PRECLINICAL TRIAL OF THE ANTIOXIDANT COMPOUND HEXAFLUORO IN A ZEBRAFISH MODEL OF USHER SYNDROME TYPE 1F

by

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A THESIS

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Usher syndrome (USH) is a genetic disorder that is the leading hereditary cause of deaf-blindness, affecting more than 400,000 people worldwide. Usher syndrome type 1 (USH1) is characterized by hearing loss from birth, and gradual vision loss beginning with reduced night vision in childhood. Mutations in the *PCDH15* gene result in Usher Syndrome type 1F (USH1F), a subcategory of USH1. These mutations create nonfunctional versions of the protocadherin-15 protein (PCDH15) which impair its function, disrupting the structure and function of hair cells in the inner ear and photoreceptor cells in the eye. Zebrafish models of USH1F have abnormal photoreceptor structure, reduced visual function, and elevated rates of cell death, symptoms that are exacerbated by bright light. A pilot study showed that the antioxidant compound hexafluoro improved visual function in young zebrafish models of USH1F raised in dim conditions. We treated USH1F mutant fish raised in a variety of daytime light conditions with hexafluoro to understand hexafluoro's effect in conditions that result in increased cell death. We used an optokinetic response assay to test the compound's effect on visual function, and analyzed its effect on photoreceptor cell death by tallying the number of photoreceptors in the central region of the retina. We found that hexafluoro improved visual function and slowed photoreceptor cell death in the USH1F mutant fish raised in all light conditions. Our data indicate that hexafluoro

has a stabilizing effect on photoreceptors in zebrafish USH1F models, and implicate oxidative stress as a possible mechanism behind the pathology of USH1F visual symptoms. These results support further investigation into the mechanism(s) underlying hexafluoro effects and application of this antioxidant in other models of USH.

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1 Introduction

1.1 Usher Syndrome is an inherited disorder caused by possessing two disease-causing copies of the gene

The central dogma of molecular biology defines the flow of genetic information from DNA which is then transcribed into RNA before being translated into the amino acid sequence of a protein (Chang and Qi, 2023). Genetic information is stored in sequences of DNA, known as genes, using a genetic code made up of four different characters. DNA sequence of a gene is transcribed into messenger RNA (mRNA), a template that can be read by the molecular machinery that translates the genetic information into the sequence of a protein. The code is read three characters at a time, known as codons, and each three-base codon codes for either one of twenty amino acids or the halting of translation (Diercks et al., 2021). Amino acids are the building blocks of proteins and when first translated from mRNA, form a chain known as a polypeptide. Polypeptides fold, and sometimes bind with other polypeptides, in a sequence-specific manner to form functional proteins. As the primary macromolecular machinery, proteins carry out most of the essential tasks for cell survival and function. A protein's role in the cell is determined by its three-dimensional structure and specific arrangement of amino acids (Diercks et al., 2021). In summary, a gene is a sequence of DNA which encodes a protein that has a specific role in the cell depending on its structure. Changes to the sequence of the gene, known as mutations, can change the structure and function of the protein that the gene encodes, and can cause disorders like Usher Syndrome (USH).

DNA is packaged into structures called chromosomes that allow the genetic material to be passed from parents to offspring. Humans have twenty-three pairs of chromosomes, receiving

one chromosome in each pair from each parent. Genes contained on the chromosomes are also in pairs. One copy of every gene comes from each parent and both are used to produce proteins.

USH gets passed from parents to children via an recessive inheritance pattern, meaning that the disorder is caused by having two disease-causing copies of a gene, one on each chromosome in a pair. Usually recessive disorders are caused by non-working copies of a gene, ones that result in a lack of protein formation or the production of non-functional proteins. One USH-inducing copy must be inherited from each parent, and for this to occur both parents must either be “carriers" or affected themselves (Figure 1). Carriers are individuals with just one disorder-causing allele but aren’t affected themselves because one working copy is enough to produce the appropriate amount of protein product. Two carrier parents of autosomal recessive disorders like USH have a 25% chance of having an unaffected child with no disease causing alleles and a 50% chance of having a child that is a carrier. There is a 25% chance in this scenario that their child will have two copies of the disease causing gene and would be affected with USH (Genetic Science Learning Center, 2014).

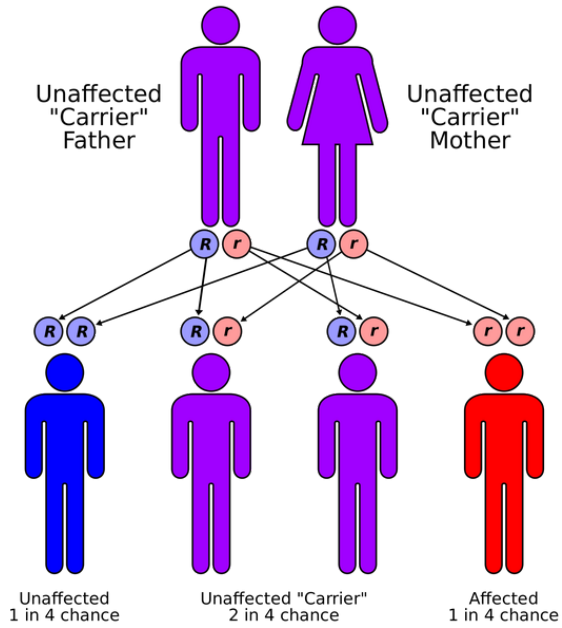

Figure 1: Inheritance pattern of an autosomal recessive disorder
In an autosomal recessive disorder, two carrier parents, who each have one disease-causing copy of a gene, have a 25% chance of having an affected child. The disease-causing copy of the gene is represented by the lowercase 'r' in a red circle while the non-disease-causing copy is represented by the uppercase 'R' in the blue circle. Source: Rosalind

1.2 USH1F symptoms are caused by the loss of functional PCDH15 protein

Usher syndrome was first described in 1914 by Scottish ophthalmologist Charles Usher. The disorder has three clinical types, 1, 2, and 3, which are characterized by when symptoms first appear and their severity. Five different genes have been linked with USH1. Between ten and twenty percent of USH1 cases are caused by mutations in *PCDH15* which is categorized as Usher syndrome type 1F (USH1F) (Chen et al., 2022). The gene *PCDH15* encodes a protein called protocadherin-15 (PCDH15). PCDH15 belongs to a class of integral membrane proteins and is found in the retina and the inner ear of humans (Chen et al., 2022). Individuals with USH1F lack a functional PCDH15 protein, resulting in the disease symptoms described below.

People with USH1F are born deaf, due to abnormalities with the hair cells of their inner ear which also causes balance defects, and experience the onset of vision loss in childhood. Retinitis pigmentosa (RP) is part of the visual symptoms of USH1F and is a type of retinal degeneration, where the cells in the eye that detect light, which are known as photoreceptors, degrade and die. What makes RP distinct from other types of retinal degeneration is that the rod photoreceptors are lost first, then the cone photoreceptors (Berni et al., 2023). RP gets its name from the splotchy, dark appearance of the retina that is observed during ophthalmological examination, caused by the pigmented cell layer in the back of the eye protruding through the degenerating photoreceptor layer (Figure 2). Photoreceptor loss in USH1F is gradual and follows a spatial pattern, starting at the periphery and moving inward. Vision loss starts with night blindness in childhood and the daytime visual field narrows over the course of decades, with the cone-rich central vision being preserved the longest. (Delmaghani and El-Amraoui, 2022).

