IDENTIFICATION OF MOLECULAR INTERACTIONS BETWEEN USHER SYNDROME TYPE 2 PROTEINS IN THE ZEBRAFISH RETINA

by

TAYLOR ANN RUMOHR HOWAT

A THESIS

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Monte Westerfield, PhD

Usher syndrome is the most common cause of hereditary deaf-blindness. Usher type 2A is the most common form of the disease and is caused by mutations in the gene *USH2A*, which encodes the large multi-domain protein Usherin. Mutations in Usherin are also the most common cause of an inherited form of retinal degeneration called Retinitis Pigmentosa. Other genes associated with Usher type 2 (USH2) include *ADGRV1* and *WHRN*. Physical protein-protein interactions among all three USH2 proteins have been demonstrated in other systems, and the cooperative function of this multi-protein complex is thought to be required for retinal cell function. This study characterizes zebrafish models of USH2 using strains with mutations in *ush2a* and *adgrv1*, alone and in combination. In the single *ush2a* mutant, Whirlin protein localization in the retina is notably defective, providing us with important information regarding what regions of Usherin are most important for maintaining a stable USH2 protein complex. Subsequently, these studies showed that the stability of this complex in the *ush2a* mutant background fluctuates according to time spent in daylight. This study further explored light-dependent effects on retinal cells and revealed that elevated

light levels exacerbate retinal cell death in young fish defective in either *ush2a* or *adgrv1*. Collectively, this thesis research has enriched our understanding of the functional roles and relationships of the USH2 proteins Usherin, Adgrv1, and Whirlin and provided new data to direct preventative and therapeutic efforts toward improving clinical outcomes in patients with mutations in USH2 genes.

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Introduction

Usher syndrome (USH) is a hereditary condition that affects vision, hearing, and often balance. It is reported that as many as 1 in 6,000 Americans and 1 in 23,000 worldwide are affected by this disease, making it a more common affliction than ALS or Cystic Fibrosis (Kimberling et al., 2010; Yang et. al, 2012). Clinically, Usher syndrome is classified into types 1, 2, and 3, each differing in the severity and age of onset of the symptoms (Petit, 2001; Williams, 2008; Millan et. al, 2011). Hearing aids and cochlear implants can assist with hearing deficits, but there are currently no clinical interventions to address the progressive blindness caused by USH. Emerging treatments that aim to address the underlying molecular causes of the disease, such as gene therapy, may present the best hope for future treatment and prevention of USH-related vision loss.

Genes and Gene Therapy

Deoxyribonucleic acid, or DNA, is the genetic material that encodes instructions for building proteins and other molecules that perform vital functions within all cells. Genetic disease results when errors in the DNA code, defined as a mutation, impair the body's ability to produce functional molecules. The Online Mendelian Inheritance in Man database (https://www.omim.org) lists over 5000 genes that contribute to human disease when mutated. Ongoing research into various therapies targeting the genetic defects that underlie these diseases falls into three broad categories. The first method involves delivering a replacement copy of the genetic information affected by the mutation, providing cells with a new template from which to produce a functional gene

product. This technique is currently constrained by the physical size of the replacement gene because the factors that package and deliver the replacement genetic information can accommodate genes only up to a certain size, and there are many disease genes, including several of the USH genes, that exceed this maximum size limitation.

The second approach involves using molecular methods of skipping over the errors in the genetic code to restore the ability to generate a functional product. The types of mutations that this method could address are limited by the location and type of change to the DNA, and thus it is not a suitable strategy for many disease-causing mutations. The third technique, colloquially known as gene editing, seeks to repair the error permanently at the DNA level. The limitations associated with gene editing include variable success in targeting the desired DNA sequence precisely, as well as the possibility of off-target effects, meaning that interference could occur at an unwanted position in the genome. Because each method is highly dependent on the type of mutation as well as the size and complexity of the coding region of the gene, the optimization of a gene therapy to treat a disease requires a thorough understanding of the molecular genetics of the disease. Understanding the various options of gene therapy relates to the research I conducted on specific mutations modeling Usher syndrome type 2 because the data I have generated in these studies can inform the selective process of developing the most effective treatments for patients with USH2.

Pattern of Inheritance

Usher syndrome results from changes in any one of a number of documented genes corresponding to each clinical subtype. Usher syndrome is a recessive disorder, meaning that an individual must inherit one mutated copy of the gene from each parent

to show symptoms (Figure 1). Individuals who have one mutated copy of the gene and one normal copy are not affected, but are considered carriers of the disorder, as their genetic defect can be passed on to offspring. In most cases, two different mutated copies of the gene are inherited by people who suffer from recessive disorders, although in populations with reduced genetic diversity, the exact same mutation can be inherited from both parents. Knowing the location and type of mutation(s) in a patient with a recessive genetic disease informs what type of gene therapy would be most appropriate for treatment because only one copy of a gene without the mutation is needed to restore normal function.

Figure 1

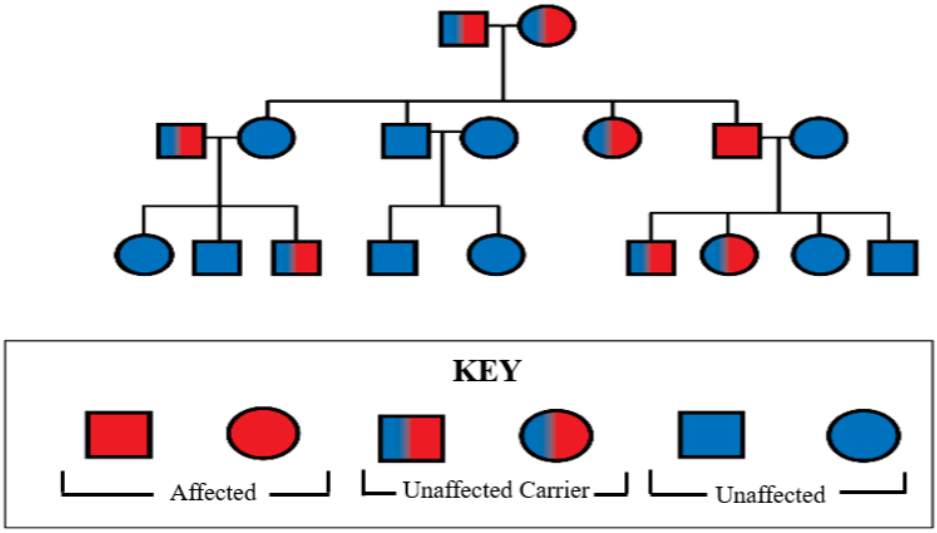

Figure 1: Pedigree of Usher syndrome-affected family. The partial and full red shading show the pattern of inheritance for a disease gene moving through a family tree. At the top, the parents are both unaffected carriers of the gene having both blue (wild type) and red (mutant) copies of the gene. They pass these genes on to their offspring at random (middle), producing two offspring who have only wild-type versions of the gene, one offspring who is an unaffected carrier, and one child who has inherited mutated copies of the gene from both parents and will therefore be affected by the disorder. Mutant copies of the gene appear in the third generation (bottom), passed on by affected or carrier parents. (Image Adapted from: Jerome Walker, Wikipedia Human Genetics)

USH Type 2

Usher syndrome type 2 (USH2) is the most common form of USH (Spandau and Rohrschneider, 2002). In USH2 patients, hearing loss is present at birth and varies in severity from moderate to severe. This differs from Usher type 1, in which deafness is present at birth and Usher type 3 where hearing is normal at birth and worsens later in life. In USH2, vision loss progresses more slowly compared to the other types and the onset is not apparent until adolescence (NIDCD: "Usher Syndrome," 2017). No balance

issues are associated with this subtype, which further distinguishes it from type 1 and type 3. My studies focus on the retina because of the prolonged window of opportunity to understand the progression from a functional cell, to dysfunctional, and ultimately degeneration, as well as the extended opportunity this protracted disease progression provides for therapeutic interventions.

The retina contains light sensitive cells called photoreceptors whose main function is to detect light and convert that detection into an electrical signal that is relayed through the visual system. Photoreceptors are highly specialized sensory cells with functional and structural asymmetry. Numerous functional molecules are made and processed in the inner segment (Figure 2) and then trafficked to the outer segment to participate in the complex process of light detection and response. The connecting cilium, a hair-like structure located within the photoreceptors, is a main conveyor system between the inner and outer segments, forming a bi-directional conduit for cargo to travel between the segments of the cell. Many Usher proteins colocalize in the region surrounding the connecting cilium and are thought to form a protein complex essential for loading cargo onto the ciliary trafficking system (Yang et al., 2012; Sorsuch et al., 2017, Sahly et al., 2012). When mutations exist in USH genes, the resulting impairment to USH protein function leads to photoreceptor cell degeneration. The clinical symptoms of retinal degeneration in USH are caused by photoreceptor dysfunction and death. Rods, photoreceptor cells located in the periphery of the primate retina, are responsible for peripheral vision and light detection in low-light environments. These cells are the first ones affected in USH, which manifests clinically as night blindness and gradual loss of peripheral vision that progresses toward the center of the retina. This

progressive dysfunction and degeneration results in worsening tunnel vision over time and ultimately results in legal blindness in middle age (Sandberg M.A. et al., 2008). The relatively late age of onset of retinal degeneration in USH patients presents an excellent window of opportunity for preventative treatments that could slow or even halt retinal degeneration. My research focused on Usher syndrome type 2 models, exploring the three documented USH2 proteins, Usherin, Adgrv1, and Whirlin, and their localization in different mutant backgrounds.

