible for the most common form of Usher syndrome. For zebrafish to be a good model of *USH2A*, the zebrafish models of *ush2a* must have defects similar to those seen in the human disease.

The main retinal cell type affected by Usher Syndrome is the photoreceptors. These are specialized sensory cells that consist of two main parts, the inner segment and outer segment, which are connected by the connecting cilium. Proteins that are made in the inner segment are trafficked to the outer segment, where the majority of photoreceptor cell function occurs. Previous studies have established that Usherin localizes at the base of the connecting cilium in photoreceptor cells (figure 1) (Yang et al. 2012). Usherin is tethered to the cell membrane surrounding the cilium via its transmembrane domain (figure 2A). Here, Usherin forms a complex with other USH proteins that is hypothesized to facilitate the loading of molecular cargo onto the ciliary transport system (Liu et al. 2007). When the function of Usherin protein is defective, progressive photoreceptor degeneration is the result.

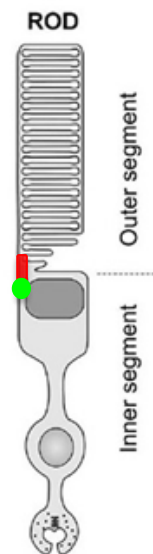


Figure 1. This image depicts a photoreceptor cell in the retina. Usherin is shown with a green dot where it is known to localize at the base of the connecting cilium between the outer and inner segments (red). Other USH proteins are also known to localize at the base of the connecting cilium.

Although gene therapies for some forms of Usher syndrome are being actively tested, current gene replacement methods are not feasible for *USH2A* patients due to the large size of the *USH2A* gene, which is comprised of 72 exons encoding over 5,200 amino acids (Williams 2008). Having a better understanding of the different regions of the Usherin protein will assist future researchers in identifying the critical functional domains that would need to be included in gene replacement therapy efforts.

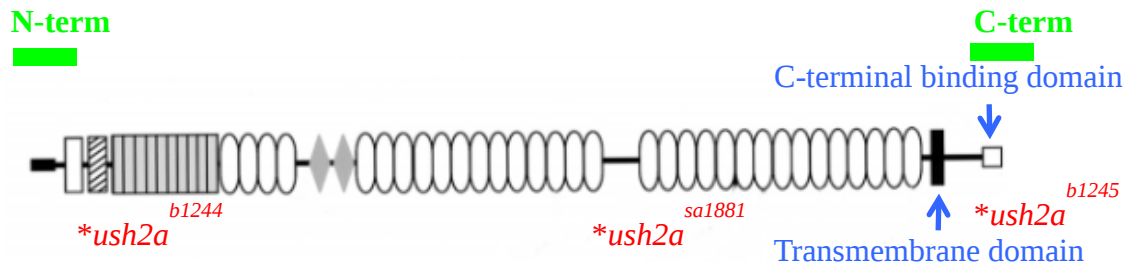
In addition to careful clinical characterization of Usher syndrome patients, the cellular and molecular mechanisms of the disease are studied using animal models, including both mice and zebrafish. The *Ush2a* mutant mouse model has provided valuable information on the role of Usherin in the auditory system (Adato et al. 2005), but limited data on its function in the retina due to differences between mouse and human retinal phenotypes. In mice, vision defects occur late in life and are also milder relative to human visual defects (Williams 2008). Zebrafish are useful model organisms as they have all the same Usher genes as humans, are able to produce hundreds of offspring in a single mating, and are amenable to a variety of genetic and molecular manipulations. A zebrafish model of *USH2A* will provide an additional resource for Usher researchers to understand the function of Usherin better. This model could be used to test different treatment therapies or potential cures. While hearing loss is congenital in human USH patients, retinal degeneration is slow and progressive and there is more time for potential treatment options designed to preserve vision or slow degeneration to be delivered. For this reason, I focused my project on the retina.

Usherin Localization Experiments

The domain at the C-terminal end of Usherin is known to interact with other USH2 proteins in photoreceptor cells, but other domains may also be important for normal protein function. Mutations have been generated in three distinct regions of the zebrafish *ush2a* gene, each of which results in a shortened protein (see figure 2). One of these, the *ush2a*^{b1244} allele, disrupts the beginning of the gene (see figure 2B). This mutation results in a transcript encoding an extremely short protein that is missing virtually all of its functional domains. Another allele, *ush2a*^{sa1881}, has a defect in the mid-region of the gene, which would produce a mutant protein of intermediate length if translated (see figure 2C). A third allele, *ush2a*^{b1245}, harbors a mutation near the end of the coding region, resulting in a slightly truncated Usherin protein that lacks only the C-terminal binding domain but leaves the transmembrane domain intact (see figure 2D).

All proteins are comprised of a chain of amino acids, joined together through peptide bonds made between the Amino group (N-term) and Carboxyl group (C-term) of each amino acid. The first and last amino acids of a protein, therefore, are unbound on one side and thus the beginning and end of the protein are called the N- or C-terminal, respectively. I looked at Usherin protein localization in all three mutants by using reagents that recognized either the N-terminal or the C-terminal of the protein, to learn whether either or both ends of the protein were present.

A. WT Usherin protein



B. *ush2a*^{b1244} (mutation in the beginning of the gene)



C. *ush2a*^{sa1881} (mutation in the mid region of the gene)



D. *ush2a*^{b1245} (mutation in the end of the gene)



Figure 2. A diagram of the Usherin protein, modified from van Wijk et al. (2004). The N and C-terminals are marked as well as the locations of the 3 mutations studied in this thesis (marking the *ush2a*^{b1244}, *ush2a*^{sa1881}, and *ush2a*^{b1245} alleles), the transmembrane domain, and the C-terminal binding domain (figure 2A). Figure 2B shows the truncated protein resulting from the *ush2a*^{b1244} mutation, 2C shows the truncated protein predicted in the *ush2a*^{sa1881} mutant, and 2D shows the slightly truncated protein in the *ush2a*^{b1245} mutant.

The USH2 Protein Complex

In the mouse retina, Usherin has been shown to co-localize with other proteins that are associated with Usher syndrome type 2 at the base of the photoreceptor connecting cilium. These proteins are thought to act in a complex to help load ciliary cargo onto the connecting cilium to move molecules from the inner segments to the outer segments (Liu et al. 2007). Usherin, GPR98, and Whirlin form a protein complex at the base of the connecting cilium through interactions between different regions of each protein (see figure 3B). The loss of any one of these three proteins due to mutations in the corresponding genes has been shown to cause the mislocalization of the other two proteins in mouse photoreceptors (Yang et al. 2012). This suggests that the proteins are dependent on each other for proper localization to the base of the connecting cilium.

Mutations in the *GPR98* and *DFNB31* genes cause Usher syndrome type 2C (USH2C) and Usher syndrome type 2D (USH2D), respectively. Aside from these three main USH2 proteins, another protein has been shown to interact with this complex (Zou et al. 2014). *PDZD7* is a gene that encodes a protein with multiple binding domains. When mutated, *PDZD7* contributes to Usher syndrome when other mutated USH2 genes are also present (Ebermann et al. 2010). The PDZD7 protein has been shown to affect localization of the USH2A/GPR98/Whirlin protein complex in the ear, although research in mice suggests that it may have a different role in the retina (Zou et al. 2014). PDZD7 has been shown to bind directly to USH2A and GPR98 *in vitro* (Yang et al. 2012). All of these proteins localize to and are thought to interact at the base of the connecting cilium in photoreceptor cells (Ebermann et al, 2010; Yang et al. 2012).