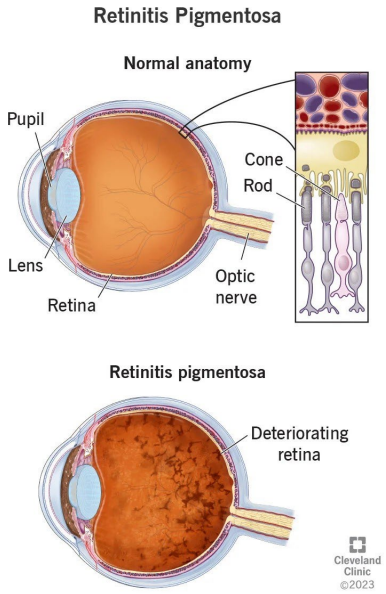

Figure 2: Appearance of Retinitis Pigmentosa
An eye with retinitis pigmentosa has a characteristic splotchy appearance caused by the pigmented layer (represented by purple and red circles) behind the photoreceptor layer (shown above as rods and cones) moving forward towards the lens and poking through the degenerating photoreceptor layer. This appearance is observed during an eye exam. Source: Cleveland Clinic

USH1F is more prevalent among Ashkenazi Jews than many other populations, and over 60% of cases are caused by a particular allele (alternative form of a gene) of *PCDH15.* One in fifty Ashkenazi Jews are carriers for this allele known as R245X. It's a mutation of *PCDH15* which leads to the premature stop in production of protein PCDH15 (Ben-Yosef et al., 2003). When protein production is halted early, it creates a truncated form of PCDH15 which is nonfunctional (Figure 3).

Figure 3: R245X mutation produces a truncated PCDH15 protein
Top: Full-length PCDH15 protein represented graphically. The beginning of the protein is labeled with an N and the C denotes the end of the protein. The blue blocks represent regions of PCDH15 that interact with other proteins outside of the cell, while the orange block shows what part of the protein interacts with cell membranes and allows the protein to embed within it. Proteins inside the cell interact with the region of PCDH15 that is colored purple.
Bottom: The truncated PCDH15 protein that results from the R245X mutation causing a premature halt to protein production. This truncated protein no longer has the blue, orange, or purple regions, rendering it nonfunctional as it's unable to interact with other proteins. Source: Phillips et al., 2021.

Now that the symptoms of USH1F have been reviewed, let's zoom in on the human eye and take a look at the anatomy of the retina to get a better understanding of the structures and what occurs in USH1F on a cellular level. Since this paper focuses on the visual symptoms of USH1F, the focus will be on retinal anatomy and not the anatomy of the inner ear.

1.3 The retina contains the cells that detect light and their dysfunction causes the vision loss associated with USH1F

When light waves enter the eye, they are first focused by the outermost transparent layer, known as the cornea, onto the lens. The lens focuses light onto the tissue of the back of the eye known as the retina. There, the light is absorbed by the photopigments in the photoreceptors of the outer retina and converted into electrical signals. The electrical signals travel through a

complex array of neurons in the inner retina, ultimately arriving in the ganglion cells layer where they are consolidated in the optic nerve for transmission to the visual center of the brain (Chhetri et al., 2014) (Figure 4).

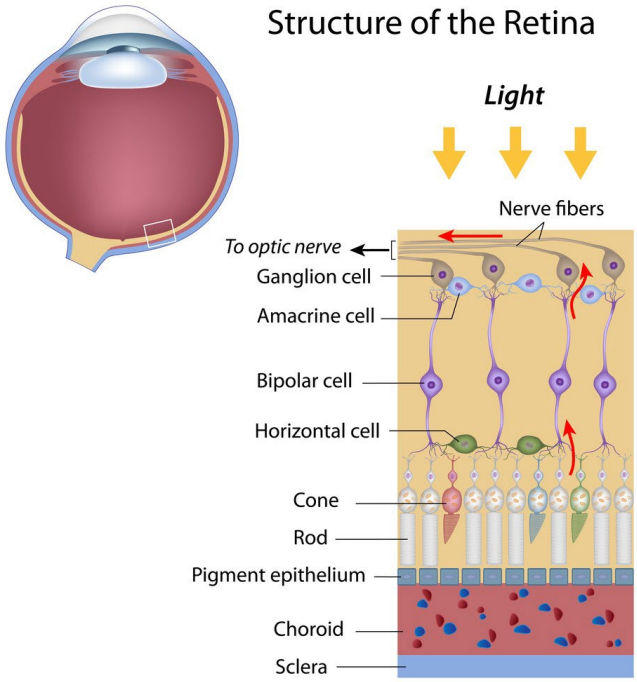

Figure 4: The structure of the human retina
Light passes through the retina and is absorbed by rods and cones in the photoreceptor layer, which get excited and convert that light into an informational signal which travels along the path denoted by the red arrows. This information travels through the bipolar cells to the amacrine cells, then the ganglion cells which eventually coalesce to form the fibers of the optic nerve. This nerve carries the visual information to the brain. Source: Discovery Eye Foundation

Photoreceptors have inner and outer segments, each of which contain different cellular components (Figure 5). The inner segment houses the machinery that keeps photoreceptors alive like mitochondria which produce energy to power the cell and the endoplasmic reticulum (ER) which processes the proteins that carry out cellular tasks. The outer segment is much larger than the inner segment, with enough room to hold an ordered stack of membranes containing the proteins that detect light (opsins) and carry out the resulting signal transduction cascade (Zang

and Neuhauss, 2021). This flood of directed signals is a biochemical pathway that turns light waves into biological information.

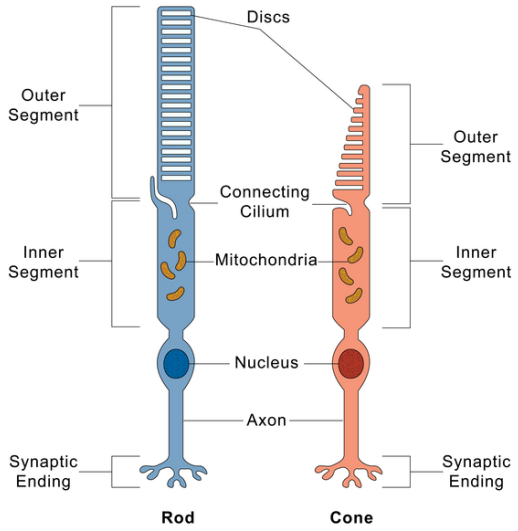

Figure 5: Anatomy of Rods and Cones
Rods and cones are structurally similar. The mitochondria that power the cell are located in the inner segment and discs containing the pigments that absorb light are in the outer segment. The outer segment points towards the RPE and the synaptic ending points towards the bipolar cells. Source: MedlinePlus Genetics

In the human retina, the base of the outer segments of photoreceptor cells has a collar of small finger-like projections known as the calyceal processes (CP) (Figure 6). It has been suggested that the CP have a structural role, reinforcing the junction between inner and outer segments. PCDH15 is known to localize in the CP and elevated levels of the protein are also found at the inner-outer segment junction of rods in humans (Sahly et al, 2012). PCDH15, like other USH1 proteins, likely plays a key role in maintaining the connection of CP to the OS. Therefore, when PCDH15 loses its function due to USH1F, CP have structural abnormalities and photoreceptor survival decreases (Sahly et al., 2012).