Figure 2

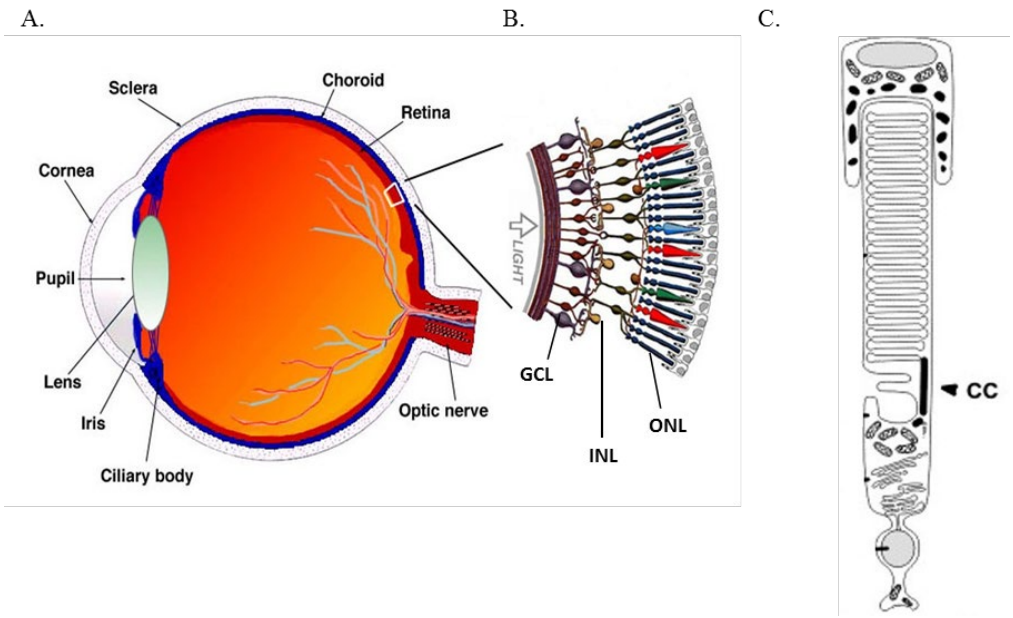

Figure 2: A schematic diagram of the photoreceptor cell in the retina. Retina is a tissue in the back of the eye (A) made up of three layers: ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL). The ONL is comprised of rod and cone photoreceptor cells (B). Photoreceptors are neurons that receive and relay visual information. USH2 proteins have been found to localize adjacent to a structure called the connecting cilium (CC) of the photoreceptor (C). (Image Adapted from: http://webvision.med.utah.edu/imageswv/Sagschem.jpeg and Reiners, 2006)

The USH2 Protein Complex

The most common form of all types of USH results from mutations in a gene called *USH2A,* which encodes the large, multi-domain protein Usherin (van Wijk et al., 2004). Usherin localizes at the base of the connecting cilium in the photoreceptor cells (Yang et al. 2010). Previous studies conducted in a mouse model of USH2A have demonstrated that the interactions that occur between Usherin and other USH2 proteins in the sensory cells of the eye and ear are critical for vision and hearing (Lui et al., 2007; Hartel et al., 2016), suggesting that when these interactions are compromised due to mutations in *USH2A*, the resulting cell dysfunction leads to disease. Similar results have been shown in zebrafish studies using morpholinos to knock down *ush2a* and *adgrv1* (Ebermann et al., 2010; Dona et al. in preparation). USH2C and USH2D are rare form of USH caused by mutations in the genes *adgrv1* and *whrn*, respectively. Adgrv1, like Usherin, is a large protein with an extracellular region containing multiple functional domains, whereas Whirlin is a smaller protein with multiple interaction domains that can act as a 'scaffold' for assembling a multi-protein complex (Yang et al., 2010). Adgrv1 and Whrn, like Usherin, have established binding relationships with other USH proteins (Lui X et al., 2007, Mearker et al., 2008, van Wijk et al., 2006).

In mice and zebrafish Usherin and Adgrv1 both localize to the cell membrane at the base of the connecting cilium with Whirlin (Figure 4B) (Yang et al., 2010, Sorusch et al., 2017). Studies conducted on USH2 mouse models showed that a loss in any one of these USH2 proteins due to mutation affected the localization of all other USH2 proteins in photoreceptors, suggesting that the members of this protein complex depend on one another for sustained localization in this region of the cell (Yang et al., 2010).

This is further supported by clinical evidence that defects in Usherin, Adgrv1, and Whirlin result in similar levels of visual impairment; thus, a tractable gene-specific therapy to replace a defective component of the USH2 complex would need to restore function of the entire complex. A deeper understanding of the interacting domains of each protein in the complex will increase the successful development of such interventions.

Figure 3

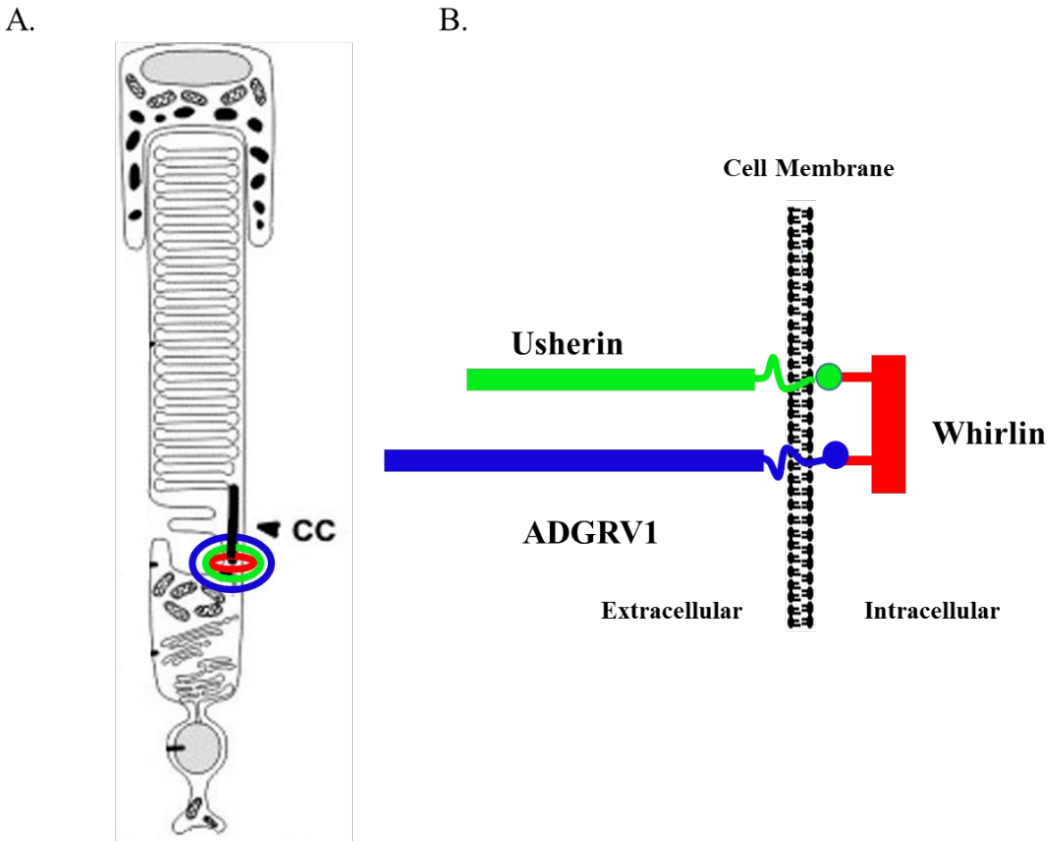

Figure 3: The Usherin, Adgrv1, and Whirlin protein complex. Usherin (green) and Adgrv1 (blue) bind to Whirlin found in photoreceptor cells of the retina. A: shows the location of these proteins along the cells found in the retina. B: depicts a model for how these proteins interact to form a multi-protein complex (Adapted from Yang et al., 2010 and Lerner, 2015).

Experimental Design

Using Zebrafish to Study Usher Syndrome

Before considering clinical trials to test therapies on human patients, extensive preclinical trials are required to test potential treatments using animal models. In our laboratory, we replicate Usher syndrome using the zebrafish model. Zebrafish have Usher genes and sensory organs similar to humans (Ernest et al., 2000; Seiler et al., 1999; Phillips and Westerfield, 2014). The anatomical and functional composition of the visual system is similar to that of humans. Specifically, zebrafish and humans have conserved photoreceptor arrangements reflecting their preference for daytime activity (Link & Collery, 2015; Raymond et al., 2014). Zebrafish are amenable to genetic manipulation, have large clutch sizes, and quick generation times making them a convenient study system. Mouse models have been used previously to study Usher syndrome, providing many valuable insights into how USH proteins contribute to the development and function of the cells involved in hearing and balance. However, the contribution of mouse models to our understanding of the visual defects in USH has been limited by the fact that most of them do not exhibit visual symptoms comparable to the human condition. Many mouse models have no detectable retinal defects at all, and those that do often show milder and/or later onset defects (Lui X et al., 2007; Lagziel et al., 2009; Slijkerman et al., 2015). Because my research focuses on USH protein function in the retina, the zebrafish proves a suitable animal model for studying retinal degeneration and Usher syndrome.