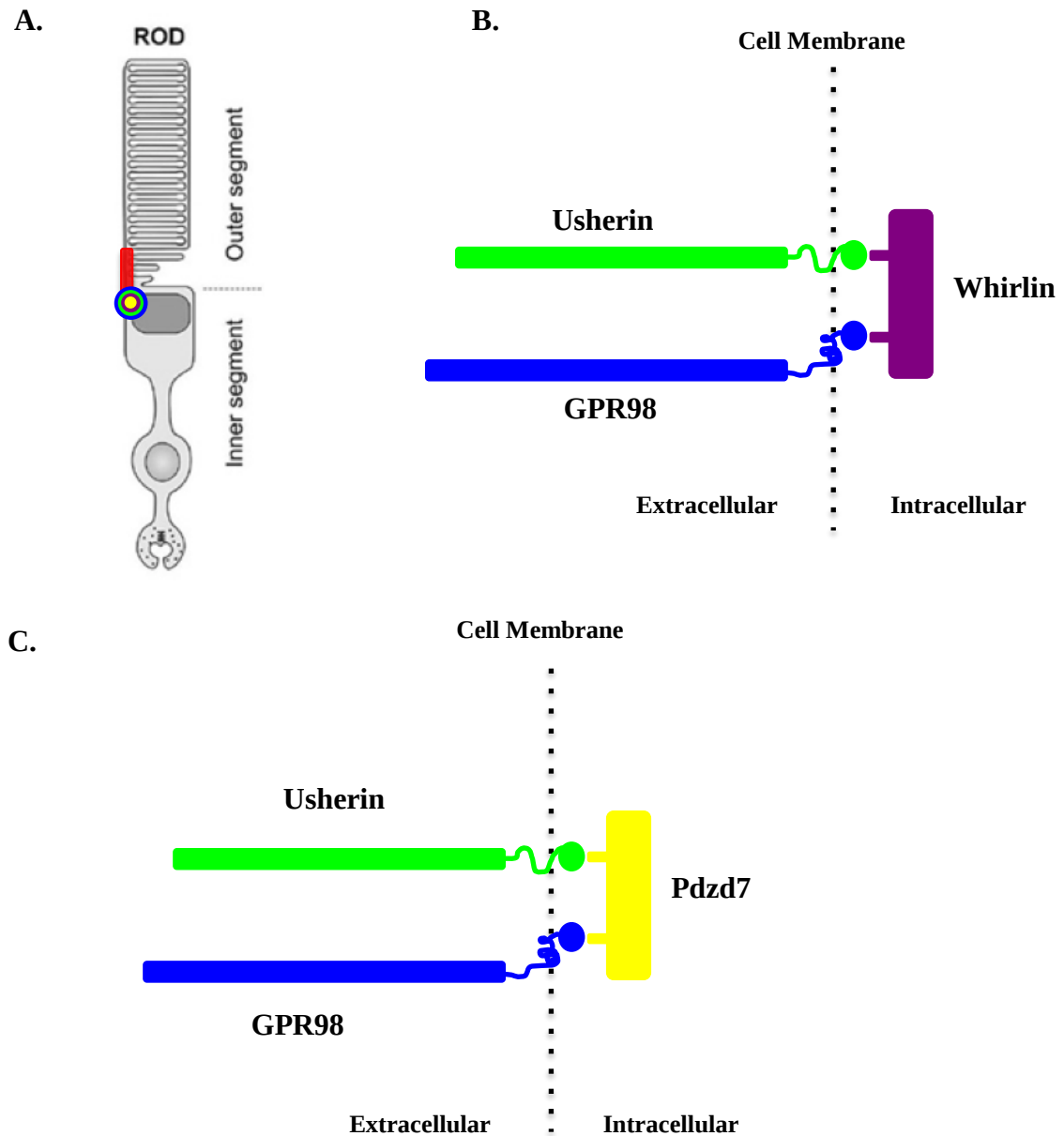


Figure 3. Adapted from Yang et al. (2010). Usherin (green), GPR98 (blue), and Whirlin (purple) localize at the base of the connecting cilium (red) in photoreceptor cells (3A). These proteins interact at the cell membrane (3B). Pdzd7, another USH2 protein, is an alternate binding partner for Usherin and GPR98 at the base of the connecting cilium (3C).

Constant Light Experiment Background

Retinal degeneration is slow in human USH2A patients and generally is not clinically detectable until the second decade of life. Night blindness is one of the first symptoms of photoreceptor loss, and over time, the field of vision narrows to tunnel vision (“Usher Syndrome” 2014). This timeline may explain why retinal defects are so attenuated in mouse models of USH, given the much shorter lifespan of mice (approximately 2 years). Zebrafish, similarly, have a lifespan of only a few years. Additionally, unlike humans, zebrafish maintain a population of retinal stem cells that continually add new functional retinal cells throughout life (Fischer et al. 2013). They also have the ability to regenerate retinal cells to replace photoreceptors that are lost due to injury (Thummel et al. 2007, Kassen et al. 2007). Ebermann et al. (2010) showed that using a morpholino against *ush2a* could induce retinal cell death, however, *ush2a*^{sa1881} mutant zebrafish do not show significant larval retinal cell death.

Constant bright light exposure has been shown to induce photoreceptor cell death in both mouse and zebrafish models of Usher syndrome (Rajaram et al. 2014; Peng et al. 2011; Tian et al. 2014; Taylor et al. 2012; Wasfy et al. 2014). Intensive light exposure resulted in photoreceptor degeneration in a zebrafish model of Usher type 1B where a phenotype was not previously seen (Wasfy et al. 2014). Mice with USH1B were also shown to have significant photoreceptor damage after intensive light exposure (Peng et al. 2011). In a similar study, mouse models of USH2D also showed severe light-induced photoreceptor degeneration, whereas photoreceptor degeneration was previously not seen in mouse models of USH2D raised under normal light conditions (Tian et al. 2014).

In our experiments, we applied moderate, but constant, light to our zebrafish *ush2a* mutants. We hypothesized that if Usherin function is impaired in these mutants, retinal cell function would be significantly compromised by increased light exposure compared to wild-type (WT) fish raised under the same conditions. Most of the research done on light-induction of photoreceptor degeneration has been performed using adult zebrafish, but our research focused exclusively on zebrafish larvae. If a significant phenotype can be seen in larvae, the experimental process would be expedited. The constant light experiment can be completed in 8 days, as opposed to having to wait for adult zebrafish to grow up before performing the experiment. Also, a greater sample size and more data can be obtained from zebrafish larvae, as hundreds of larvae can be kept in a relatively small light cabinet as opposed to keeping about 8 adult zebrafish in the same sized space.

METHODS AND EXPERIMENTAL APPROACH

Animal Use and Practices

The *ush2a*^{sa1881} zebrafish line was obtained from the Zebrafish International Resource Center (ZIRC). The *ush2a*^{b1244} and *ush2a*^{b1245} fish lines were generated using CRISPR gene editing technology in the Westerfield lab. To obtain embryos for experiments, adult fish were placed in small tanks overnight and embryos were collected in the morning. All of the fish were raised in the zebrafish facility at the University of Oregon, and the light cabinet experiment was also carried out in the zebrafish facility. Larvae used in all experiments were kept in petri dishes in embryo medium, which was renewed daily. The temperature of the light cabinet was similar to that of the main facility, about 28-29°C. The Institutional Animal Care and Use Committee (IACUC) protocols were followed for all procedures, including all protocols for minimizing pain or discomfort.

Visualizing Proteins within Retinal Cells

The method that I used to evaluate the tissue was immunohistochemistry. This is a process of fixing and staining tissue so that the protein of interest can be labeled and evaluated. Larvae preserved in a chemical fixative were embedded in gelatin blocks, frozen, cut into thin slices and put onto microscope slides. 5 days or 8 days post-fertilization (dpf) larvae were euthanized and fixed in 4% paraformaldehyde. The tissue was rehydrated in a descending methanol series (first into 66% Methanol (MeOH) and 33% Phosphate buffered saline with tween (PBST), then into a solution of 33% MeOH and 66% PBST, and finally into PBST with 10% sucrose.) The tissue was then

cyroprotected in 30% sucrose overnight. This process protects the tissue from freezing damage.

After sectioning, the tissue was rehydrated in 2 washes of PBST for 5 minutes. The slides were then put in sodium citrate and heated in the pressure cooker for 8 minutes to expose antibody binding sites that may have been molecularly blocked during the fixation process. To reduce the background staining and nonspecific labeling in later imaging, the slides were blocked with a solution designed to bind the reactive molecules present in the tissue. There are different types of blocking solutions, but I used 10% non-fat dry milk (NFDM) dissolved in PBST. Slides were washed in PBST 3 times for 5 minutes each before going into block with 10% NFMD in PBST for 30 minutes.

Then, the tissue was incubated with primary antibodies to the proteins of interest overnight at 4°C. The slides were washed 3 times in PBST for 10 minutes each and then incubated with a secondary antibody (with a fluorescent tag) that recognized the primary antibody so that the protein localization could later be visualized. The slides were exposed to secondary antibody for 2 hours at room temperature and then washed 3 times in PBST for 10 minutes each. Slides were coverslipped and imaged using confocal microscopy.

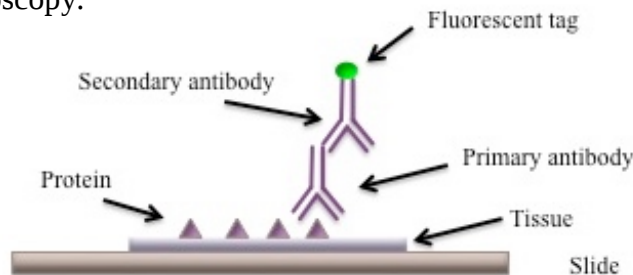


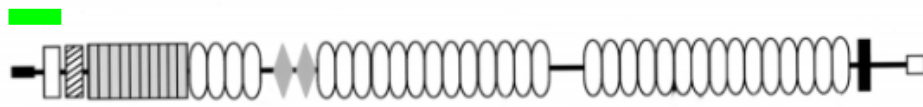
Figure 4. Schematic representation of immunohistochemistry

A similar protocol to the above procedure for looking at larval eye tissue was followed for evaluating adult eye tissue. Adult 10-month old zebrafish were put into ice water in small tanks with MESAB (a fish anesthetic) to euthanize them. The eyes were removed from each fish and put into PBST, and then the lenses were removed from each eye. The eyes were put into a 4% paraformaldehyde fixative to preserve them. The eyes were washed 2 times in PBST for 5 minutes, placed in 10% sucrose for 30 minutes, then placed in new 30% sucrose overnight at 4°C. Embedding, sectioning, and staining proceeded as described above.

Usherin protein localization in *ush2a*^{b1244}, *ush2a*^{sa1881}, and *ush2a*^{b1245} mutants

To determine how the Usherin protein was affected in each of these mutants, they were analyzed using antibody labeling of histological sections and fluorescence microscopy.