Figure 6: Location of calyceal processes
The finger-like projections of the calyceal processes (green) extend from the top of the inner segment to the bottom of the outer segment on photoreceptors. Calyceal processes help to maintain the connection between the inner and outer segments and this is where PCDH15 can be found. Source: Modified from MedlinePlus Genetics.

There are two different types of photoreceptors: rods and cones. Rods are capable of detecting dim light but do not detect color. Rods are highly sensitive, meaning it doesn't take much light to excite them and they can pick up subtle shifts in light caused by movement. They are also able to respond really quickly to these minute stimuli. The sensitivity and speed of rods comes from the structure of the proteins in rods that absorb light (rhodopsin) and the wiring patterns of the nerves that accept signals from rods (Kolb, 2011). Detecting dim light means that rods are capable of firing at night, producing night vision in the dark conditions. The ability to pick up small shifts in light makes rods great motion detectors, a feature that is used in peripheral vision. Rods are more dense in the periphery of the retina, farthest away from the center. This is an adaptation that allows humans to detect movement or a threat approaching from the side, in the edge of their visual field, with ease and speed (Zang and Neuhauss, 2021). RP causes the

rods to die first, producing the tunnel vision experienced by people with USH1F as they gradually lose their sense of sight. The vision loss starts at the edge of the visual field as the rods in the periphery of the retina die first, and moves inward over decades as the cones begin to die second (Figure 7).

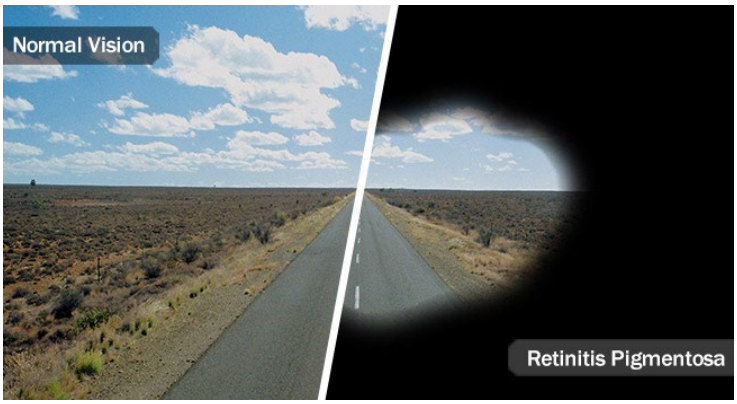

Figure 7: Vision loss caused by retinitis pigmentosa
Retinitis pigmentosa causes a pinholing effect on the visual field as seen in the panel in the right. Vision loss begins in the periphery of the visual field and moves inward over decades. Source: Lions Eye Institute

Unlike rods, cones function under bright light, detecting color and luminance. Cones are less sensitive than rods, requiring more light before it can be absorbed and initiate a signal cascade. Despite decreased sensitivity and slower firing time, cones are more accurate than rods. Cones are really dense in the central retina which supports forward facing human eyes and produces the most accurate vision in the center of the visual field (Zang and Neuhauss, 2021).

The layer behind the photoreceptors, closer to the back of the eye, is known as the retinal pigmented epithelium (RPE). The RPE acts as a selective barrier, controlling the flow of nutrients and waste products to and from the photoreceptors, and supports the photoreceptors and retina as a whole. Cells in the RPE are known for their melanin granules, structures that have the pigment melanin which helps to protect photoreceptors. The RPE is the aforementioned

pigmented layer that comes into the degenerating photoreceptor layer in RP, producing the splotchy, pigmented appearance of the eye that gave RP its name.

The type and distribution of photoreceptors in a vertebrate eye are adapted to its environment. Animals like humans and zebrafish that are awake and active in the daytime have photoreceptor ratios that are suited to bright light (Peichl, 2005). The center of the human retina is cone dense, similar to the retina of zebrafish which is made up of about 60% cones and 40% rods. A higher percentage of cones suits perception of daylight and colors caused by sunlight that is useful to animals awake during the day. The retinas of nocturnal animals like mice and rats have retinas made up of 97% rods and only 3% cones, a favorable ratio for seeing well in the dark (Zang and Neuhauss, 2021).

1.4 Zebrafish are great model organisms for studying the ocular symptoms of diseases like USH1F

Studying complex concepts in live organisms can help experimental biologists make new discoveries. Organisms from microbes, to fruit flies, to mice are used as 'model organisms' which gives scientists the opportunity to explore biological processes in one lifeform and learn new information that can be applied to another, like humans. Studies done in model organisms teach biologists about genetics, development, disease mechanisms, and evolution. These discoveries can explain processes occurring in humans that cannot be directly studied in the human body (Matthews and Vosshall, 2020). Generally, model organisms are easy and inexpensive to grow and maintain in a laboratory, have quick generation times, and possess genomes that can be easily altered by experimental genetic tools (Matthews and Vosshall, 2020).

The zebrafish (*Danio rerio*) (Figure 8) was established as a vertebrate model organism at the Institute of Molecular Biology at the University of Oregon by George Streissinger (Irion and Nüsslein-Volhard, 2022). These small tropical fish have many features that make them good model organisms including their small body size, ease of maintenance, and the fact that they produce lots of embryos when bred. Over 70% of human genes have a zebrafish version, making zebrafish a strong option for studying genetic disorders (Howe et al., 2013). The large size of their eyes relative to their body, the structure of their retina, and the early development of the eye make zebrafish good models for studying ocular development, function, and disease symptoms like those associated with USH1F (Zang and Neuhauss, 2021). The zebrafish retina is quite similar to that of humans, increasing the confidence of scientists that discoveries made in zebrafish are applicable to humans. Zebrafish have retinas composed of three nuclear layers and two layers where neurons make their connections to one another. This layering is established by 3 days post fertilization (dpf) and contains six neuronal cell types along with a population of neuronal support cells called glia (Gestri et al., 2012). Zebrafish have four different types of cones: capable of detecting blue, UV-, red-green-wavelengths, respectively. Again, the retinas of zebrafish and humans are adapted to daytime living (Richardson et al., 2017). Rapid development of the visual system means that visual function can be assessed within the first week of life and this increases the ease of assessing visual function relative to other organisms like human children (Zang and Neuhauss, 2021).

Figure 8: Zebrafish
A male adult zebrafish with characteristic yellow abdomen and darkly colored anal fin. Zebrafish are typically about 2-4 cm in length, roughly the length of 1-2 quarters. Source: News Medical: Life Sciences

Young zebrafish exhibit characteristic visual behaviors that can be observed and recorded to assess visual function. The optokinetic response (OKR) is a reliable behavioral test where eye movements track a visual stimulus of moving stripes. This behavior is observed and dependable at 5dpf, requiring a relatively simple set up to test (Zang and Neuhauss, 2021).

1.5 Zebrafish models of USH1F have decreased visual function and increased photoreceptor cell death

Zebrafish have two *pcdh15* genes, *pcdh15a* and *pcdh15b*. Pcdh15a protein contributes to the structural and functional integrity in the inner ear. Pcdh15b protein is found in the retina and inner ear, meaning that the typical structure and function of photoreceptor cells and the ability to balance are severely impaired by the loss of functional Pcdh15b (Seiler et al., 2005; Miles et al., 2021; Phillips et al., 2021).