I studied three zebrafish strains with particular mutations in the *ush2a* and *adgrv1* genes. The *ush2a*^{b1245} mutant was generated in the Westerfield laboratory by gene editing that introduced a premature stop codon in the 71st exon of *ush2a*. This mutation results in a slightly truncated version of Usherin lacking the PDZ binding motif known to interact directly with Whirlin. The extracellular functional domains and the transmembrane domain are left intact, which enables the truncated protein to traffick to and integrate within the cell membrane proximal to the connecting cilium (Figure 3B).

The second mutant model, the *adgrv1*^{sa10539} allele, contains a mutation in exon 28 of the *adgrv1* gene, producing a truncated Adgrv1 protein (Figure 3D). The *adgrv1*^{sa10539} mutant is a point mutation, which converts a DNA code for a particular protein subunit to a premature stop codon. This mutant has not yet been extensively studied, but experiments in the Westerfield laboratory using an antibody generated against the C-terminal portion of the Adgrv1 protein have shown a complete lack of signal in the mutant background, indicating that production of full-length Adgrv1 protein is ablated. The third strain of fish I used in my studies was obtained by mating fish bearing each of these mutations to generate a double mutant line with the *ush2a*^{b1245} and *adgrv1*^{sa10539} alleles in the same lineage. The third protein involved in the USH2 protein complex is Whirlin. Previous studies in mouse have shown that Whirlin directly interacts with Usherin and Adgrv1 in photoreceptors. (Yang et al., 2010). In zebrafish, this gene is duplicated, and the two *whirlin* genes are known as *whrna* and *whrnb*. For my work, I used antibodies generated against specific regions of the Whrna and Whrnb proteins to visualize their localizations in the *ush2a* mutant backgrounds.

Figure 4

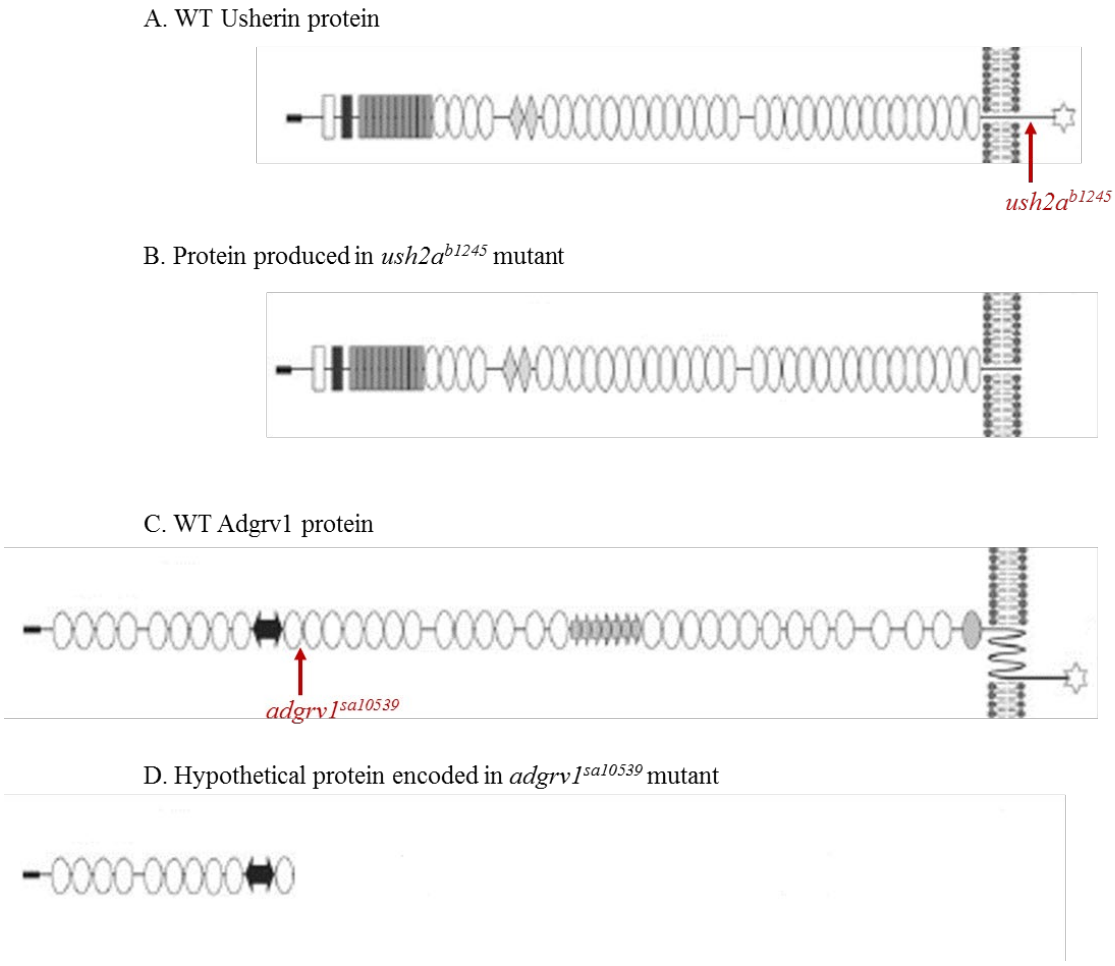

Figure 4. Schematic representations of the Usherin and Adgrv1 protein structures. A: The wild type Usherin protein with all functional domains, including extracellular, transmembrane, and PDZ-binding domains. B: The protein produced in the *ush2a*^{b1245} mutant is slightly shortened due to a premature termination of translation causing the protein to lack the PDZ-binding domain. C: The wild type Adgrv1 protein including numerous extracellular domains, a multi-pass transmembrane domain, and a C-terminal PDZ-binding domain. D: The hypothetical truncated protein that would be encoded in the *adgrv1*^{sa10539} mutant (Adapted from van Wijk et al., 2004, and Lerner, 2015).

Variability of Whirlin Localization in ush2a *Mutant Backgrounds*

Research conducted in the Westerfield laboratory has shown variable results in the localization of USH2 proteins in the photoreceptors of zebrafish *ush2a* mutants. In some experiments, localizations of Whrna, Whrnb, and Adgrv1 were reduced in *ush2a* mutant backgrounds, whereas in other experiments with the same strains and reagents, these localizations appeared normal. In an *ush2a* mutant background with a truncating mutation early in the protein coding sequence, Whirlin localized properly (Lerner, 2015). This result was in contrast to previous reports in mouse USH2 models, where mutations in Usherin reduced the normal localization of Whirlin as well as Adgrv1. This difference in the localization of Whirlin in the zebrafish model motivated us to see whether Whirlin localization was affected in the $ush2a^{b1245}$ mutant, where the truncated form of Usherin is still able to anchor in the cell membrane, but lacks the binding domain necessary for interaction with Whirlin. In the $ush2a^{b1245}$ mutant background, I conducted experiments to visualize the localization of both Whrna and Whrnb. The variability seen in localization of Whrnb in these experiments prompted us to take a closer look at other factors that might influence this result.

Whirlin Time of Collection Experiment

Experiments performed in the course of this thesis and subsequently replicated by our collaborators showed that the localization of Whrna in $ush2a^{b1245}$ photoreceptors was partially reduced or absent, but Whrnb localization was unaffected. However, repetitions of this experiment using the same procedures and reagents gave variable results. In each experiment, larval zebrafish were euthanized at 6 days post-fertilization and put immediately into fixative to preserve the cellular conditions of the moment.

Thus, the variability in the results might be explained by capturing different degrees of stability in these independent experiments. One factor we had not previously controlled was the time of day that fixation took place, so we devised an experiment to determine whether this variable could influence the localization of Whirlin. Normal fish husbandry practices specify a light cycle for fish of all ages, with a 14-hour day beginning at 9am and a 10-hour night beginning at 11pm as the standard of care. There are well-documented differences in the cellular behavior of dark-adapted vs. light-adapted retinal cells. Therefore, we theorized that the time of euthanasia relative to the time of day might influence localization of Whrn proteins in the *ush2a* mutant retinas.

Two independent experiments were conducted in which the fish were separated into three groups and euthanized under the following times and conditions: 9am immediately after the facility lights came on, 2pm after being dark adapted for approximately 15 hours, and 2pm under normal light conditions (Figure 5).

Figure 5

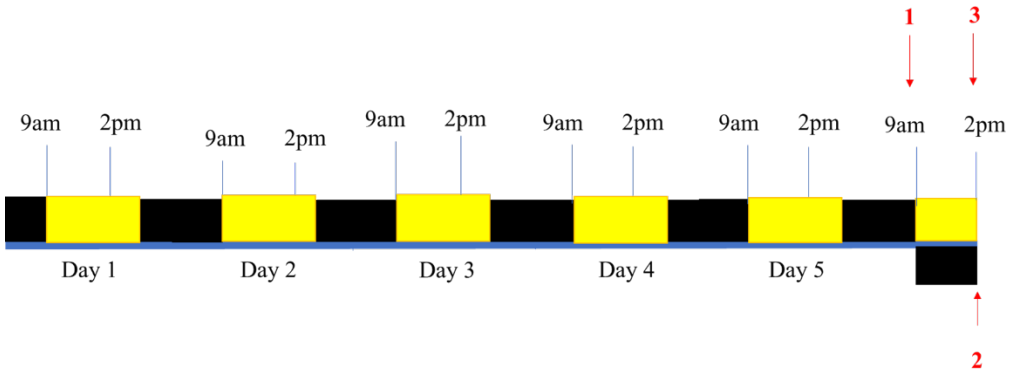

Figure 5: Schematic of the time of collections for three groups of fish: 1. 9am when the facility lights came on, 2. 2pm dark adapted, 3. 2pm light adapted.