A. N-term antibody



B. C-term antibody

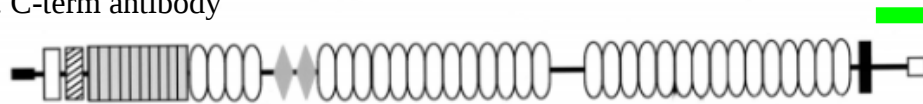


Figure 5. Diagram of where the N-terminal and C-terminal antibody binding sites on the Usherin protein. **5A** corresponds to the region recognized by the N-terminal antibody, and **5B** shows the region recognized by the C-terminal antibody. Both are labeled in green, because in the images taken using confocal microscopy (figures 6 and 7) Usherin is labeled in green.

Acetylated tubulin (AT) was used to visualize the photoreceptor connecting cilia, but also used as a marker of retinal structure, as tubulin is an abundant protein in all retinal neurons.

Slides were stained using an AT primary antibody diluted 1:800 in block along with either a 1:500 N-term Usherin or 1:800 C-term Usherin primary antibody overnight at 4°C. After washing, all slides were then incubated with a 1:800 dilution of anti-mouse 568 secondary antibody (recognizing AT). The number 568 corresponds to the wavelength of the fluorescence emitted, and in this case the light emitted from the secondary antibody (corresponding to the location of AT) is in the red part of the color spectrum. Therefore, wherever AT appears in the images taken using fluorescence microscopy it will appear in red. N-term slides were also stained with a 1:400 anti-guinea pig 488 secondary antibody (which recognizes the only the N-term primary antibody), and C-term slides were stained with a 1:800 anti-rabbit 488 secondary antibody (which only recognizes the C-term primary antibody) for 2 hours at room temperature. The number 488 corresponds to the wavelength of green light, so Usherin will appear on the images in green. Confocal microscopy was used to analyze the slides.

Localization of other Usher proteins

A similar protocol to the immunohistochemistry staining protocol above was used; in this case the AT primary antibody and either the GPR98 primary antibody, PDZD7 primary antibody, Whirlin a primary antibody, or Whirlin b primary antibody was used at a dilution of 1:800. For all USH antibodies, the secondary antibody used was anti-rabbit 568, diluted 1:800. For AT, the secondary antibody used was anti-mouse

488, diluted 1:800. Again, confocal microscopy was used to analyze the slides. In larval retinas, AT staining will appear in red, while GPR98 and PDZD7 staining appears in green. In the adult retinas, AT staining again will appear in red, and GPR98, Whirlin a, and Whirlin b staining appears in green.

Constant light exposure and photoreceptor cell death

Mutant larvae alongside wild-type controls were raised in either elevated constant light or normal light cycle conditions, to determine if there is a significant difference in photoreceptor cell death caused by increasing the luminance.

I performed an experiment with compound heterozygous mutants (*ush2a*^{sa1881} crossed with *ush2a*^{b1244}, resulting in offspring with two different deleterious alleles of *ush2a*) to optimize the conditions for when I performed this experiment again. A second experiment was performed using *ush2a*^{sa1881} homozygous mutants and WT larvae. Larvae were raised to 8dpf in constant 24-hour light starting from day 0. Control larvae were also raised to 8dpf in a normal 14-hour light/12-hour dark cycle. The light cabinet ranged from 2,000-2,300 lux and the control normal light condition was 300 lux.

At 8 dpf, larvae were sacrificed and processed for immunohistochemistry to assay photoreceptor cell death. Slides were labeled with an antibody recognizing caspase-3, a marker for cells undergoing apoptosis (programmed cell death). Each cell positive for caspase-3 appears as a fluorescent (green) dot. The slides were visualized using confocal microscopy, and cell death was counted manually in the outer nuclear layer (ONL; the layer containing the photoreceptor cells), inner nuclear layer (INL), and ganglion cell layer (GCL) of each eye in sequential sections of each whole eye.

Statistical Methods

A student's t-test was performed to determine if two sets of data were significantly different from each other. From this a p-value was determined, and if it was below 0.05 the data were determined to be significant. A p-value is the calculated probability that our two sets of data are different due to random chance. Therefore, a p-value of <0.05 , which is typically accepted as statistically significant, indicates that there is less than a 5% chance that the observed difference could be explained by chance. A smaller p-value indicates higher significance. (*indicates significance, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant).

RESULTS

Localization of Usherin in *ush2a* mutants

I looked at Usherin localization in the various mutants using antibodies recognizing the N- or C-terminal regions of the protein. The presence of the antibody signal would indicate that the protein is present and able to localize to its normal place within the cell. We needed to establish whether the mutant Usherin proteins were able to localize correctly in the zebrafish *ush2a* mutants.

We hypothesized that normal localization of the Usherin antibody against the C-terminal region of the protein would be disrupted in *ush2a*^{b1244} and *ush2a*^{b1245} mutant tissues, given that the C-terminal region should not be translated to protein due to the insertion of a premature stop codon. The premature truncation in the *ush2a*^{b1244} mutant occurs so early in the coding region that it is unlikely that a persistent protein would be synthesized. More likely, the precursor molecules to this mutant protein are degraded, so we would not see localization of the C-terminal or N-terminal antibody.

The premature stop codon in the *ush2a*^{sa1881} mutant might produce a protein of intermediate length, but it is unknown whether it would be stable enough to avoid targeted degradation by cellular quality control mechanisms. Even if stable, this mutant protein would lack the transmembrane domain as well as the C-terminal binding domain, and would not be expected to localize normally. We do not expect to see evidence of either the N-terminal or C-terminal antibody, because the protein is unlikely to localize normally.

The *ush2a*^{b1245} mutant encodes a protein that includes the transmembrane domain but lacks the C-terminal region recognized by the C-terminal antibody. We

tested the hypothesis that this mutant protein would be able to localize normally via the transmembrane domain by evaluating Usherin N-terminal antibody localization. We do not expect to see the C-terminal Usherin antibody, since this protein lacks the C-terminal region.

In retinas from wild-type larvae, both the N-terminal and C-terminal antibodies localize to the region at the base of the photoreceptor connecting cilium, whereas the C-terminal antibody was not detectable in the *ush2a*^{b1244}, *ush2a*^{sa1881}, and *ush2a*^{b1245} mutant backgrounds (figure 6A). This confirms that the proteins encoded by the three Usher mutants lack the C-terminal binding domain, and are thus unable to form a complex with other Usher proteins via this protein interaction domain (figures 6B, 6C, 6D). By just looking at the C-terminal antibody, we do not see any evidence that the protein is produced, because we do not see a signal from any of the mutant proteins.

The N-terminal antibody signal was also undetectable in retinas from both the *ush2a*^{b1244} and *ush2a*^{sa1881} mutant backgrounds. Therefore, we can conclude that the mutant protein in the *ush2a*^{b1244} and *ush2a*^{sa1881} backgrounds was likely degraded, as we see no antibody signal anywhere in the cell.

Conversely, the N-terminal Usherin antibody was detected at the connecting cilium in *ush2a*^{b1245} larvae (figure 7C), which indicates that protein interactions mediated by the C-terminal binding domain are not required for normal localization of Usherin in photoreceptors.

In all *ush2a* mutants, the overall retinal structure revealed by visualizing AT appears normal when compared to WT. This indicates that disrupted Usherin function does not affect the shape or patterning of the retina.

Localization of other Usher type 2 proteins

Usherin has been shown to co-localize in mammalian retinas with other Usher type 2 proteins at the base of the photoreceptor connecting cilium. I looked to see whether localization of other USH2 proteins, specifically GPR98, Pdzd7, or Whirlin was affected in the different *ush2a* mutant backgrounds in both larvae and adults. In humans, Whirlin protein is encoded by the *DFNB31* gene. Zebrafish have two copies of this gene that encode Whirlin a and Whirlin b, respectively, both of which localize to the base of the connecting cilium.

I hypothesized that normal localization of other Usher type 2 proteins may be disrupted in mutants lacking the C-terminal binding domain of Usherin. This analysis was carried out in the *ush2a*^{sa1881} mutant background and compared to WT controls.

I first assayed localization of both Gpr98 and Pdzd7 in larval retinas at 5dpf. Both proteins localized normally in *ush2a*^{sa1881} mutant retinas (figure 8B and 8D). To test whether this localization might be affected by chronic absence of Usherin over time, I conducted further antibody staining in 10-month old zebrafish retinas using antibodies against Gpr98, Whirlin a, and Whirlin b. All showed normal protein localization in the mutant background (figure 9). So, contrary to my hypothesis and to results seen in *Usha2a* mutant mice, these results suggest that the localization of other USH2 proteins in zebrafish retinas does not require Usherin.

Constant light exposure and photoreceptor cell death

To establish the conditions of the light cabinet, I first used mutants obtained from a cross between two alleles, *ush2a*^{sa1881} and *ush2a*^{b1244}, along with WT controls to determine that larvae could be raised in the light cabinet and remain healthy. Although a statistically significant increase in photoreceptor cell death was observed in both the WT and mutant backgrounds in constant elevated light conditions compared to normal light conditions (figure 10), the increase was statistically greater in the mutant retinas compared to wild-type. This experiment also told us that the light cabinet environment had no adverse affects on overall larval health during the course of the exposure period. Larvae that were raised in the light cabinet showed a more extreme difference in cell death than larvae that were raised in the normal light cycle.