A handful of zebrafish models of USH1F have been described to date. Using the gene editing technique CRISPR/Cas9, Phillips et al. (2021) produced a zebrafish with a mutation in

exon 8 of the *pcdh15b* gene producing an allele (alternative form of gene) known as b1257 (Figure 9). This mutation models the R245X mutation seen in the Ashkenazi population and just like the R245X allele, produces a truncated pcdh15b protein in zebrafish caused by a premature halt in protein production (Phillips et al., 2021).

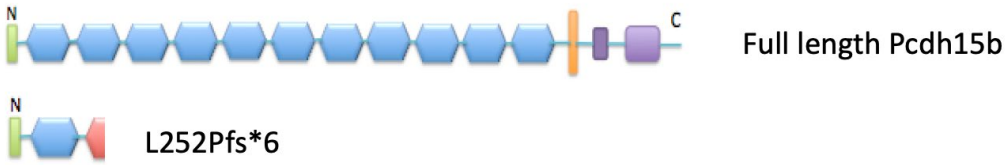

Figure 9: Truncated Pcdh15b protein models R245X mutation in humans
Top: Full length pcdh15b protein found in zebrafish. Like PCDH15, it has regions that interact with other proteins outside of the cell (blue blocks), regions that interact with proteins inside the cell (purple blocks), and a region that embeds within the cell membrane (orange block). N denotes the start of the protein and C denotes the end.
Bottom: CRISPR/Cas9 technology caused a gene alteration known as L252Pfs*6 that models the R245X mutation seen in Ashkenazi populations, producing a truncated pcdh15b protein that lacks similar components as the truncated PCDH15 protein. Source: Phillips et al., 2021.

The b1257 mutants have balance defects, which cause them to swim in an abnormal fashion, and visual defects. Balance defects are caused by structural deficiencies in the hair cells of the inner ear. At 5 dpf, b1257 mutants can be identified by their abnormal swimming, characterized by a looping and twirling motion. This is the same day that visual function can be assayed and the mutant fish are found to have reduced visual ability in comparison to their normal, also known as wild-type (WT), siblings. The b1257 mutants have abnormally structured outer segments of their photoreceptors. Their calyceal processes (CP) are also irregularly shaped and less of them are intact than in the wild type (Figure 10) (Phillips et al., 2021; Ivanchenko et al., 2023). Antibodies raised against human PCDH15 recognize both zebrafish pcdh15 proteins and label the CP in wild-type fish. This labeling is absent in the b1257 mutants, suggesting that the mutant does not make a full length, functional pcdh15b protein and validating its presence and role as an essential component of CP stability. Miles et al. (2021) also produced a zebrafish

model of USH1F, known as uot14, by making a seven base pair deletion in exon 7 of *pcdh15b*. These uot14 fish have defective CP and disorganized outer segments, similar to the b1257 mutants.

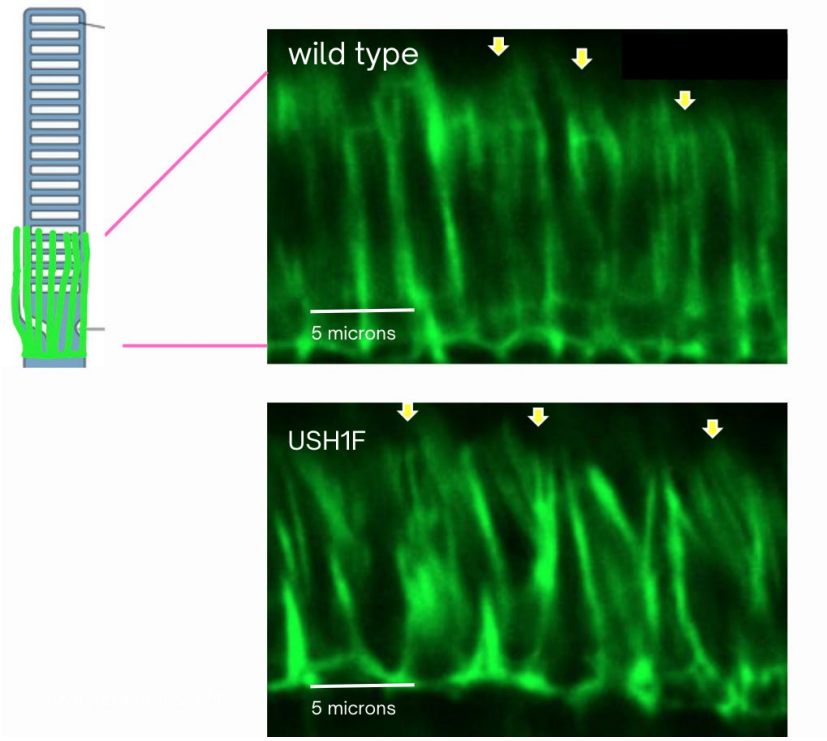

Figure 10: Calyceal processes are abnormally shaped in b1257 mutants
Phalloidin binds to the actin in the calyceal processes and makes them fluoresce green.
Top: A zoomed in blue photoreceptor shows the location of the calyceal processes in green on the left. In wildtype fish, the calyceal processes are a fine and organized fringe surrounding photoreceptors (right).
Bottom: In b1257 mutants (labeled as USH1F), calyceal processes are disorganized and have structural abnormalities.
Source: Phillips et al., 2021.

1.6 Rearing in bright light exacerbates photoreceptor cell death in USH1F mutants

The b1257 mutants have higher rates of photoreceptor cell death than their normal siblings and cell death increases when fish are reared in brightly lit conditions (Figure 11) (Phillips et al, 2021). Similar to the b1257 mutants, uot14 mutants have a decreased number of

intact CP and an increased percentage of deformed outer segments when raised in bright light. They experience attenuation of the severity of photoreceptor defects when raised in a dark environment (Miles et al., 2021). The early and strong phenotype of the zebrafish models of USH1F make them valuable tools for studying disease pathology and testing potential therapies.

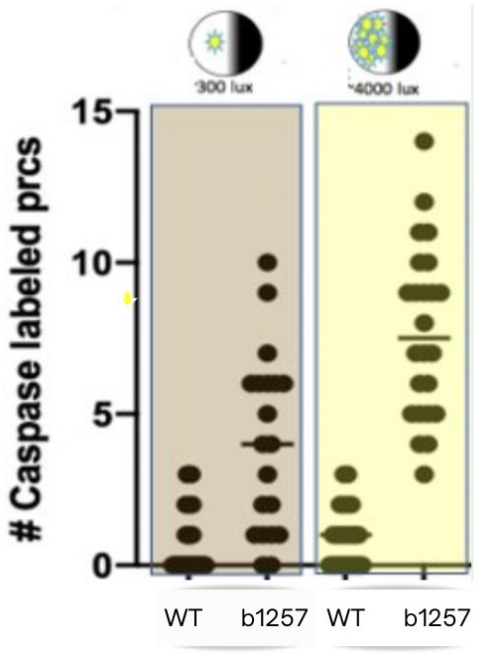

Figure 11: Elevated cell death in b1257 mutants is exacerbated by bright light
The number of caspase labeled cells is a measure of cells undergoing cell death, the number of which is increased in mutants in ambient indoor lighting (300 lux) and even higher in mutants raised in lighting similar to outside on an overcast day (4000 lux). Source: Phillips et al., 2021.