The Effects of Elevated Light on Retinal Cell Death

Retinal degeneration in USH2 patients is usually not clinically detectable until adolescence, and then progresses slowly over several decades. The much shorter lifespan of the mouse is one possible explanation for why the phenotype is attenuated in mouse USH models. Another possible contributor is the facility conditions in which mice are raised for research. Because mice are nocturnal animals, their eyes are evolved for vision in low light, resulting in facility conditions designed with minimal illumination. In contrast, the zebrafish is a diurnal organism that prefers activity during the day time, similar to humans. However, zebrafish facility light levels are much lower than natural fish environments. Previous studies conducted on mouse USH models have demonstrated that short exposure to an elevated light intensity significantly increases retinal degeneration, suggesting a vulnerability to light-induced photoreceptor damage (Peng et al. 2011; Tian et al. 2014). In zebrafish models of USH1B, intensive light exposure produced a mild retinal degeneration phenotype especially in the outer nuclear

layer (Wasfy et al., 2014). Finally, unpublished data from the Westerfield laboratory has documented increased retinal degeneration in several USH zebrafish models.

To study retinal degeneration in our USH2 models, we exposed larvae to an elevated light level. Previous studies done in the Westerfield laboratory showed that increasing the light intensity tenfold above normal facility illumination on a 14-hour light /10-hour dark cycle resulted in significant levels of photoreceptor death. In my experiments, I exposed homozygous *adgrv1*^{sa10539} and *ush2a*^{b1245} mutants and *adgrv1*^{sa10539}*;ush2a*^{b1245} double homozygotes to an elevated intensity of between 4500 and 5000 lux on a light cycle. Having already established that photoreceptor degeneration is escalated under similar conditions in *ush2a* mutants, the goal of this experiment was to evaluate retinal cell death for the first time in *adgrv1* mutant larvae and to test whether the loss of Adrgv1 function in addition to the loss of PDZ-binding mediated activity of Usherin would affect the degree of photoreceptor degeneration compared to each of the single mutants and to wild type. If the double mutant demonstrates higher levels of cell death than the singles, this would suggest that the further destabilization of the USH2 complex affected in the absence of both functional inputs leads to more rapid and widespread photoreceptor degeneration. If, on the other hand, there is no increase in cell death in the double mutants, this would indicate that there is no additive effect of lacking multiple components of the USH2 protein complex. Through this research, I hope to gain greater insight into how the USH2 protein complex is affected in various mutant backgrounds and ultimately aid in tailoring treatment options to USH2 patients.

Methods and Experimental Approach

Zebrafish Animal Model Practices

The zebrafish lines used in these experiments are maintained in the zebrafish facility at the University of Oregon. To procure larval fish for experiments, adult fish were paired in mating crosses overnight, and the embryos were collected in the morning. The embryos were raised to the desired age in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$), which was refreshed daily. For these experiments, the retinal tissue was evaluated from larval fish at 6 days post fertilization (dpf). These experiments are approved and regulated by IACUC (Institutional Animal Care and Use Committee) to ensure optimum animal health and humane treatment.

Zebrafish Strains

The *ush2a*^{b1245} zebrafish mutant was generated by CRISPR/Cas9 gene editing in the Westerfield laboratory, targeting the gene sequence encoding the intracellular domain of Usherin and inducing a deletion within that region that results in a premature stop codon. The *adgrv1*^{sa10539} mutant was generated by the Zebrafish Mutation Project at the Sanger Institute and obtained from the Zebrafish International Resource Center. It features a point mutation that introduces a premature stop codon within exon 28 (out of 96 total exons). Both lines were outcrossed several times to Oregon AB* wild type fish to reduce random mutations in the genetic background and to isolate only the mutation of interest prior to in crossing homozygous viable mutants of each stock to create the *adgrv1*^{sa10539}*;ush2a*^{b1245} double mutant strain.

Protein Visualization

Immunohistochemistry (IHC) is the process of visualizing proteins within their native tissues using antibodies designed to recognize and bind to proteins of interest. At 6dpf, larvae were euthanized and preserved in a chemical fixative, 4% paraformaldehyde, to stop cellular activity and prevent degradation of the cellular organization and structure. The tissue washed twice in phosphate buffered saline with 20% Tween-20 (PBST) for 5-10 minutes each on a shaker, followed by a 10-minute wash in methanol to dehydrate and further stabilize the tissue. A second volume of methanol was added to the tissue and samples were stored in a freezer at -20°C for at least 24 hours and up to six months before rehydration. To rehydrate the tissue, the samples were washed in a descending series of methanol: 66% methanol in 33% PBST, followed by a solution of 33% methanol and 66% PBST, and 100% PBST. The process of cryoprotecting the tissue prior to section began with a 30-minute incubation in 10% sucrose in PBST, followed by incubation in 30% sucrose in PBST overnight at 4°C. The dark pigmentation surrounding the eye tissue provides a challenge to observing cell structure and protein localization within the retina, therefore the fixed larvae were embedded in a stiff gel matrix, frozen, and cut into thin sections to reveal retinal cells beneath the pigment.

Once the tissue had been sectioned onto slides and dried, the slides could be stored at -20°C until use. When ready to commence IHC experiments, the sectioned tissue was rehydrated in 2 washes of PBST for 5 minutes each. To optimize the condition of the tissue for antibody staining, the slides were immersed in 10 mM sodium citrate and heated in a pressure cooker for 8 minutes. This step is designed to

enhance the antibody binding affinity and overall staining quality and by unmasking binding sites on the proteins of interest which may have been blocked during fixation. The slides were allowed to cool to room temperature once removed from the pressure cooker and were then washed three times in PBST for 5 minutes each. Next, a block composed of 10% nonfat dry milk (NFDM) diluted in PBST was applied to the tissue for at least 30 minutes at room temperature. The block masks undesired binding sites before the antibody is applied and encourages more specific binding of the primary antibody to the protein of interest.

Antibody Staining

Primary antibodies are designed to recognize and physically bind to a specific part of the protein of interest. Secondary antibodies are used to detect the primary antibody and part of their structure includes a fluorescent molecule that can be activated by light of a particular wavelength. When activated, the molecule emits a fluorescent glow that can be detected by microscopy (Figure 6).

Figure 6

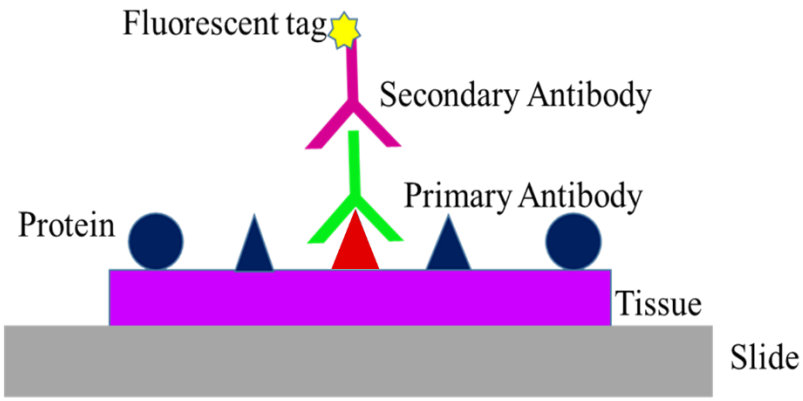

Figure 6: A representation of antibody binding (Adapted from: Lerner, 2015).

The primary antibodies used for these experiments were designed to recognize USH2 proteins or structural proteins found within the tissue of interest. In addition to each of the USH2 proteins visualized, I used a primary antibody designed to recognize a protein called acetylated tubulin, a component of a wide range of neural cell structures as well as microtubule-based structures called cilia. The connecting cilium of the photoreceptor is one such structure. I also used an antibody recognizing a protein called centrin, which localizes to a structure called the basal body, from which the connecting cilium emanates. Use of these markers helped me to orient to the anatomy of the photoreceptors at high magnification, and to evaluate the localization of the USH2 proteins I was visualizing relative to these cellular landmarks.

To visualize the USH2 proteins in the larval retina, I used antibodies recognizing the N-terminal and C-terminal regions of Usherin, the C-terminus of Adgrv1, and unique sites within the zebrafish proteins Whirlin A and Whirlin B. All

primary antibodies were diluted to a 1:1000 concentration in 10% NFDM in PBS-T, and tissues were incubated in primary antibody solutions overnight at 4℃. Between applications of the primary and secondary antibodies, the tissue was washed three times in PBST for 10 minutes. In all IHC experiments, the secondary antibodies were selected so that the USH proteins and the structural markers of the cilium (centrin or tubulin) were labeled with contrasting fluorescent colors. I used a secondary antibody with a fluorescent wavelength of 488nm to label the primary antibody of the structural markers (centrin and tubulin), which emits light in the green range of the visible light spectrum. To recognize the primary antibody of the USH2 proteins, I used a secondary with a fluorescent wavelength of 568nm, which emits light in the red range of the spectrum. Secondary antibodies were diluted to 1:800 concentrations in block and slides were incubated overnight at 4℃. Tissue was washed three times in PBST for 10 minutes each at room temperature to remove unbound secondary antibody solution. The slides were mounted with anti-fade mounting media (Vectashield) and coverslipped before viewing on a Zeiss confocal microscope.