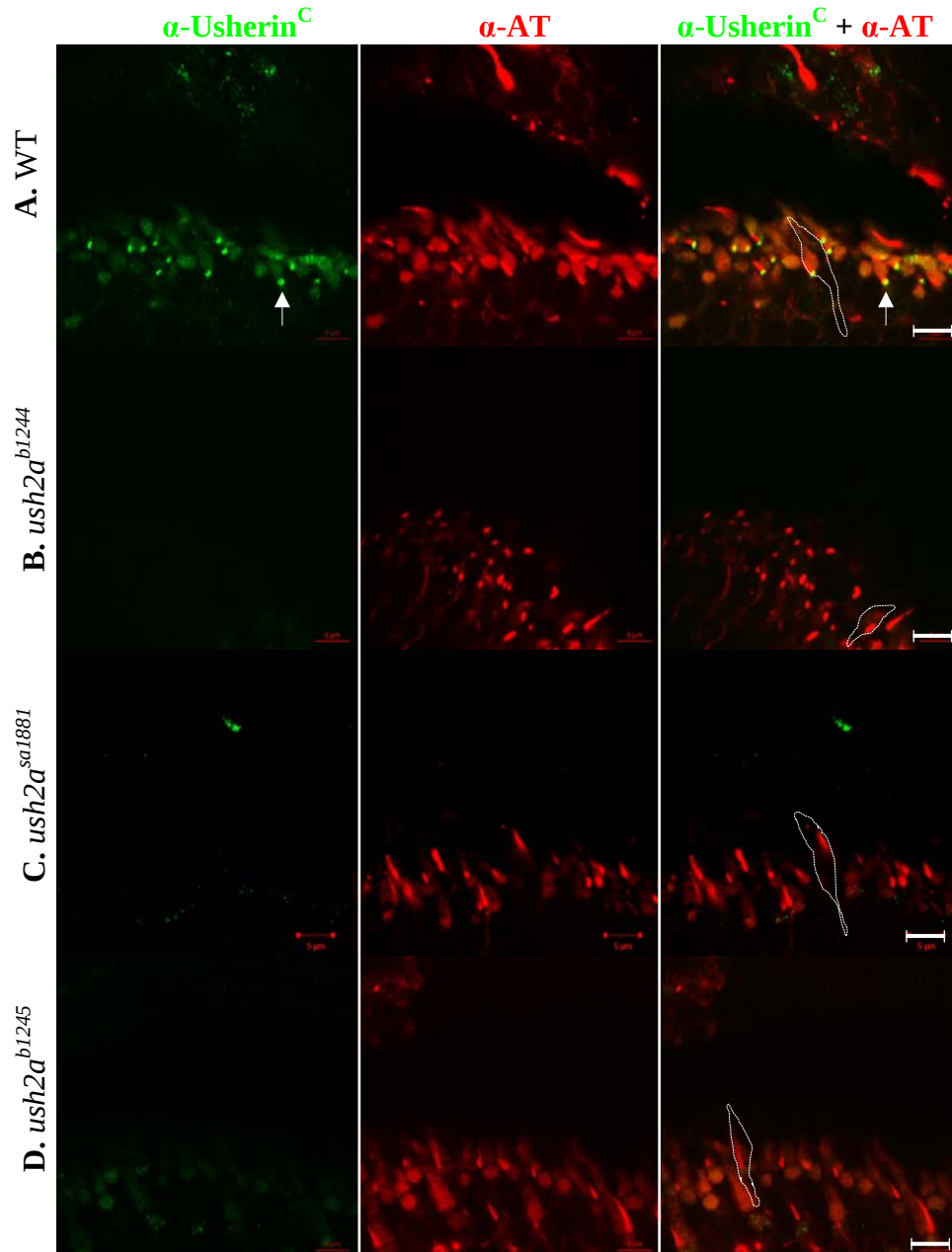
I next performed this experiment using *ush2a*^{sa1881} homozygous mutants and their WT siblings. Usherin protein was undetectable in the *ush2a*^{sa1881} mutant, which suggests that Usherin function in the retina is completely abolished in this background. Because Usherin is localized specifically in the photoreceptor cell layer, we hypothesized that we would see the greatest phenotypic change in photoreceptors of *ush2a*^{sa1881} mutants.

An overwhelming amount of light exposure would likely cause widespread cell damage across all regions of the retina, which would not help us determine if the *ush2a* mutants responded differently to constant light than WT larvae. To avoid this, we wanted to identify a light level that would produce a difference between WT and mutant larvae, but would not cause an enormous increase in cell death in WT larvae.

There was no significant difference in photoreceptor cell death between WT and mutant fish that were raised in the normal light cycle (figure 12B). This confirmed our suspicion that some combination of the relatively low light levels of the fish facility, the young age of the fish, and the regenerative potential of the fish retina might make it difficult to detect photoreceptor death in these models. However, a significant increase in photoreceptor cell death was detected in *ush2a*^{sa1881} mutants raised at constant, higher intensity light levels compared both to WT controls at constant light and to mutants raised in normal light (figure 11). While we also detected an increased in photoreceptor cell death in WT controls raised at constant light compared to controls reared in normal light conditions, this increase, while statistically significant, was a much smaller magnitude than the difference observed in mutants raised in the two light conditions.

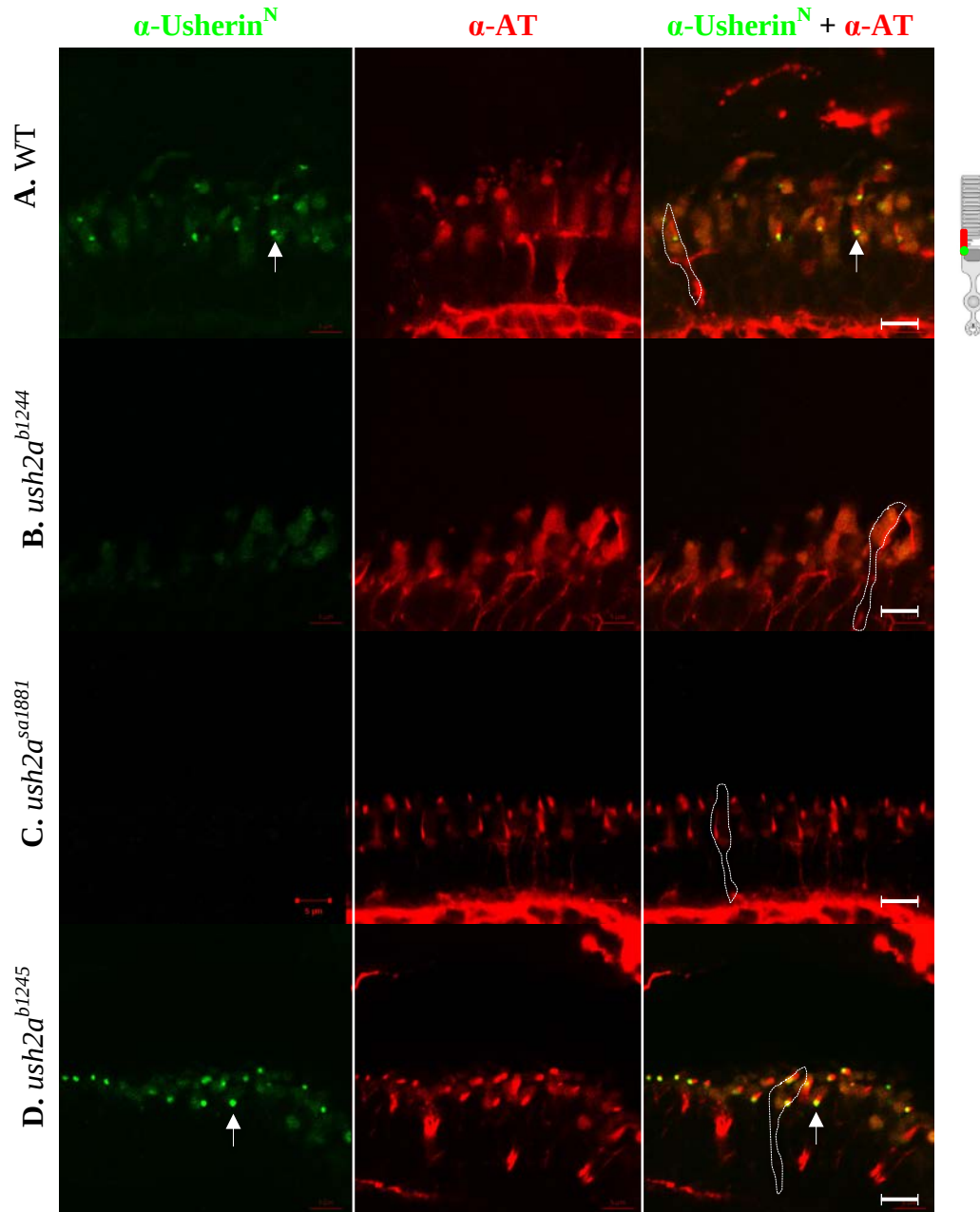
C-term Usherin localization is disrupted in all *ush2a* mutant backgrounds

Figure 6. C-term Usherin antibody localization in WT larvae at 5dpf (A), *ush2a*^{b1244} (B), and *ush2a*^{b1245} (C) photoreceptor cells. A single photoreceptor is outlined in white in the right column. The C-term antibody (green; left column) is located beneath the connecting cilium (red; middle column), and the right column shows a merged image. C-term antibody localization (indicated with white arrows) is apparent in WT (A), but not in *ush2a*^{b1244} (B), *ush2a*^{sa1881} (C), or in *ush2a*^{b1245} (D) mutants. A single photoreceptor cell is outlined to the right for reference depicting where Usherin (green) and connecting cilium (red) are located. Scale bar (5μm) is shown in white in the bottom right corner of the merged image.