Establishing this zebrafish model of USH1F is a great step forward and it allowed us to ask new questions. This thesis is but a small part of larger lab projects and goals, and in the Westerfield lab, we seek to understand how we can slow photoreceptor death caused by USH1F and other forms of USH, as well as why the loss of pcdh15b leads to cell death in our zebrafish model.

1.7 Oxidative stress is a possible mechanism for the death of photoreceptors in USH1F

Mitochondria are organelles that serve as primary energy producers within cells. One normal consequence of the production of energy in mitochondria is superoxide production. A cell has mechanisms in place that it utilizes to maintain a balance between the production and neutralization of reactive oxygen species (ROS) like superoxide (Shu et al., 2023). Superoxide dismutase (SOD) is an enzyme that converts negatively charged superoxide molecules into hydrogen peroxide and oxygen. Enzymes catalase and glutathione (GSH) address hydrogen peroxide, neutralizing it so it is no longer toxic to the cells (Siddiqui et al., 2023). If ROS and/or hydrogen peroxide builds up due to increased production or decreased antioxidant function, the inappropriate oxidation of lipids, proteins, DNA, and metabolites can occur in a process known as oxidative stress. Oxidation of these molecules can disrupt gene expression, decrease protein function, and induce cellular dysfunction. If damage reaches a level the cell can't recover from, oxidative stress can induce the pathways that lead to programmed cell death known as apoptosis (Shu et al., 2023).

In recent studies, oxidative stress has been shown to have some importance to the pathogenesis and disease progression of RP. A study by Campochiaro et al. (2015) revealed that human patients with RP show evidence of ocular oxidative damage and ongoing oxidative stress. Endogenous oxidative stress produced by regular retinal metabolism, like DNA damage or lipid peroxidation, has been demonstrated to contribute to photoreceptor cell death, which occurs progressively in people with RP. Exogenous sources of oxidative stress, like exposure to sunlight, can also lead to increased photoreceptor cell death (Domènech and Marfany, 2020). This link between oxidative stress and RP presents a promising mechanism of disease progression for USH1F that can be targeted with therapeutics. Assessing antioxidant compounds

capable of reducing oxidative stress is an important avenue for the development of potential treatments for RP and USH1F.

1.8 Antioxidant hexafluoro addresses oxidative stress and might slow photoreceptor cell death

Honokiol is a naturally occurring compound found in the bark of the magnolia tree, *Magnolia grandiflora,* which is endemic to Asia. Honokiol is commonly used in traditional Asian medicines and previous studies have shown that it activates proteins like sirtuin 3 (SIRT3) that are involved in countering mitochondrial ROS production (Balasubramaniam et al., 2022). SIRT3 gene expression occurs in the retina of zebrafish and can be activated to reduce OS in the mitochondria by pharmacological compounds (Sheng et al., 2018). As a protein, SIRT3 works by removing the acetyl group from two other proteins: Fox03a and superoxide dismutase 2 (SOD2). SOD2 is one of three SOD proteins and is specific to the mitochondria (Shu et al., 2023) Deacetylating Fox03a activates it to increase SOD2 expression and deacetylating SOD2 increases its activity. SOD2 reduces levels of superoxide, a ROS, so increasing the amount of SOD2 in the cell, as well as increasing its activity, decreases levels of ROS in the cell (Song et al., 2017).

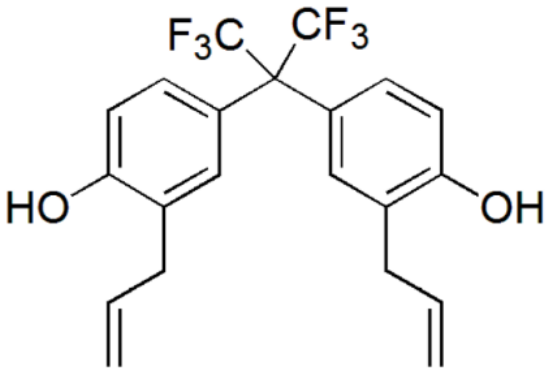

Figure 12: Chemical structure of hexafluoro
This figure represents carbons bonded to other carbon molecules with lines. The six fluorine atoms give the compound its name and the OH groups make the compound soluble in water. Its antioxidant capabilities also come from the two OH groups. Source: Medra et al., 2016.

Hexafluoro-honokiol (hexafluoro) is a synthetic version of honokiol that has fluorine atoms added on (Figure 12). It is proposed to operate via the same mechanism as honokiol, increasing SIRT3 protein expression levels and its antioxidant response to excess ROS production (Figure 13) (Balasubramaniam et al., 2022).

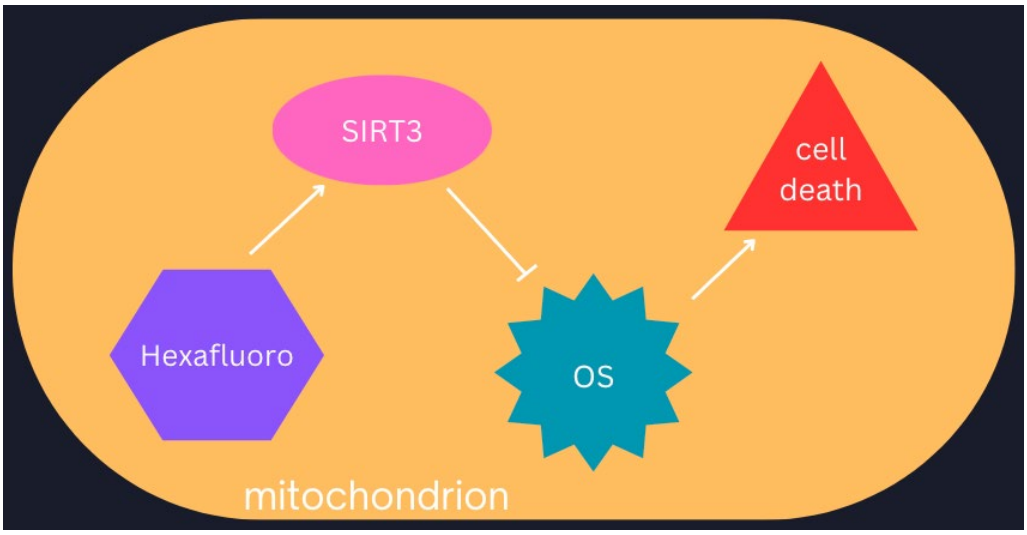

Figure 13: Mechanism of action for hexafluoro
Hexafluoro activates SIRT3 and oxidative stress (OS) activates cell death, activation represented by a line with an arrowhead. SIRT3 inhibits oxidative stress, represented by a line with a blunt head. If SIRT3 stops oxidative stress from activating cell death, activation of SIRT3 by hexafluoro stops cell death due to oxidative stress. Figure made in Canva.

Since previous studies have shown that oxidative stress can lead to some forms of RP, we are investigating an antioxidant compound like hexafluoro to assess its ability to mitigate oxidative stress and address the RP symptoms caused by USH1F. If oxidative stress is part of the molecular pathology of USH1F, the hexafluoro could ameliorate it.