High Magnification Confocal Imaging

To detect the location of the labeled antibodies, light of a particular wavelength was focused on the tissue to activate the fluorescent signal of the secondary antibodies as the slides were viewed through the microscope either by eye or by laser confocal imaging. The activated fluorescence signifying the position of the proteins of interest was captured by imaging software. In my images, the green signal shows the location of the photoreceptor connecting cilium when labelled with acetylated tubulin antibody and

the basal body when labelled with centrin, while the red signal denotes USH2 protein localization.

Elevated Light

For elevated light treatment, the embryos were raised from 0dpf to 6dpf in transparent petri dishes under either normal facility lighting (300-450 lux) or an elevated light source (4,500-5,000 lux). Regardless of illumination intensity, all larvae were maintained on a light cycle with 14-hour day and 10-hour nights. Embryo medium was refreshed daily. At 6dpf, the fish were euthanized and underwent the preparations for immunohistochemistry. To visualize cells positive for cell death, the primary antibody against a protein produced during apoptosis, caspase-3, was used. Use of a secondary antibody in the 568 wavelength reveals the presence of caspase as a fluorescent red signal under high magnification microscopy. The number of labeled cells in each region of the retina were manually counted and tabulated.

Statistical Analysis

To analyze and compare the data gathered from the elevated light experiment, a student's t-test was performed. A p-value was calculated to determine if the data were statistically significant. A p-value of less than 0.05 indicates that the data are significant and suggests that there is strong evidence to reject the null hypothesis.

Results

Localization Variability of Whirlin A in the ush2a^{b1245} mutant background

I analyzed the localization of Whirlin A and B in the mutant background of *ush2a*^{b1245}. The presence of the Whirlin proteins was indicated by a positive signal corresponding to either the Whirlin A or B antibody concentrated at the base of the connecting cilium and, in the case of Whirlin A, at the photoreceptor synapse. It was previously shown that the truncated form of Usherin produced in this *ush2a* mutant still localizes to the membrane surrounding the connecting cilium, so we now investigated whether the lack of the intracellular binding domain of this truncated form of Usherin would affect the normal localization of Whirlin A and/or Whirlin B.

I repeated this experiment multiple times because I obtained inconsistent results of Whirlin A localization (Figure 7). The localization of Whirlin B in this mutant background was consistently unaffected in each analysis. This variability observed within Whirlin A was different between experiments, but appeared invariable within a given experiment.

For example, in a group of *ush2a* larvae labeled with the Whirlin A antibody the localization was severely attenuated in all mutant retinas, across multiple slides, compared to wild-type controls. In other iterations of this experiment, however, normal localization would be observed in all mutant retinas.

Variability between experiments but not between tissues from the same experiment suggested that we might be observing some periodic process of stabilization and destabilization of the USH complex, so we conducted an experiment to assay whether Whirlin localization or stability in the *ush2a*^{b1245} background was influenced by the time of day at which the larvae were collected.

Figure 7

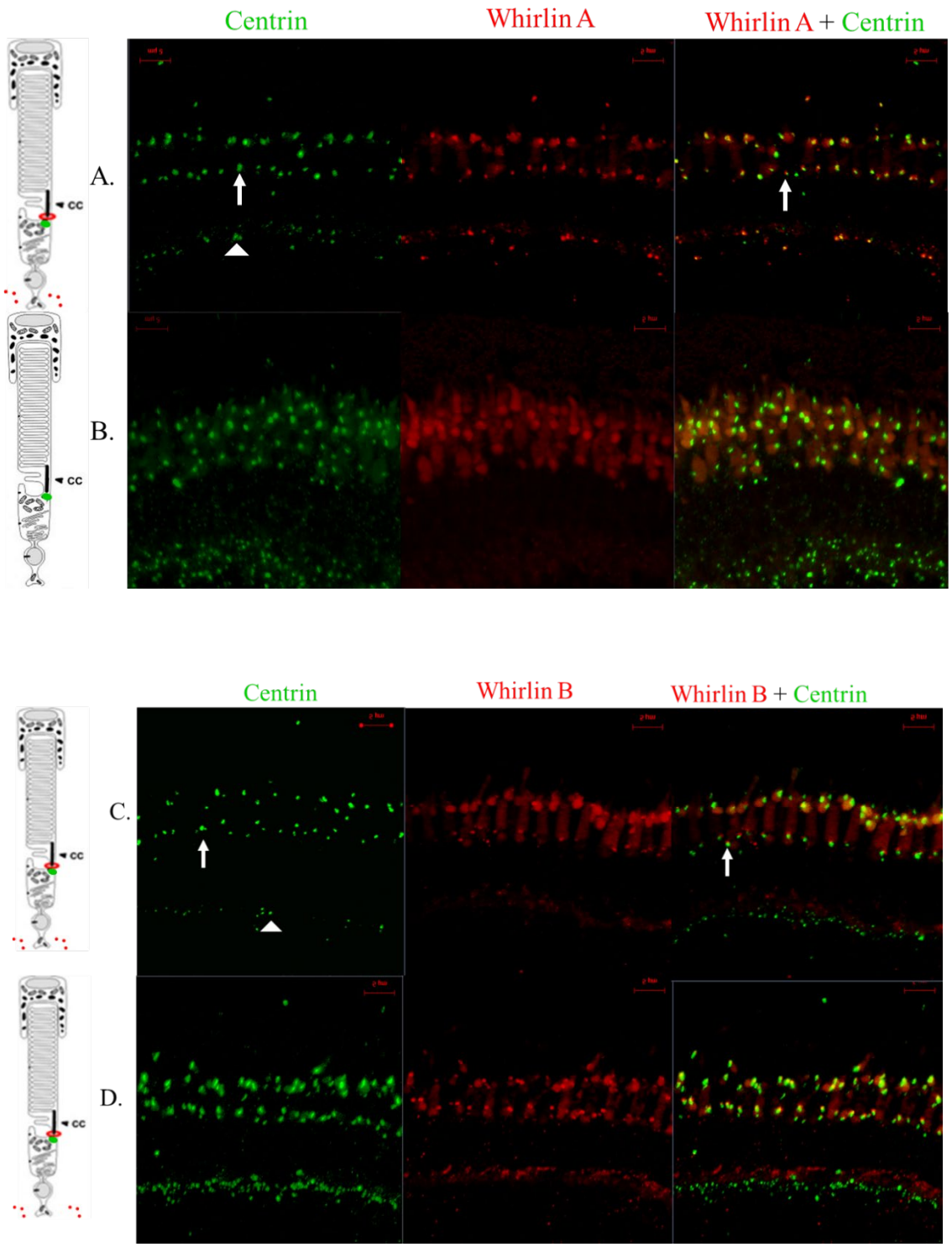
Centrin
Whirlin A
Whirlin A + Centrin
A.
B.
CC
CC
Centrin
Whirlin B
Whirlin B + Centrin
C.
D.
CC
CC

Figure 7: The localization of Whrna and Whrnb in the *ush2a*^{b1245} mutant. Centrin (green) marks the basal bodies from which the connecting cilium emanates (arrowhead in A and C, left column). Whirlin A and B localization is indicated by the fluorescent red dots located at the bottom of the connecting cilium and at the synapse. A: Wild type localization of Whrna in the ush2a mutant. B: No localization of Whrnb observed in the ush2a mutant. C: Wild type localization of Whrnb, D: Normal localization of Whrnb. Scale bar = 5µm.

Whirlin A Localization variability within the 2pm light condition

I designed an experiment to examine the effects of fixation at different times of day and with different durations of light exposure. Mutant and wild-type larvae were raised to 6dpf and euthanized under three different conditions: 9am as the facility lights came on; 2 pm after having spent the hours between 9 and 2 in normal light conditions, and 2pm after having been placed in a dark box the night before (Figure 5). Under these conditions, the localization of Whirlin A was unaffected in *ush2a*^{b1245} mutants collected at 9am at normal conditions and at 2pm after dark adaptation, (N=10 for each, data not shown).

However, for the mutant larvae collected at 2pm after five hours of light adaptation, the localization of Whirlin A was diminished (Figure 8). Curiously, in contrast to previous experiments in which the presence of Whirlin A was either normal within every mutant retina or diminished within each mutant retina, in two replicate experiments under these conditions I found variability within the 2pm light adapted mutant group, in which I documented retinas with Whirlin A localization that was normal, reduced, or gone (Figure 8). I observed normal localization 45% of the time, reduced localization 20% of the time, and absent localization 35% of the time. The occurrence of a reduced and absent localization combined was more than the occurrence

of retinas where normal localization was observed. This result suggested that light adaptation influences the stability of Whirlin A localization in the absence of functional Usherin, but the variability in these experiments suggest that additional factors likely also contribute to the stability of the USH2 complex as well.