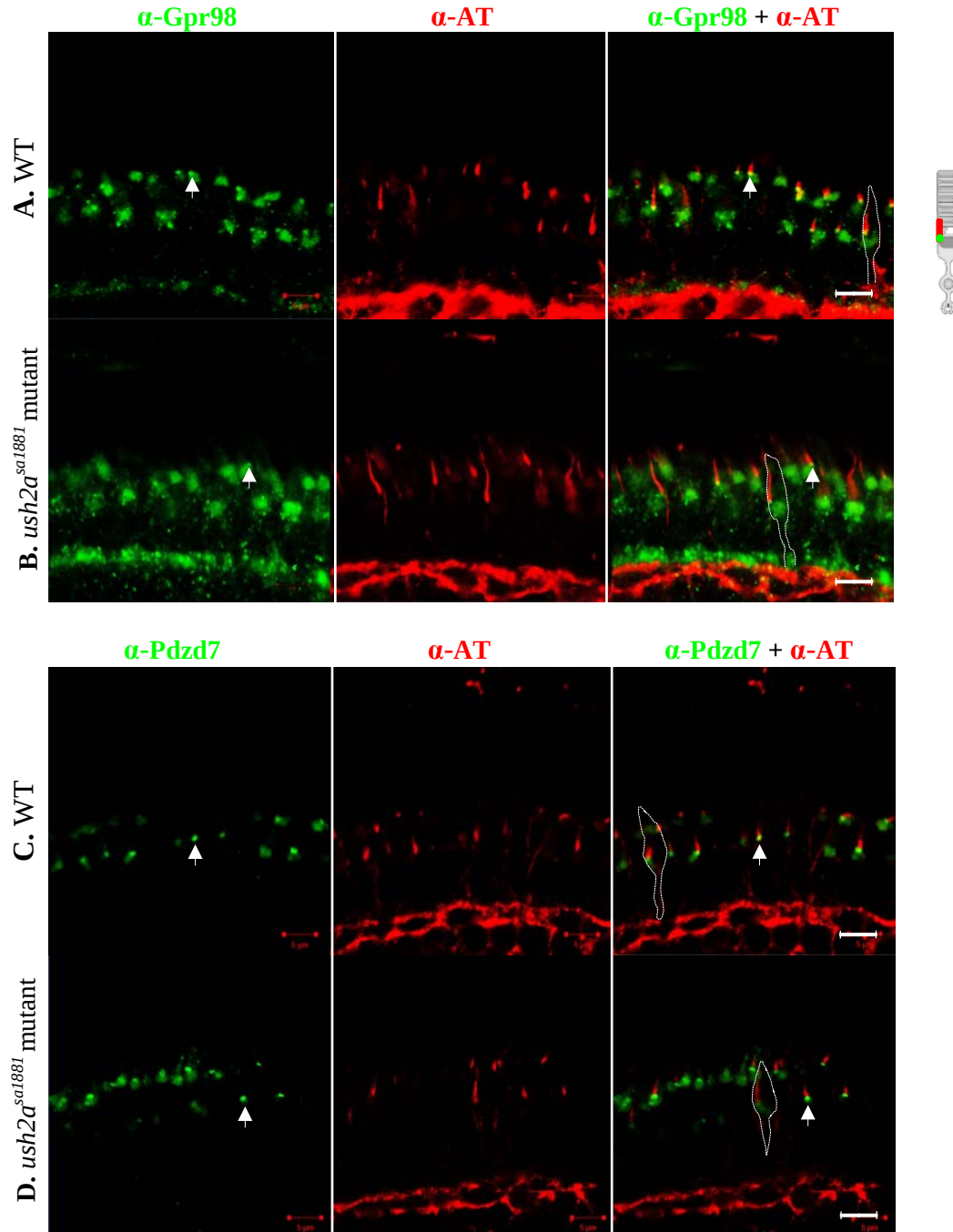
N-term Usherin localization is disrupted in some, but not all, mutant backgrounds

Figure 7. N-term Usherin antibody localization in WT larvae at 5dpf (**A**), *ush2a*^{b1244} (**B**), and *ush2a*^{b1245} (**C**) photoreceptor cells. A single photoreceptor is outlined in white in the right column. The N-term antibody (green; left column) is located beneath the connecting cilium (red; middle column), and the right column shows a merged image. N-term antibody localization (indicated with white arrows) is apparent in WT (**A**) and in *ush2a*^{b1245} (**D**) mutants, but not in *ush2a*^{b1244} (**B**) or *ush2a*^{sa1881} (**C**) mutants. A single photoreceptor cell is outlined in white for reference depicting where Usherin (green) and connecting cilium (red) are located. Scale bar (5μm) is shown in white in the bottom right corner of the merged image.



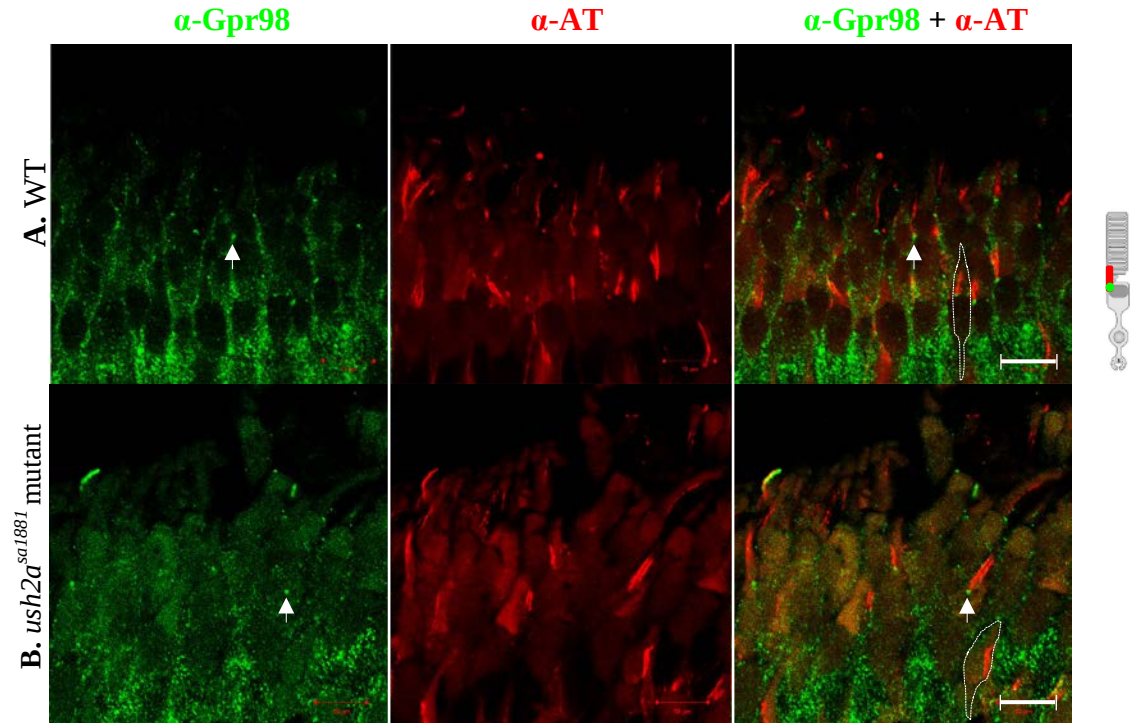
Localization of other Usher proteins (5dpf larvae)

Figure 8. Localization of other Usher proteins to the base of the connecting cilium in 5 dpf larvae. Both WT (A) and *ush2a*^{sa1881} homozygous mutants (B) show normal GPR98 localization (indicated with white arrows). Similarly, both WT (C) and *ush2a*^{sa1881} homozygous mutants (D) show normal Pdzd7 localization. Reference for the connecting cilium (red) and both GPR98 and Pdzd7 localization (green) is shown in a cartoon photoreceptor cell to the right. Photoreceptors are outlined with a dotted line in the merged image. Scale bar (5μm) is shown in white in the bottom right corner of the merged image.



Localization of other Usher proteins (10-month adults)

Figure 9. Localization of Usher proteins in 10-month adult *ush2a^{sa1881}* retina. Localization of GPR98 was visualized in both WT (A) and homozygous mutant (B) backgrounds. Similarly, localization of Whirlin a was seen in both WT (C) and homozygous mutant (D) backgrounds, and localization of Whirlin b was seen in both WT (E) and homozygous mutant (F) backgrounds. Next to the image is a cartoon photoreceptor for reference to location of the connecting cilium (red) and the location of GPR98, Whirlin a, and Whirlin b (green). An individual photoreceptor cell is outlined with a dotted white line in the merged image. Localization of GPR98, Whirlin a, and Whirlin b is indicated with white arrows. Scale bar (10 μ m) is shown in the bottom right corner of the merged image in white.