1.9 Hexafluoro improves visual function but not cell death in USH1F mutants raised in dim light

In a previous Westerfield Lab study, Buchner (2022), WT and b1257 mutant zebrafish were raised in low light (40 lux, light intensity similar the lighting of a living room) and treated the fish with hexafluoro or a DMSO (solvent without hexafluoro) control. Hexafluoro's effect on visual function and cell death was assessed after four days of treatment since it is known that

mutants have reduced visual function and increased cell death compared to their WT siblings (Phillips et al., 2021). Treatment with hexafluoro significantly improved visual function in the mutant zebrafish compared to the untreated mutants, although not quite to the level of WT siblings. There was no significant difference in the visual function of treated and untreated siblings, indicating that hexafluoro does not have a detrimental effect on visual function. No significant difference was found in the amount of cell death in treated and untreated mutants but there was a trend towards higher cell counts in the treated mutants (Buchner 2022). It is possible that the dark conditions were so protective that it was difficult to see robust cell rescue. This meant that the next step was to repeat the experiment in brighter light conditions to see if it would change hexafluoro's effect.

1.10 Purpose of This Thesis

After the promising results of the pilot experiment, we wondered what effect hexafluoro would have on the retinas of mutants raised at brighter light intensities. Hexafluoro may have no effect on visual function or cell death in any light condition, but instead act only to improve visual function rather than cell survival. Alternatively, the known elevated photoreceptor death in b1257 mutants caused by brighter light brings up two other possibilities: hexafluoro may protect against cell death at higher light intensities, or hexafluoro may be unable to keep up with the increasing rates of cell death as the light intensity increases.

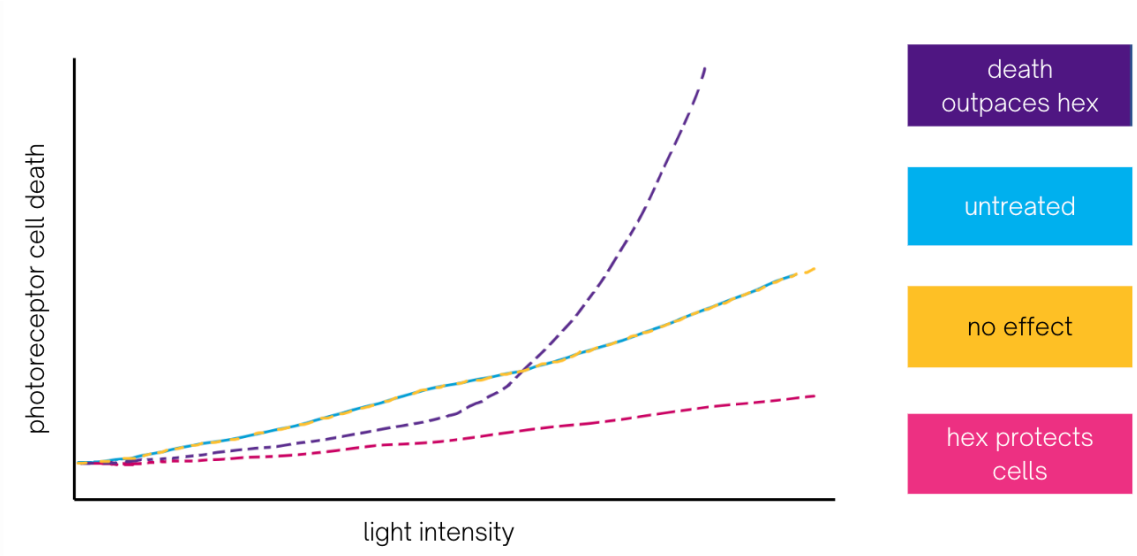

Figure 14: The potential effects (represented by dotted lines) of hexafluoro on photoreceptor cell death in USH1F mutants as light intensity increases. It could have no effect (yellow line) and produce the same outcome as no treatment (blue line), it could protect cells and slow death as light increases (pink line), or we could see a protective effect at mid-range environmental illumination (500 lux) that wears off as brightness increases and death outpaces hexafluoro (purple line). Created in Powerpoint.

Testing hexafluoro's efficacy at illuminations of 500 lux, which is like office lighting, and 5000 lux, which is like the lighting outside on an overcast day, is important considering the recipients of this potential therapy. People with USH1F work in offices and enjoy spending time outside, so it is imperative to make sure that hexafluoro is effective at these light levels.

In addition to assessing hexafluoro's efficacy in normal daylight environmental conditions, this thesis includes the beginning stages of an investigation of a potential mechanism behind hexafluoro's observed effect. Based on the documented correlation between oxidative stress and inherited RP, this thesis seeks to understand if treatment with hexafluoro-honokiol will activate SIRT3 which could decrease levels of oxidative stress, causing the preservation of visual function and slowing of photoreceptor cell death in a zebrafish model of USH1F.

2 Methods

2.1 Zebrafish Husbandry

The zebrafish facility at the University of Oregon is maintained at 28.5°C and kept on a light schedule of 14 hours of light, 10 hours of darkness. All experiments performed on zebrafish are approved by the Institutional Animal Care and Use Committee and any personnel working with zebrafish must complete institutionally mandated training on the safe and humane handling of vertebrate animals.

To obtain the young fish used in the following experiments, adult female and male fish are mated. The sexes are differentiated visually, with females having round white bellies, while males are identified by their amber colored bellies and darker colored anal fins. For mating purposes, pairs of male and female fish are placed in small tanks of water with a basket insert that allows the fertilized eggs to fall through and out of reach of the adult fish. Green netting is added to the basket with the fish to simulate river grass and promote mating behavior. Zebrafish breed at the beginning of the light cycle and the fertilized eggs are collected around 10:00 am PST.

The embryos obtained are placed in Petri dishes filled with embryo medium (EM), a buffered salt solution that is optimized for the health and development of larvae. The dishes are checked and cleaned daily, including the removal of non-viable embryos and debris and replacement of EM.

2.2 Preclinical Screening of Hexafluoro

To perform this experiment, adult carriers of the b1257 mutation were bred and offspring were collected as described above. Based on what we know about autosomal recessive inheritance patterns and crosses of carriers of these alleles (described in literature review), we predicted that about 25% of our offspring from these crosses will get both USH1F mutant alleles and have the disease characteristics. Ten Petri dishes containing about 50 embryos each were placed on a baker's rack in the fish facility for 2 days post-fertilization (dpf).

At 2 dpf, 6 dishes were selected to go onto our experimental rack set up in the fish facility. Each of the six dishes represented a different group. Three of the dishes were treated with hexafluoro, but they were subjected to different light conditions. One was subjected to high light intensity (3000-5000 lux), sitting directly under a light source. The second dish was subject to medium light intensity (300-500 lux), similar to standard office lighting, and the last drug-treated dish was subject to low lighting which was the dimmest of the three. The other three dishes were evenly spread out among the three light levels just like the drug-treated dishes but these ones were the control and were only administered DMSO. The experimental group was treated with a solution containing hexafluoro, added to the EM in the Petri dishes. We added 6.6 µL of a 1 mg/mL solution of hexafluoro diluted in dimethyl sulfoxide (DMSO) to 60 mL of EM for a final concentration of 0.11 µM. This is the maximum dose shown to be tolerable to wild-type embryos and larvae in earlier dosage testing experiments. The control fish received 6.6 µL of DMSO with no active ingredient added. The groups received their designated solution every day from 2dpf to 6dpf.