Figure 8

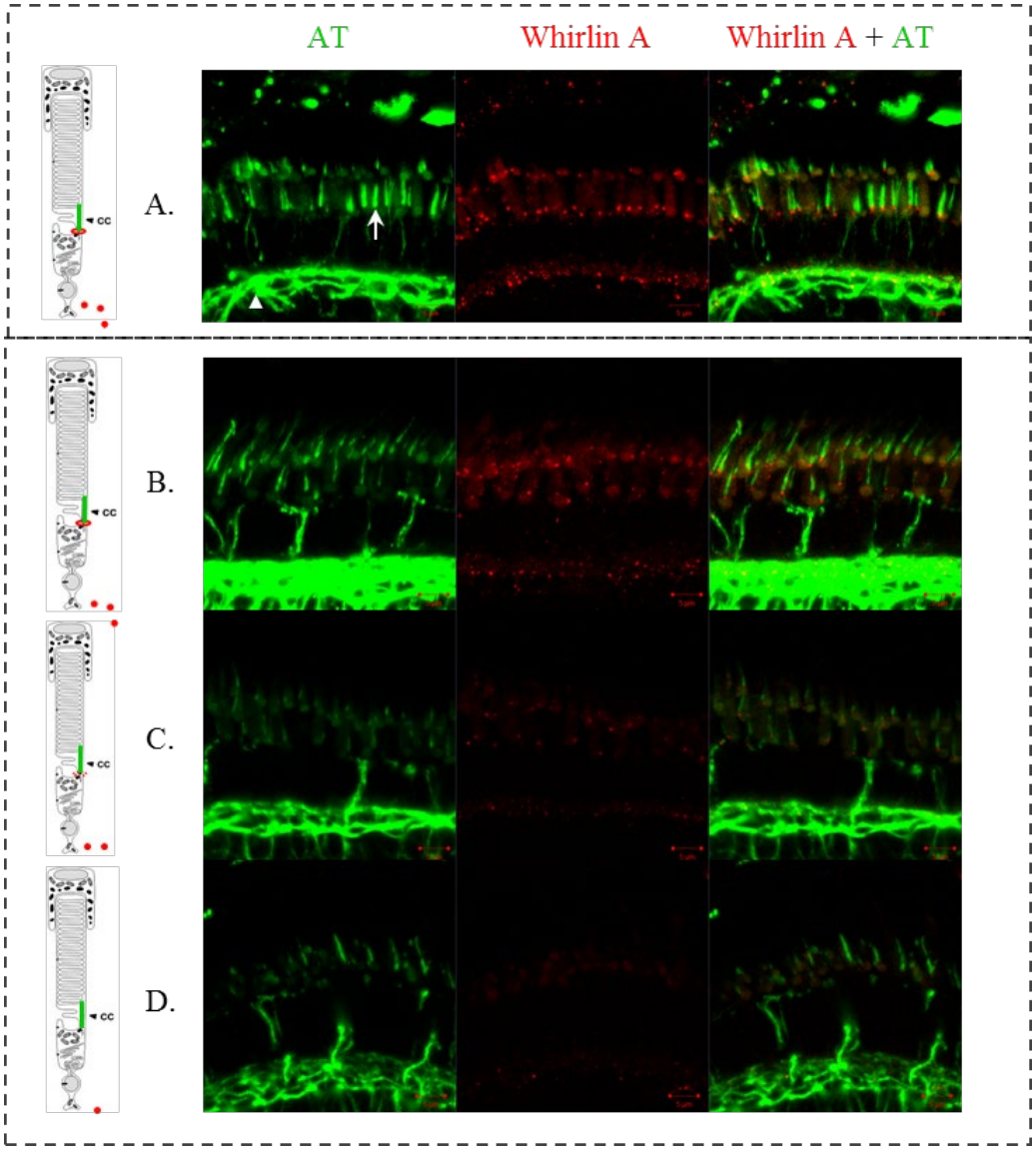

Figure 8: Variable localization of Whirlin A in the 2pm light condition in the *ush2a*^{b1245} mutant. Acetylated tubulin (green) marks the synaptic connections between photoreceptors and second order neurons (arrowhead in A) and the connecting cilium of the photoreceptors (arrow in A). Whirlin A localization is indicated by the fluorescent red dots located at the bottom of the connecting cilium and at the synapse. A: Whirlin A localization in light-adapted wild-type retinas collected at 2pm is normal. Rows B and D show examples of variable Whirlin A localization in light adapted retinas collected from the same group of fish: either unaffected (B), reduced (C), or undetectable (D). Localization appears equally affected at the ciliary membrane and at the synapse. Scale bar = 5 µm.

Truncated Usherin localizes to the ciliary membrane in adgrv1^{sa10539};ush2a^{b1245} double mutants.

Like Usherin, Adgrv1 has a large extracellular region with multiple domains for which the functions are largely unknown. Given the similarity in subcellular localization of these two proteins at the ciliary membrane, it is possible, though not proven, that the extracellular domains interact, either directly or indirectly. Previous experiments in the Westerfield lab have shown that the localization of an adgrv1-specific antibody is completely lost in *adgrv1^{sa10539}* retinas. Therefore, I designed an experiment to test whether the previously documented presence of truncated Usherin protein in at the ciliary membrane in *ush2a^{b1245}* mutants was dependent on the presence of normally localized Adgrv1. I obtained larvae from the *adgrv1^{sa10539};ush2a^{b1245}* double mutant line and performed immunocytochemistry with an antibody that recognizes the N-terminal region of Usherin. I observed that the N-terminal Usherin antibody signal was present at the base of the connecting cilium and unchanged compared to wild type and to *ush2a^{b1245}* single mutants (Figure 9). This result indicates that protein interactions mediated by the presence of the full-length Adgrv1 protein are not required for normal localization of Usherin at the connecting cilium.

Figure 9

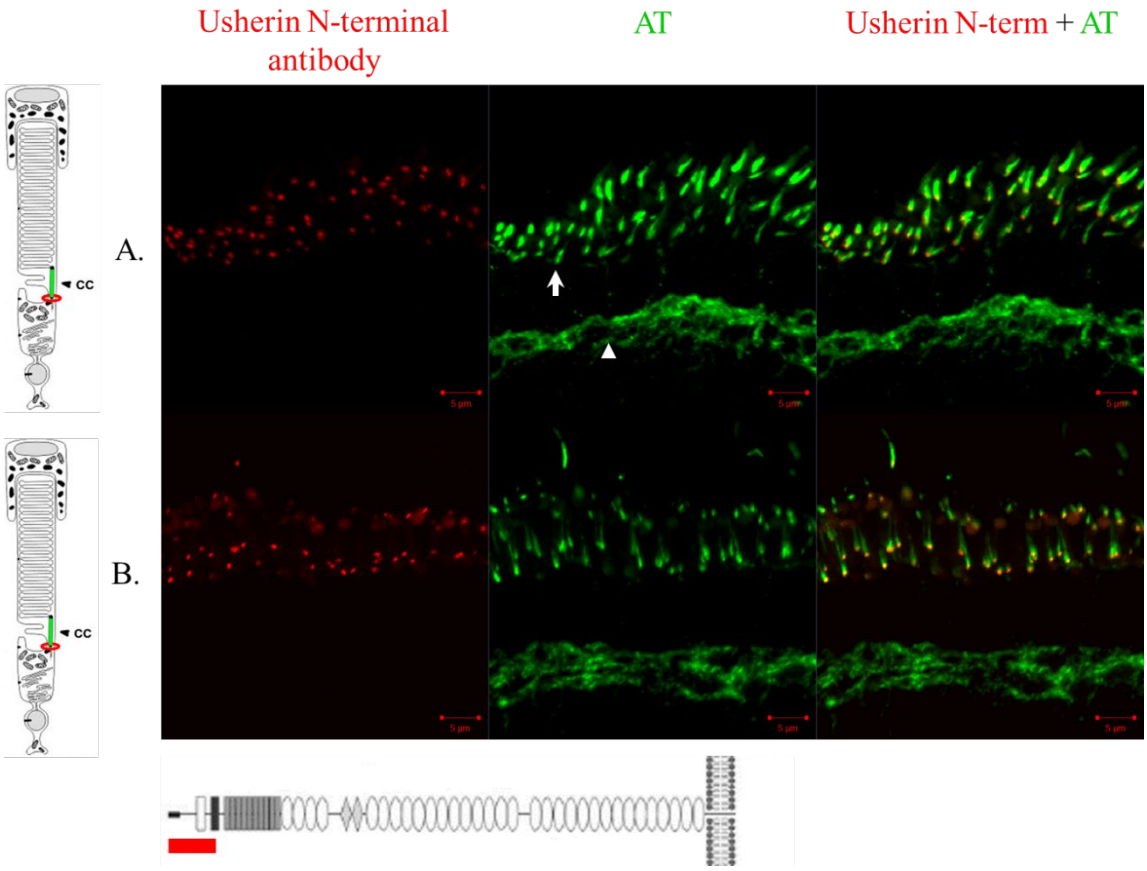

Figure 9: Usherin N-terminal localization in the *adgrv1*^{sa10539};*ush2a*^{b1245} mutant. A: Localization of Usherin in the wild type, B: Localization of Usherin in the *adgrv1*^{sa10539};*ush2a*^{b1245} double (dbl) mutant. The presence of the N-terminal Usherin antibody is indicated by a fluorescent red concentric circle. Acetylated tubulin (AT) marks the structural landmark of the connecting cilium within the photoreceptors. Scale bar = 5µm.