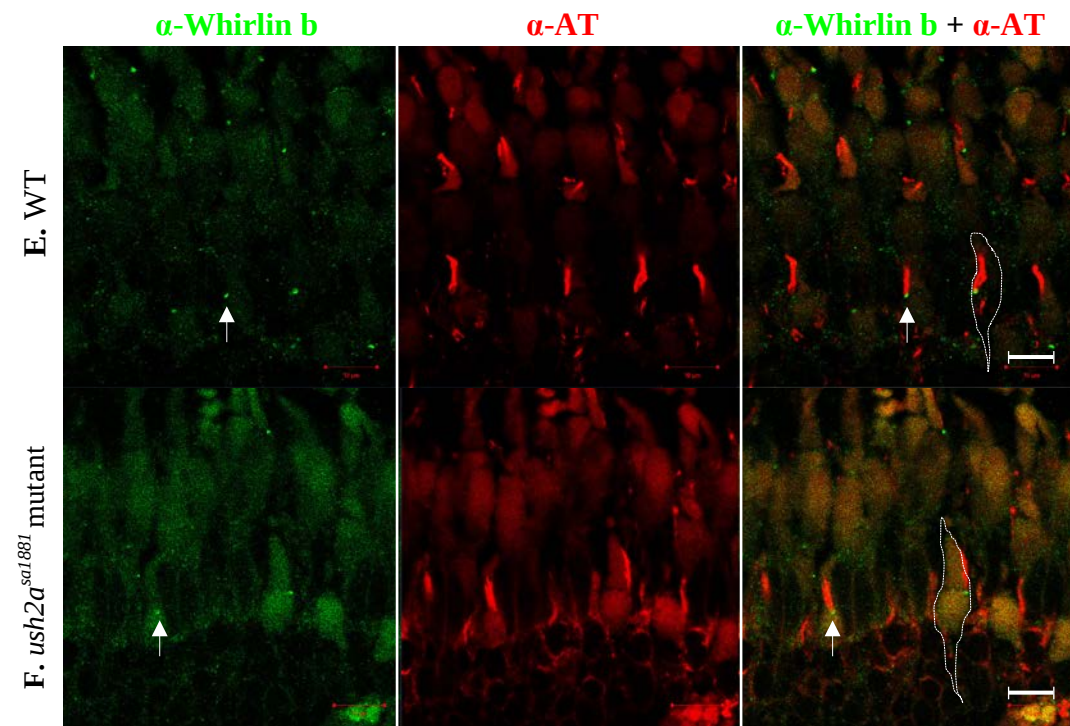
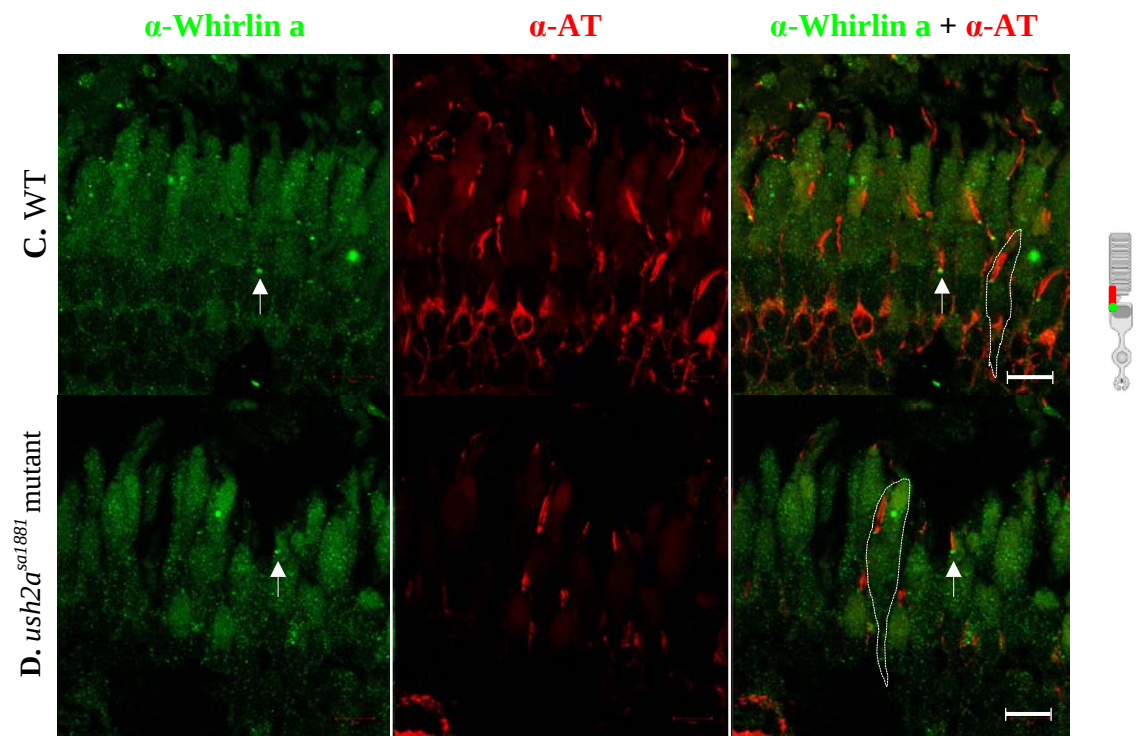


Figure 10. Photoreceptor cell death in light-reared larvae. Comparison of compound heterozygous (*ush2a*^{sa1881} x *ush2a*^{b1244}) larvae with WT larvae in normal light cycle and constant light conditions. The graph below refers to a comparison of cell death between larvae of the same genotype in different light conditions (larvae raised in the normal light cycle, larvae raised in constant, elevated light levels from day 0-8, and larvae raised in constant, elevated light from day 4-8). * indicates significance, p < 0.05; ** p < 0.01; *** p < 0.001; ns = not significant.

Comparison of cell death within the same genotype:

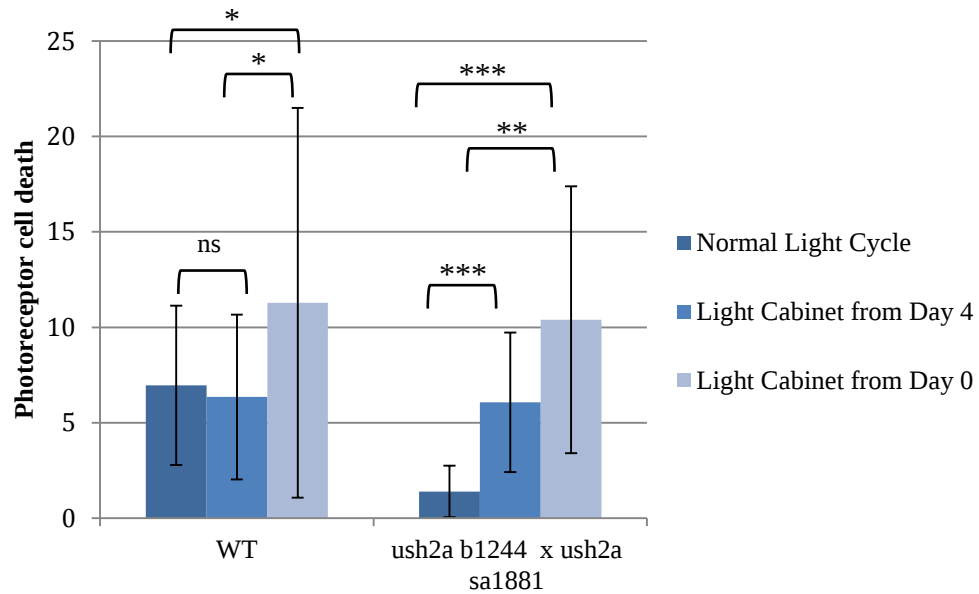
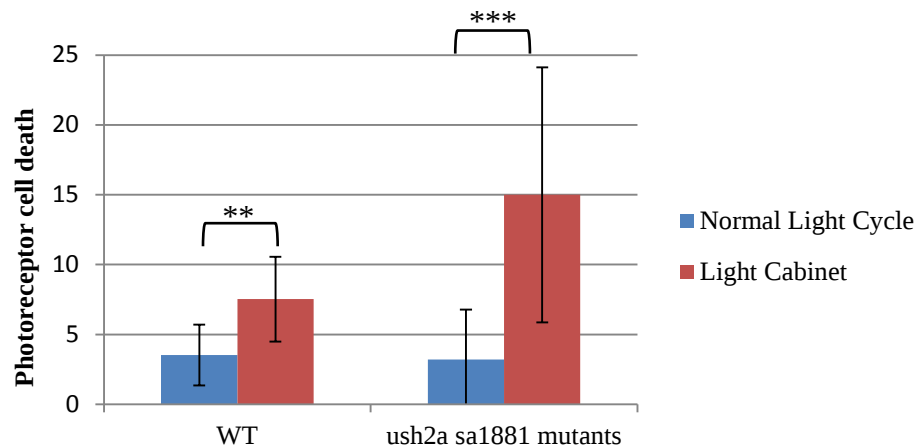


Figure 11. Photoreceptor cell death is significantly increased in *ush2a* mutants under constant light conditions. Comparison of 8dpf *ush2a*^{sa1881} homozygous mutants and WT larvae in normal light cycle and intensive light cabinet conditions. Figure **11A** refers to a comparison of cell death between larvae of the same genotype in different light conditions. Figure **11B** is a representation of the same data, only this time comparing different genotypes in the same light condition. *** indicates significance; p < 0.001; ns = not significant.