Mutant swimming behavior was observed at 5 dpf by tapping loudly on the side of the Petri dish, startling the larvae. Mutants identified in this manner were kept, along with an equal number of unaffected siblings, for the duration of the experiment.

2.3 Optokinetic Response Assay

At 5dpf, a visual function test known as an optokinetic response assy (OKR) was performed. In a small Petri dish, fish were placed in a few drops of methyl cellulose, a gelatinous substance that keeps the fish wet but holds them in place, and oriented in an upright position, dorsal side up, with the aid of a soft probe. The Petri dish was then placed on the pedestal in the center of a drum lined with a series of regularly spaced black and white stripes. For one minute, the drum rotated at 9rpm clockwise and the number of saccades (controlled eye movements) were recorded as the immobilized fish viewed the rotating stripes in turn. This was repeated in the counterclockwise direction. The wild-type siblings underwent this visual test first to give a baseline for the average number of targets a fish with normal vision can track. Three to six mutants and three to six WT siblings were tested from each DMSO- and drug-treated dish.

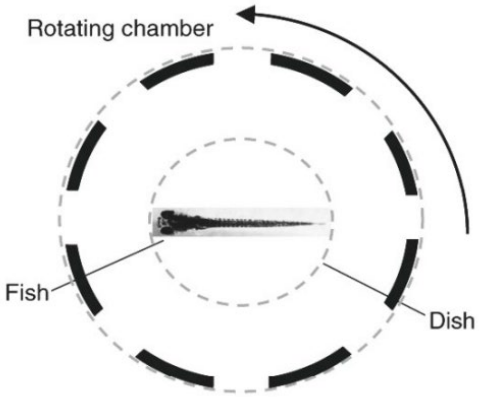

Figure 15: Optokinetic response assay
A 5dpf zebrafish is placed in immobilizing, viscous liquid in the center of a rotating drum that has black and white panels. A microscope is set up over the apparatus to view the eye movements as fish track the rotating panels. Source: Lagnado, 2005.

2.4 Histological Analysis

At 7dpf, mutant and WT fish from each experimental group were put into separate microcentrifuge tubes. The tubes were placed in a bucket of ice to humanely euthanize the fish via hypothermic shock. This euthanasia protocol is approved by the Institutional Animal Care and Use Committee (IACUC) and only trained individuals are allowed to perform it. Once the fish were euthanized, as much liquid as possible was removed from the tubes and 1mL of 4% paraformaldehyde, a fixative, was added. The fish remained in the fixative overnight at 4℃. The next day, the tissues were rinsed three times in PBS-T to remove the 4% PFA and to prevent the larvae from sticking together. After the tissues were rinsed, they were placed in methanol for ten minutes at room temperature and then the old methanol was replaced with fresh methanol before the fish were placed in the freezer at -20℃ for at least one hour and were stored there until we needed to use them. Methanol prevents the formation of ice crystals in preserved tissue at below-freezing temperatures, which protects the structural integrity of the sample. When the tissues

were ready for processing the fish were removed from the freezer and rehydrated with rinses that contained descending concentrations of methanol.

Photoreceptor Cell Counts

For the tissues being used for photoreceptor cell counts, the fish were rehydrated and then embedded in water-soluble acrylic resin blocks and hardened at 55°C for 24 hours. Then, using a sharp metal blade, the blocks were cut into 2-micron sections, and transferred to microscope slides.

A toluidine blue solution was prepared by dissolving 1 g of borax and 1 g of toluidine blue in 100 mL of water. This solution stains acidic compounds like nucleic acids, found in cell bodies, a deep indigo. This was how we identified individual cells. A plastic pipette was used to transfer a few drops of the solution onto the microscope slides until the sectioned tissue was covered. The slide was placed on a heat block for about one minute, or until the edges of the blue solution started to dry, after which excess stain was removed by washing the slides in several changes of deionized water. Next, increasing concentrations of ethanol were used to dehydrate the slides followed by clearing in xylene, a chemical solvent that makes the tissue more transparent so that everything other than the specifically stained regions is clear and colorless. Following the dehydration and clearing process, slides were treated with a mounting medium (Permount) and cover-slipped (Buchner, 2022).

Once slides were complete, they were observed under a microscope and coronal sections that include the optic nerve were imaged and saved for analysis. These photos were opened in ImageJ, an image processing software. Three boxes, one in each of the dorsal, central, and ventral regions of the outer nuclear layer, about 50 micrometers in length and 17-20 micrometers

in width, were created in each image. The three boxes were utilized to sample as much of the population of cells without being too labor intensive, making cell analysis manageable and reproducible. The number of photoreceptors in each rectangle were recorded. A photoreceptor was identified by its indigo-colored nucleus, a result from toluidine blue staining the DNA inside the nucleus a dark color. Wild-type siblings were analyzed first, followed by DMSO mutants, and then hexafluoro-treated mutants.

The base statistical package Graph pad in the software Prism was used to complete statistical analysis. A nonparametric Mann-Whitney test was used to calculate the p-values for all cross-group comparisons listed. A p-value of less than 0.05 was used to describe the result of a comparison as statistically significant, meaning that the difference between two groups is not due to chance but due to a specific cause, for example, hexafluoro being administered.

3 Results

3.1 Optokinetic Response

Both untreated and treated WT fish had visual responses that were between 10-12 saccades per minute (spm), with the average being 11.1 spm (gray rectangle, Figure 16). Treated mutants had better visual responses than untreated mutants in both lighting conditions. Visual function in the treated mutants did not completely return to the level of WT fish, consistent with previous findings, but treated mutants raised at 5000 lux had an average number of saccades/min that was closest to the all-sibling average.

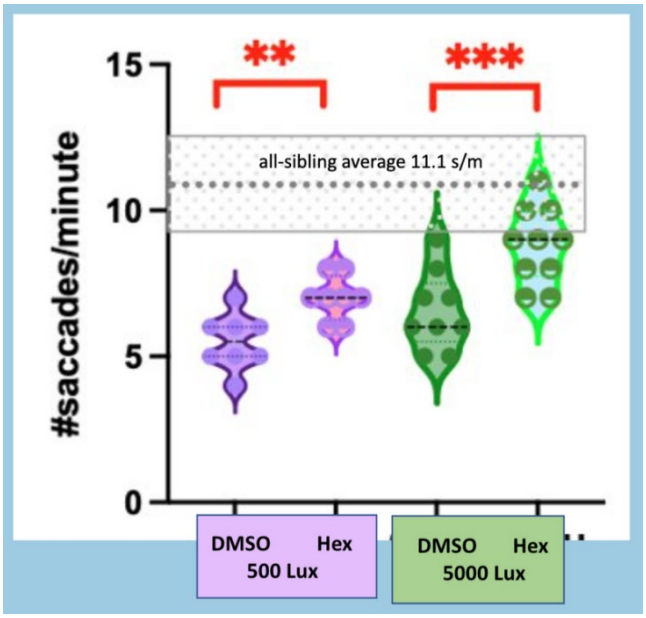

Figure 16: The number of saccades per minute increases in b1257 mutants with hexafluoro treatment.
Plots of treated and untreated mutants raised at office lighting conditions (500 lux) and at conditions consistent with the outdoors on an overcast day (5000 lux) show that treatment with hexafluoro (Hex) increases the number of saccades demonstrated by mutant fish. Visual function of treated mutants raised in 5000 lux approaches that of wildtype siblings. ** and *** both represent $p < 0.05$. $N \geq 10$ for all groups.