Elevated Light Experiment

Understanding the USH2 protein interactions revealed by these localization studies is an important component of defining the molecular pathology of this retinal disease, but for my final experiment for this thesis project, I wanted to evaluate the effects of these mutations on retinal degeneration, the key clinical finding in cases of

USH2 as well as *USH2A*-related RP. To test the extent to which depletions of different USH2 proteins impacted levels of retinal cell death, I raised *ush2a*^{b1245} and *adgrv1*^{sa10539} single mutants alongside double mutant and wild-type controls in elevated light conditions. The fish were raised beginning at 2-4 hours post-fertilization, during the late cleavage or early gastrula stages onward, at an elevated light exposure of 5000 lux on a standard light/dark cycle, and compared to stage-matched fish of the same genotypes raised under normal facility conditions. Previous work with the single *ush2a* mutant raised in constant light from 5dpf to 8dpf showed significant photoreceptor degeneration. These fish had an average 25 labeled photoreceptors per eye compared to an average of 9 labeled cells per wild-type retina under the same conditions. A slight increase in cell death in the inner nuclear layer was also observed in this previous experiment, with an average of 5 INL cells per eye labeled in mutants vs. less than one labeled cell per eye in wild-type larvae under the same conditions.

The elevated levels of cell death in photoreceptors detected in previous experiments with *ush2a*^{b1245} were recapitulated in this experiment, with a light dependent increase of caspase labeled cells noted in both the photoreceptor (ONL) layer and the INL compared to wild type (figure 10, first four groups). The level of photoreceptor cell death in this mutant was not as elevated as what was previously observed and described above, but photoreceptor health was consistently impaired in both experiments.

Cell death in the *adgrv1* mutant line had not been previously analyzed. In my analysis, the *adgrv1* mutants raised under normal facility conditions exhibited an increase in photoreceptor cell death as well as INL cell death compared to the wild type

raised under the same conditions. This increase in INL and ONL death was also in excess of the slight increase observed in these same cell layers in *ush2a* mutant retinas in normal light conditions. For both the *adgrv1* and *ush2a* single mutants, the data showed an upward trend for the light-enhanced cell degeneration with *adgrv1* looking generally worse than *ush2a*.

Based on the results in the single mutants under these conditions, we speculated several outcomes of the elevated light exposure on the double mutants. One possibility would be that the double mutant would have a higher level of photoreceptor degeneration than either of the single mutants, which would indicate that the loss of two functional USH2 proteins in tandem had an additive effect on the stability and function of the USH2 complex. Another potential outcome would be no observed difference between the levels of the photoreceptor degeneration in the double mutant, compared to the 'worst case' single mutant condition, which would indicate that losing multiple components of the USH2 complex is no worse than losing only one. A third possibility would be an observation of less cell death in the double mutant than in either of the single mutants, suggesting that a more dysfunctional cellular condition is created when one of these two large proteins is disabled than when both are impaired simultaneously.

The results from the double mutant raised under normal light conditions continued the trend of increased photoreceptor death seen in the outer nuclear layer. This increase was seen to a greater extent compared to the *ush2a* and *adgrv1* single mutants and the wild-type controls raised under normal light, conforming to the 'additive effect' hypothesis. However, there was an unexpected decrease in the level of cell death observed in the double mutant raised under elevated light conditions

compared to the double mutant raised in normal light as well as the single mutants raised under either condition.

The retinal cell death observed in each group of mutant larvae had high statistical significance compared to the corresponding wild-type groups (($p < 0.001$). However, although the average number of labeled cells increased in the single mutant larvae raised in elevated light conditions compared to those raised in normal light, there was a large degree of variance around the mean in these groups, and these increases were not statistically significant ($p > .05$). The lack of significance could be attributed overall to the low sample size of all the groups. Specifically, for the ush2a mutant, compared to a previous experiment, this result may be due to the amount of days the larvae were raised in the elevated light and a later onset of the elevated light exposure could have made a significant difference between the normal and elevated light groups. Since this is the first-time retinal cell death in the *adgrv1* mutant has been analyzed, this result may be due to some other background mutation(s) in the line contributing to the cell death sensitivity that we need to breed away from for future experiments.

Figure 10

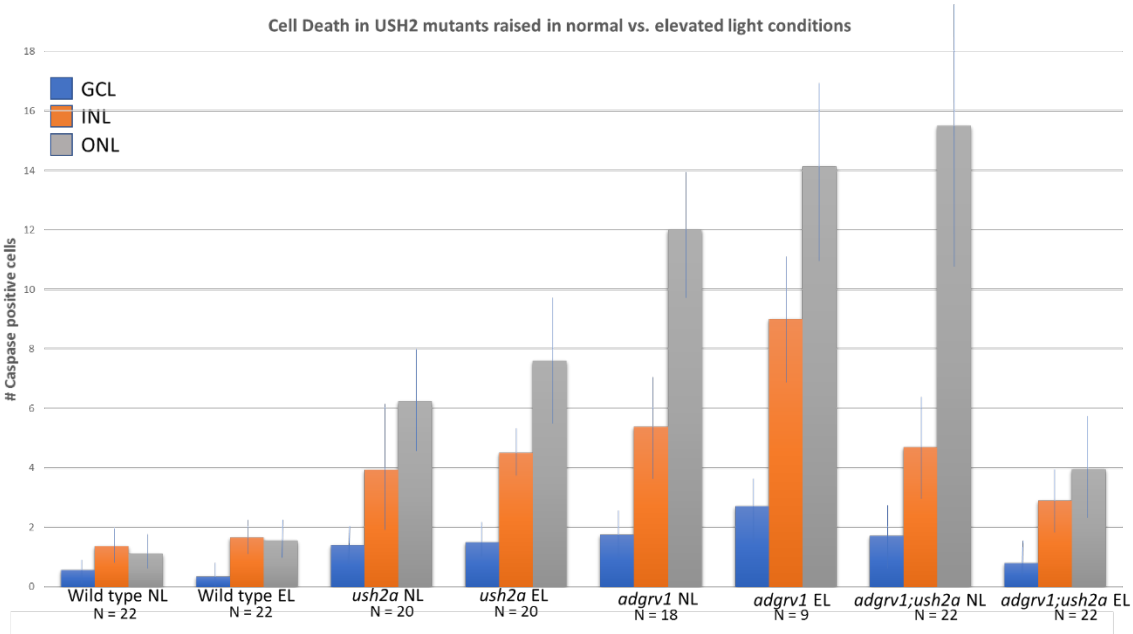

Figure 10: Retinal cell degeneration recorded by retinal cell layer in normal (NL) versus elevated (EL) light conditions. Positive caspase-3 antibody signals within each layer were counted and summed. Each column represents the mean number of labeled cells for the particular genotype, cell layer, and condition. Standard deviation is shown with a thin blue line on each bar. GCL (blue): ganglion cell layer, INL (orange): inner nuclear layer; ONL (grey): outer nuclear layer.

Figure 11

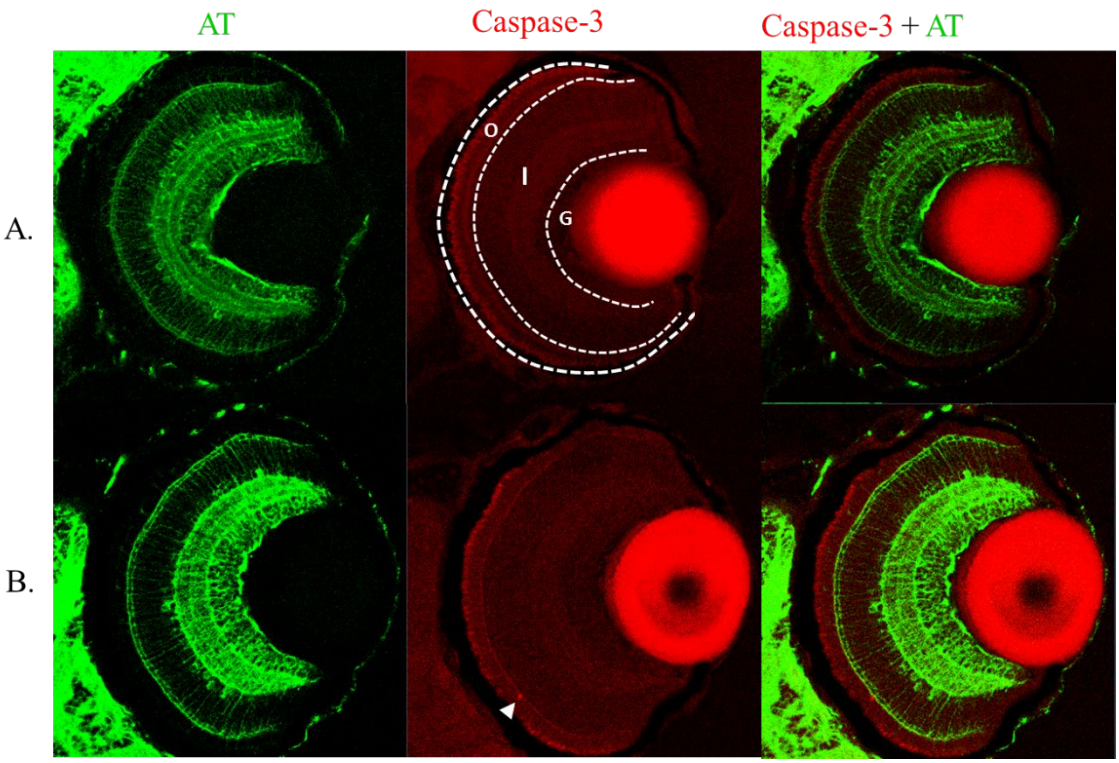

Figure 11: The caspase-3 labelling of photoreceptor cell death. A: A healthy wild type retina where no cell death is detected. B: ush2a mutant retina raised in normal light where there is one positive caspase labelled cell in the ONL.