A. Comparison of cell death within the same genotype:



B. Comparison of cell death within the same light condition:

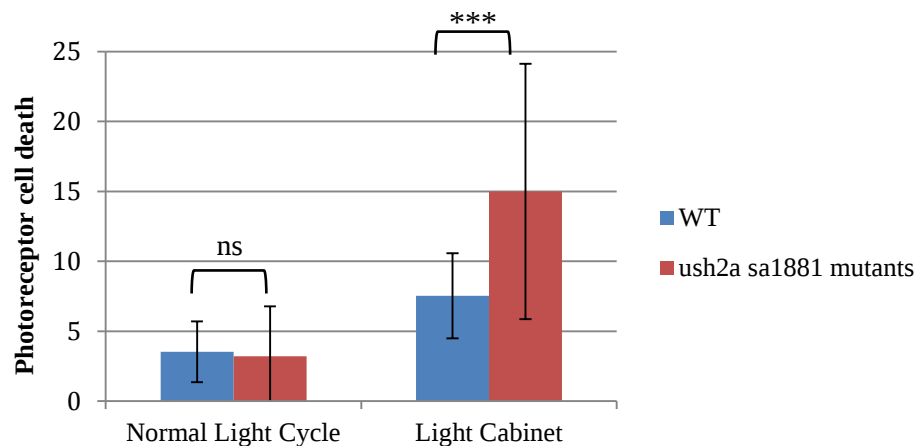
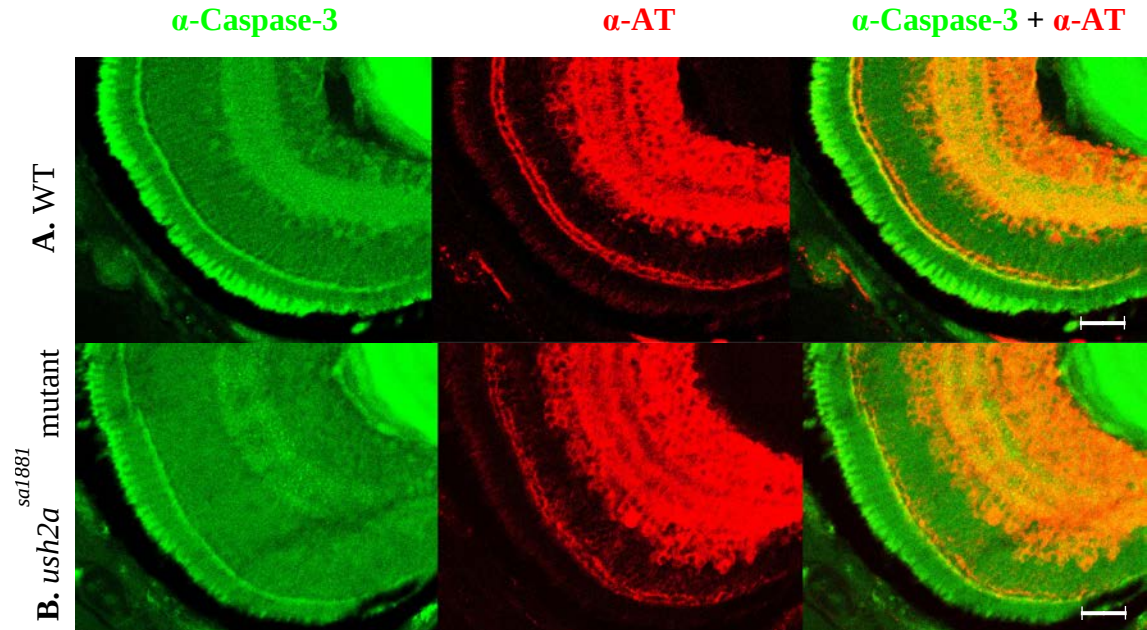
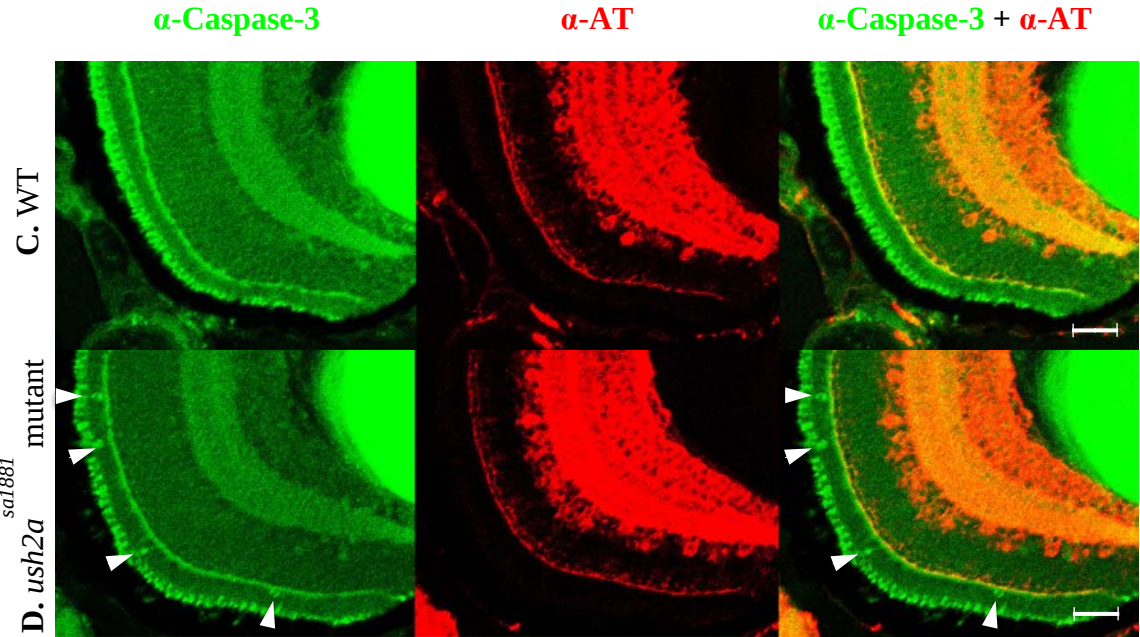


Figure 12. The caspase-3 antibody labels dying photoreceptor cells. 8dpf WT and homozygous *ush2a*^{sa1881} mutant retinas in both normal light cycle and intensive light cabinet conditions were stained with a cell death marker (caspase-3; green) and an antibody against AT showing microtubule-based structures in the retina (red). Photoreceptor cell death in the ONL is indicated with white arrows. Figures **12A** and **12B** show representative eyes from both WT and homozygous *ush2a*^{sa1881} mutants that were raised in the normal light cycle. Figures **12C** and **12D** show representative eyes from WT and homozygous *ush2a*^{sa1881} mutants that were raised in the light cabinet. There is noticeable photoreceptor cell death in **12D**. Scale bar (20μm) is shown in bottom right corner of the merged image in white.

Normal Light Condition



Constant Light Condition (light cabinet)



DISCUSSION

Localization of Usherin in *ush2a* Mutants

Three different mutations that affect different regions of the *ush2a* gene were evaluated in this study. We found that Usherin localization is lost in *ush2a*^{b1244} homozygous mutants, as evidenced by the absence of both N-terminal and C-terminal antibody signal (figures 6B, 7B). This confirmed our hypothesis that the *ush2a*^{b1244} mutant, with such an early truncation, would not generate a functional protein that is able to localize normally. Similarly, the *ush2a*^{sa1881} mutant did not show localization of either the N-terminal or C-terminal of the Usherin protein (figures 6D, 7D). This indicates that a mutation at the mid region of the gene causes failure to produce a stable Usherin isoform.

The N-terminal antibody detected a signal at the base of the connecting cilium in the *ush2a*^{b1245} mutant (figure 7D), although the corresponding labeling with the C-terminal antibody was negative. This indicates that an intact transmembrane domain is sufficient to localize Usherin to the connecting cilium, and that the C-terminal binding domain is not required for this process. This set of experiments confirmed that all three zebrafish *ush2a* alleles will provide useful models for studying the effects of loss of normal Usherin function. Because we see a difference between the *ush2a*^{b1245} allele compared to the *ush2a*^{b144} and *ush2a*^{sa1881} alleles, we can use these models to understand better how having a partial, truncated form of Usherin compares to having no detectable Usherin protein at all. We can look specifically at how having an intact transmembrane domain, but no C-terminal binding domain, in the *ush2a*^{b1245} mutant background compares to having no protein present in either the *ush2a*^{b1244} or *ush2a*^{sa1881}

mutant backgrounds. From this, we can determine how important C-terminal binding activity is compared to other potential extracellular interactions.

Localization of Other Usher type 2 Proteins

Usherin colocalizes with a number of other Usher proteins at the base of the connecting cilium and forms a complex *in vitro* (Yang et al. 2012). Based on the experiments done on the mouse retina by Yang et al. (2010), we hypothesized that if Usherin was unable to localize normally in the mutant retinas, then the complex would be unable to form and localization of the other USH2 proteins would be affected.