3.2 Photoreceptor Counts

Due to variability in the amount of photoreceptors in the dorsal and ventral regions of the eye caused by embedding and slicing, only the central region, which is naturally the most densely packed with photoreceptors, was analyzed (Figure 17).

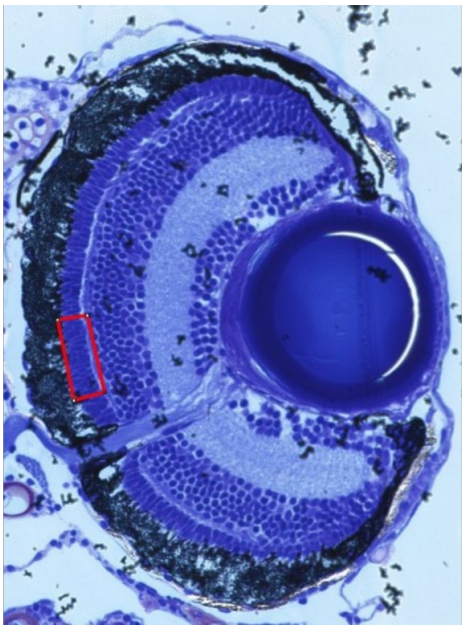

Figure 17: Location of photoreceptor counts that were assessed
Zebrafish eye stained with toluidine blue has a red box in the central region of the photoreceptor layer of the retina. The number of photoreceptors in this box were tallied and used to assay for hexafluoro's effect on cell death.

Untreated mutant fish raised at 500 lux had a significantly lower number of photoreceptor cells in the central retina than untreated siblings, consistent with previous findings ($p = 0.001$). There was no significant difference in the number of photoreceptor cells observed in treated siblings versus mutants, meaning that the treated mutants had an equal amount of photoreceptors as the treated siblings. This meant that treatment returned the number of photoreceptors in the mutants to wild-type levels. The number of photoreceptor cells in treated versus untreated mutants was statistically significant ($p = 0.0001$), with the treated mutants possessing more cells

than the untreated mutant fish (Figure 18). At 5000 lux, there was a statistically significant difference in the amount of photoreceptors of untreated sibling and mutant fish. The untreated siblings had more cells than their untreated counterparts ($p = 0.0009$). When comparing the treated and untreated mutants, a statistically significant increase in the number of photoreceptors was observed in the treated mutants ($p = 0.0038$). The number of photoreceptor cells in treated mutants and siblings is equal.

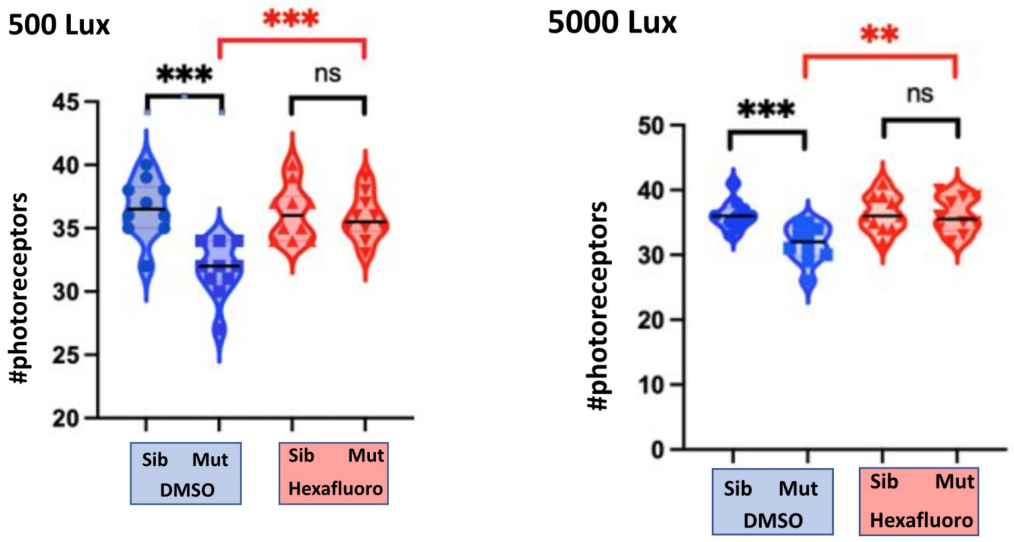

Figure 18: Treatment with hexafluoro slows cell death in b1257 mutants raised in multiple conditions.
Treated b1257 mutants have more photoreceptor cells than untreated mutants in 500 lux (left) and 5000 lux (right) light conditions. The number of photoreceptors in treated mutants is equal to the number of cells of wild type siblings. ** represents $p < 0.05$ and *** represents $p < 0.001$. $N \geq 10$ for all groups.

4 Discussion and Conclusion

Our results indicate that hexafluoro has a neuroprotective effect at illumination levels similar to those encountered by humans daily, causing improvements in visual function and photoreceptor retention. These data provide significant, reproducible evidence that photoreceptor health and function are improved by treatment with hexafluoro, despite the structural defects caused by the loss of full length pcdh15b protein. Oxidative stress is implicated as a contributing factor to the retinal degeneration of USH1F by how robust the observed rescue effect of hexafluoro is.

Continued exploration of the mechanisms behind hexafluoro's observed effect is one of the next big steps for this project. The data have indicated that oxidative stress contributes to photoreceptor cell death in USH1F. We plan to use antibodies against SIRT3 to detect the predicted increase in this factor in photoreceptor cells of treated animals. We are also curious about stress of the endoplasmic reticulum (ER), the organelle that helps polypeptides fold properly into proteins. The ER and mitochondria function in parallel but with some metabolic overlap, so oxidative stress and ER stress are often in a causal relationship (Magallón et al., 2021). Immunohistochemical assays on the zebrafish eye tissue using antibodies against key molecules of endoplasmic reticulum and oxidative stress pathways, including SIRT3 are planned in the near future. These experiments will provide additional insight into the causes of photoreceptor cell death in USH1F retinas and the molecular effects of hexafluoro.

Compounds like hexafluoro that preserve photoreceptor function survival are potentially valuable interventions for people living with retinal degeneration due to genetic disorders like USH1F. Such compounds might allow those losing their sight to RP to retain usable vision for longer and could also be important for preserving as many

working cells as possible while more targeted repair or replacement-based therapies are being developed. Hexafluoro might also have an important therapeutic role when administered alongside cell-based or gene-specific therapies. Any treatment that slows the progression of USH1F will be important to those affected by the disease because more years of usable vision might allow them to see their spouse on their wedding day, or watch their children grow up and launch into adulthood. This work is but a small contribution to the massive effort to preserve or restore the quality of life for people with USH1F, an effort that I am honored to be a part of and that I know will continue with the dedication of the wonderful Westerfield Lab at the University of Oregon.

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