Discussion

The localization of Whrna, but not Whrnb is affected in the absence of full-length Usherin.

In this study, the localization of both Whirlin A and B were evaluated in *ush2a*^{b1245} mutants. We found that the localization of Whirlin B was present and consistent across multiple trials, however, the localization of Whirlin A was variable: either present, reduced, or completely absent (Figure 7). This variation suggests that the C-terminal PDZ binding domain of Usherin, although not required for initial localization of Whrna, appears to play a role in stabilizing Whrna localization. The variable result of our initial experiments after numerous repetitions motivated us to control the time of fixation to see if this had an effect on the differences observed in localization.

Whrna localization is reduced in light-adapted photoreceptors in the *ush2a*^{b1245} mutant.

Three groups of larvae raised under normal light cycle conditions were fixed at 6dpf at either 9am, 2pm after light adaptation, or 2pm after dark adaptation. We found that only *ush2a* retinas collected at 2pm after light adaptation showed reduction in Whrna localization. This supported our hypothesis that light-adapted photoreceptors would exhibit more instability of the USH2 complex in the mutant background. The presence of Whrna was still observed in this condition but there was a majority, 55% of retinas, in which Whrna localization was abnormal, either reduced or undetectable, at the periciliary membrane (Figure 8).

N-terminal Usherin localizes to the cell membrane in the *adgrv1*^{sa10539};*ush2a*^{b1245} double mutant.

Mutations in *ush2a* and *adgrv1* were studied separately and combined in a double mutant in this study. The localization of the truncated Usherin protein produced in *ush2a*^{b1245} mutants was not disrupted in the *adgrv1*^{sa10539};*ush2a*^{b1245} double mutants as shown by the presence of the Usherin N-terminal antibody signal (Figure 9). This result suggests that localization of Usherin to the periciliary membrane does not rely on interactions with Adgrv1. Although this result is somewhat suggestive of a model in which these proteins act largely in parallel, as opposed to interactively, it was important to assay the effect on the simultaneous loss of Adgrv1 and Usherin function on the health of the photoreceptor cells.

Elevated Light Effect on Retinal Degeneration in *ush2a* and *adgrv1* single and double mutants

I evaluated the single *ush2a*^{b1245} and *adgrv1*^{sa10539} mutants as well as the double *adgrv1*^{sa10539}; *ush2a*^{b1245} mutant larvae with an elevated luminance on a light cycle from 0dpf to 6dpf. The levels in each layer for the wild-type controls between the normal and elevated light conditions were similar, indicating that this was not a damaging light level. The results showed a significant increase in retinal degeneration within the inner and outer nuclear layers of *ush2a*^{b1245} and *adgrv1*^{sa10539} mutants raised under elevated light (Figure 10). The outer nuclear layer is where the photoreceptors are found, and photoreceptors are the primary retinal cells that degenerate in human USH patients. In general, the single *adgrv1* and *ush2a* mutant retinas seemed less healthy overall and this effect of increased retinal cell death was enhanced in the elevated light conditions.

The results for the double mutant showed increased photoreceptor death in the ONL compared to the single *adgrv1* and *ush2a* mutants raised in the normal light conditions. However, there was an unexpected drop off of the retinal cell death seen in the double mutant raised in elevated light conditions. This could be due to the poor staining of this particular set of slides which made them difficult to read possibly causing some signal to be missed in the manual counting. Another reason could be that the retina in the double mutant is extremely dysfunctional that most of the cell death occurred earlier, before viewed at 6dpf. Caspase labels cells that are dying and once they are dead, the cells disappear and there is no caspase left to detect.

Analysis of these results showed that the difference between the normal and elevated light conditions within the same genotype was not statistically significant. However, the death observed in all the mutant groups compared to the wild-type controls was significantly increased ($p < 0.001$). There are detectable levels of cell death even at baseline in these Usher mutant lines and it appears that these levels are trending upward in the groups that are exposed to higher light intensities.

Through these experiments, we have gained further insight into the functional roles of USH2 proteins that will assist in directing gene therapy efforts to halt the progressive retinal degeneration experienced in USH2. We found that in the *ush2a*^{b1245} mutant, localization of Whrna exhibits more instability after longer durations of light adaptation whereas the localization of Whrnb is unaffected, and that the C-terminal binding domain of Usherin, although not required for localization to the membrane, is a crucial domain for USH2 complex stability which will need to be accounted for in emerging gene replacement therapy efforts.

Additionally, we evaluated the effect of elevated light on the *ush2a* and *adgrv1* single and double mutant retinas. We found that photoreceptor death increases in both these mutant backgrounds raised in elevated light conditions but most notably in the *adgrv1* mutant. The increase in photoreceptor death is seen even more in the double mutant raised in normal light but, unexpectedly, not in the elevated light conditions. This result suggests that increased light exposure has harmful effects on photoreceptors with faulty USH2 protein interactions. Understanding the relationships of USH2 proteins and the effects of these mutations on retinal cell function will help to inform therapeutic efforts through assaying what functional domains are critical and which method of gene therapy would be the most effective. The development and characterization of these zebrafish USH models make them appealing subjects in which to test the effectiveness of future therapies in reestablishing the USH2 protein complex and preventing retinal degeneration.

Future Directions

The results of this experiment motivate us further to understand and test the localization of USH2 proteins in these *ush2a* and *adgrv1* mutants. The *adgrv1* mutant has not been studied to the same extent as the *ush2a* mutant. Therefore, future experiments should test the localization of USH2 proteins in the *adgrv1*^{sa10539} background as well as in the double mutant. Experiments that tested the localization of Whrna and Whrnb in these backgrounds would provide insight into how Whirlin localization is affected when a functional Adgrv1 protein is not produced, and the extent to which the presence of a normal Usherin protein impacts the presence of Whirlin proteins without a functional contribution from Adgrv1

Future experiments should also look at different alleles of *adgrv1*. The allele studied in this thesis appears to be a null, with no residual Adgrv1 protein activity. The next steps to take for understanding the important functional domains of adgrv1 would be to create and test an allele that encodes a slightly truncated protein lacking only the C-terminal protein binding domain of Adgrv1 similar to the *ush2a*^{b1245} allele that allows a targeted analysis of the PDZ-binding domain of Usherin. In addition to validating the results observed with *adgrv1*^{sa10539}, experiments with these tailored alleles would illuminate the importance of this C-terminal binding region relative to other protein domains of Adgrv1 in retinal cell function and survival.

Significant results have been observed previously using *ush2a* mutants raised under increased light conditions. A future experiment should carry out this specific elevated light experiment again with an increased sample size to assist in making a more statistically significant result. Modifying the conditions of elevated light by

exposing the larvae to the increased light at different stages of their retinal development could also assist in making the result more significant.

The severe effect of cell death within the outer and inner nuclear layers of the retina seen in the zebrafish model highlights the potential for light damage and the importance for early protection of the human retina. A future direction to implement these results would be to raise the mutant fish larvae in elevated light using different filters that block particular wavelengths, to test whether filtering specific wavelengths such as UVA, UVB, or blue light could reduce photoreceptor cell death. One measure that is recommended to people living with late stage USH is wearing dark glasses when outside to reduce glare that interferes with their residual vision. An experiment that tested different filters could help inform effective measures that are recommended to USH patients and scientifically verify whether these measures assist in reducing retinal degeneration and help to slow progressive blindness.

Glossary of Terms:

Adgrv1- USH2 protein known to interact with Usherin and Whirlin
Allele- An alternative form of a gene.
Alpha Tubulin- antibody used in the staining process to show the structure and organization of neurons. Labels the connecting cilium of the photoreceptors and neuronal connections within the eye.
Antibody- a blood protein produced in response to a specific antigen (for example, a protein of interest)
Centrin- antibody used in the staining process to show the basal body, a structure at the base of the connecting cilium
Connecting cilium- hair-like structure located within the photoreceptors. It functions as the main conveyor system between the inner and outer segments, forming a bi-directional conduit for cargo to travel.
dpf- days post-fertilization
Gene- functional unit of heredity, made up of DNA, provides instructions to make proteins
Genotype- genetic makeup of an organism
Mutation- a permanent change in the nucleotide sequence of the gene
Photoreceptor- a photosensitive cell found in the outer nuclear layer of the retina
PBST- Phosphate buffered saline with tween. Solution used in many lab protocols to wash and rehydrate tissue.
Protein- molecule required for structure, function, and regulation of the body's tissues and organs
Recessive- the form of inheritance exemplified by Usher syndrome, two mutated copies of a gene must be inherited from both parents to cause the disease.
Retinitis pigmentosa (RP)- the patterns of retinal degeneration in Usher syndrome
WT- wild-type, normal function

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