In our hands, we found that all USH2 factors were normally localized in *ush2a* mutant backgrounds (figures 8, 9). These data indicate that, unlike in mouse, the localization of other USH2 proteins to the connecting cilium appears to be independent of Usherin. It is possible that although other Usher type 2 proteins still localize normally, function of the complex is compromised when Usherin is absent, or present in a truncated form. Future studies to evaluate ciliary trafficking between the inner and outer segments would determine whether there is reduced efficiency of transport in *ush2a* mutants. It would also be useful to evaluate localization of USH2 proteins in the *ush2a*^{b1245} mutant background, in which the N-terminal of Usherin does appear to localize normally, to see whether other domains (such as the transmembrane domain) are involved in interactions with the other USH2 proteins in addition to the C-terminal binding domain. Given the results of our constant light experiments, it may also be useful to look at the localization of these USH2 proteins after the larvae have been raised in constant light and show a degenerative retinal phenotype.

Constant Light Experiments

Similar versions of this experiment have been performed successfully on zebrafish and mice with other types of Usher syndrome previously (Wasfy et al. 2014, Peng et al. 2011, Tian et al. 2014). Our first attempt revealed that there was a significant increase in photoreceptor cell death in *ush2a* mutants when exposed to constant light conditions (figure 10). We also saw a lesser, but still statistically significant, increase in photoreceptor cell death in WT larvae raised in elevated, constant light. Most importantly, this experiment told us that light cabinet conditions would be sufficient to induce cell death, and allowed us to optimize conditions for our next attempt.

Mutant larvae showed significantly more retinal degeneration than WT larvae after being raised in constant, elevated light for 8 days, most particularly in the photoreceptor cell layer (figure 11). The constant light condition revealed a retinal phenotype in zebrafish similar to what is seen in human Usher syndrome patients over time. This validates the utility of zebrafish larvae as a model for the retinal degeneration characteristic of USH2A.

It is important to note that although the light cabinet experiment was designed to challenge the retinas with greater than normal light levels, the light intensity was still moderate, at about 2,000-2,300 lux. To put this in perspective, an overcast day has light levels of about 1,000 lux, and full daylight in indirect sun has a light level of about 10,000-25,000 lux (Schlyter 2009). Although most Usher patients are currently advised to wear dark glasses when outdoors, this result underscores the potential for light damage to occur even in the absence of bright sunlight. These results have implications for research using mouse models, in that similar light-rearing conditions may induce a

more significant or earlier onset retinal phenotype in mouse models of *Ush2a* as well. These data also have implications for human USH2A patients - we now have a well-characterized zebrafish model that can be used to test treatments that would reduce or delay photoreceptor cell death.

Future Directions

In an ongoing experiment at this writing, I am evaluating cell death in the *ush2a*^{b1244} and *ush2a*^{b1245} retinas after constant light exposure, and will compare the results to the data from the *ush2a*^{sa1881} larvae. We would expect that the *ush2a*^{b1244} would show similar levels of photoreceptor cell death to *ush2a*^{sa1881}, given the complete absence of Usherin localization in both mutant backgrounds. Future research will focus on the differences between *ush2a*^{sa1881} and *ush2a*^{b1245}, since these alleles show differences in Usherin localization.

These data may help us identify which domains of Usherin are most functionally important. The levels of retinal degeneration in the different mutants can be used to determine whether the particular mutations correspond to different rates of cell death. For example, if the mutation missing only the C-terminal binding domain (*ush2a*^{b1245}) gives a phenotype that is as severe as the other mutations, treatment options might focus on delivering a shorter gene sequence encoding the C-terminal region of the protein, which would be far easier to work with than the full-length gene. However, if the phenotype of the *ush2a*^{b1245} mutant was less severe than the *ush2a*^{sa1881} mutant, then further research could focus on determining which protein domains would be required to restore Usherin function. From this information and a better understanding of the

USH2A gene, treatments can be developed and optimized for human patients who suffer from *USH2A*.

A planned future experiment for the light cabinet system would be to investigate the efficacy of different filters blocking distinct wavelengths of light in reducing photoreceptor cell death. This experiment could provide information on how different lens treatments may be able to slow photoreceptor degeneration and therefore blindness. This information could lead to enhanced preventative care for *USH* patients.

Although this study focused solely on the effect of *ush2a* mutations in the retina, *USH2A* patients also have moderate to severe hearing defects. Future studies to evaluate the structure and function of the acoustic organs in the various *ush2a* mutants characterized in this study would further assist in the collective efforts to understand the roles of Usherin in hearing. Subjectively observing the live larvae used in these experiments, I noted that many of the *ush2a*^{sa1881} homozygous mutants appeared to have mild swimming and orientation impairments, which is a characteristic behavior of zebrafish with hearing defects. This behavior, along with functional and histological assays of the inner ear, should be pursued in future studies. All current published zebrafish models of Usher syndrome exhibit severe balance defects and are unable to survive to adulthood (Ernest et al. 2000; Nicholson et al. 1998; Seiler and Nicholson 1999). In contrast, the homozygous *ush2a* mutants can be raised to viable, fertile adulthood. This gives us a larger window of opportunity in which to study the effects of loss of Usherin function.

CONCLUSION

We have established a model for the visual defects in Usher syndrome type 2A. The *ush2a*^{b1244} and *ush2a*^{sa1881} alleles show no evidence of Usherin in the retina, and the *ush2a*^{b1245} allele shows evidence that a truncated form of Usherin lacking the C-terminal binding domain is able to localize normally. These models can be used to investigate Usherin function further, and can be used to test and develop treatments. We can evaluate differences between individuals lacking Usherin protein entirely and those with mutant Usherin protein localizing in the retina. We have also established that localization of other USH2 proteins at the connecting cilium does not appear to be disrupted when Usherin is absent from the retina, unlike what is observed in the mouse retina (Yang et al. 2010). These results indicate that further studies are needed to understand how the Usherin/GPR98/Whirlin complex functions. Finally, we have devised a system that is able to cause light-induced photoreceptor damage in *ush2a* mutants without damaging the other parts of the retina. This system causes a retinal phenotype similar to what is seen in human USH2A patients, which will be useful in further studies of *USH2A* using zebrafish models.

Glossary/Abbreviations

Allele – An alternative form of a gene.

Antibody – A blood protein produced in response to a specific antigen (a specific protein, in this case).

AT – acetylated tubulin; this antibody is often used in staining used to show the structure and organization of neurons. Because the eye contains a lot of neurons, this is a good protein to label the slides with.

caspase-3 antibody – This antibody recognizes a protein produced by cells undergoing programmed cell death.

CMZ – Ciliary Marginal Zone

Connecting cilium – This ciliary transport system is vital for the movement of molecular cargo between the inner and outer segments of photoreceptor cells.

CRISPR – A gene editing technique that is used (in this case) to create different alleles of the *ush2a* gene through a mutation.

dpf – days post-fertilization

Fixation (histology) – A way of preserving biological tissue (in this case, done with paraformaldehyde).

GCL – Ganglion Cell Layer

Gene – The molecular unit of heredity that is made up of DNA.

Genotype – genetic makeup of an organism.

GPR98 – A protein encoded by another *USH2* gene known to interact with Usherin.

Heterozygous – Having two different types of alleles for a single trait.

Homozygous – Having two copies of the same kind of an allele.

INL – Inner Nuclear Layer

Mutation – A permanent change in the nucleotide sequence of the genome.

Morpholino – This is a molecule that is used to temporarily reduce expression of a particular gene, allowing a short-term opportunity to study the effects of losing gene function.

NFDM – Non-fat dry milk (used as a blocking reagent).

Nonsense Mutation – A point mutation in the sequence of DNA that results in an early stop codon, so the encoded protein is truncated and incomplete.

ONL – Outer Nuclear Layer – This layer contains the photoreceptor cells of the retina.

PBST – Phosphate buffered saline with tween. This is used in many lab protocols, usually to wash or rehydrate tissue.

Phenotype – The observable characteristics of an organism, such as color, shape, size, behavior, etc.

Photoreceptor – A photosensitive cell in the retina.

Recessive – The mode of inheritance of Usher syndrome is recessive, meaning that mutated copies of the gene must be inherited from both parents.

Retinitis Pigmentosa – (RP): A degenerative disease in which there is damage to the retina through photoreceptor loss.

Stop codon – (also known as a termination codon) This is a nucleotide triplet that signals the termination of protein synthesis.

Translation – The final step in the process of turning DNA into protein.

USH2A - The human Usher gene known to encode the protein Usherin.

Ush2a – The mouse version of the human USH2A gene that encodes mouse Usherin.

ush2a – The zebrafish version of the human USH2A gene that encodes zebrafish Usherin.

Whirlin (in humans) – A protein encoded by another USH2 gene and known to interact with Usherin. Mutations in this gene cause USH2D.

Whirlin a (in zebrafish) – A protein encoded by another USH2 gene (*dfnb31a*) known to interact with Usherin.

Whirlin b (in zebrafish)– A protein encoded by another USH2 gene (*dfnb31b*) known to interact with Usherin.

WT – wild-type – the typical form as it would appear in nature (contrasts to a mutant, which is a non-standard version).

WT siblings – Fish that are WT for the gene of interest and are raised alongside mutant fish, so that they have the same genetic background (aside from the mutant gene). These are useful controls in experiments.

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