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Notch3 Expression in Zebrafish with Inherited Degeneration vs. Inflicted Damage

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ABSTRACT

Some mammalian species demonstrate the ability to regenerate functional photoreceptors after retinal injury, including those with mutations that result in non-syndromic diseases such as retinitis pigmentosa. Previous research has shown that regenerative capability exists in non-mutated animals following injury inflicted by light damage. Studies have suggested that, in mutated animals with inherited degeneration, regeneration does not occur spontaneously. However, mutated animals have exhibited regeneration following inflicted damage. Regenerative capabilities have been shown to be linked to the highly conserved Notch signaling pathway. To determine if differences in Notch signaling play a role in the lack of regeneration in zebrafish with inherited degeneration, Notch3 expression in a line of *eys*$^{-/-}$ zebrafish was quantified in relation to an unaffected heterozygous *eys*$^{+/-}$ zebrafish line, and a heterozygous line with inflicted light damage. Additionally, Notch3 expression in a line of Tg(XOPS:ntr-YFP) YFP+ zebrafish treated with metronidazole was quantified to compare regeneration capabilities of a chemical ablation damage model to those of the unaffected and light-damaged heterozygrous lines. Quantification results indicate that Notch3 expression is not upregulated in zebrafish with inherited degeneration in the way that it is in zebrafish with inflicted degeneration. Notch3 expression is downregulated in the chemical ablation model. The results are corroborated by investigation of proliferating cell counts in each group.

INTRODUCTION

While mammals possess a limited capacity for retinal regeneration, teleosts such as *Danio rerio* (zebrafish) are capable of recovering from retinal injury or cell death through stimulation of glia to induce reprogramming into neural stem-like cells (Nagashima & Hitchcock 2021). In zebrafish, retinal Müller glia engage in regeneration in response to an injury-derived signal. Müller glia cells are common between the mammalian and zebrafish retina, but regenerative response capability lies only with those in zebrafish (Goldman 2014). The reason for the functional difference between the mammalian and zebrafish models remains unknown; a greater understanding of factors in the regulatory signaling that dictates Müller glia proliferation vs. quiescence may provide the link to therapeutic options for retinal damage in humans.

It was previously shown (Campbell et al. 2020 & Conner et al. 2014) that the regenerative response to retinal damage in zebrafish is regulated by the Notch signaling pathway. This pathway is a highly conserved signaling pathway involved in cell fate determination and pattern formation in development in many organisms. The transcription-factor receptor is activated by a family of Notch ligands, which triggers a succession of two proteolytic cleavages of the receptor. The Notch intracellular domain is then able to enter the nucleus as a transcription factor (Ehebauer et al. 2006). Persistent Notch signaling causes Müller glia to remain in a quiescent state in the undamaged retina, and Notch signaling repression causes Müller glia to reenter the cell cycle (Conner et al. 2014), reprogramming them to stem-like cells that undergo division and produce multi-potent retinal progenitors, which are then able to exit the cell cycle and differentiate into replacements for the damaged neurons (Nagashima & Hitchcock 2021). The Müller glia in the zebrafish retina are capable of responding to acute injury such as noxious light and neurotoxic chemicals (Curado et al. 2008) by proliferating and regenerating lost

photoreceptors. Evidence from our lab showed that Müller cells do not proliferate in the mutants studied with inherited photoreceptor degeneration, but they do proliferate after the retina is injured (Lessieur et al. 2019, Song et al. 2020). This suggested that the cells retain the capacity to regenerate but lack the required signal to do so.

One example of inherited degeneration is the case of mutations in the eyes shut homolog (EYS). EYS is required for the survival of photoreceptors in fish, and abnormal EYS can cause autosomal recessive retinitis pigmentosa 25 (Yu et al. 2016). This model allows for the study of Notch signaling in zebrafish with inherited degenerations rather than with actively degenerating retinas. Insight into the Notch signaling pathway has the potential to contribute to treatment advancement in humans with varying types of retinal visual impairments. There are four types of Notch proteins in zebrafish, expressed by the following genes: *notch1a, notch1b, notch2,* and *notch3.* Notch3, the protein expressed through the gene *notch3,* is the most consequential of the Notch proteins in relation to retinal damage and proliferation (Campbell et al. 2020), so its measurement was the object of this study. Since existing data on light damage shows a downregulation of Notch3 expression (Lessieur et al. 2019, Song et al. 2020), the focus of the current study was to determine whether Notch 3 signaling would be similarly downregulated in 1) zebrafish expressing the nitroreductase gene following metronidazole treatment and 2) zebrafish with inherited degeneration, the *eys* mutants.

MATERIALS AND METHODS

Overview

To quantify Notch signaling in the zebrafish retina, the retina was removed and total RNA was isolated from it. Then random-primed cDNA was prepared and qPCR was run with a Notch3 expression assay in order to measure Notch3 expression. The test group was the

zebrafish with a known *eys* mutation. The retina of *eys* heterozygotes was unaffected, and they, therefore, served as the negative control group. In addition to this, two positive control groups, both of which were shown to undergo Müller cell proliferation, were tested. The positive controls included heterozygous *eys* zebrafish with inflicted light damage and a group of zebrafish expressing nitroreductase using a tissue-specific promoter with the genotype Tg(XOPS:ntr-YFP) YFP+ (Curado et al. 2008). Campbell et al. (2020) showed that Notch3 expression is downregulated after light damage or needle poke injury paradigms, so it was anticipated that the metronidazole treatment of the nitroreductase fish would have the same effect, though this had not yet been studied. Fish treated with metronidazole lost any cell expressing a nitroreductase transgene due to the production of a cytotoxic product that induces cell death; in this case, cell death occurred specifically in rod photoreceptors. The measurement of Notch3 expression was accompanied by imaging retinas from corresponding fish with a variety of stains. The following primary antibodies were used in imaging: zpr1 for visualization of cone inner segments, peanut agglutinin (PNA) for visualization of cone outer segments, zpr3 for visualization of rod outer segments, proliferating cell nuclear antigen (PCNA) for visualization of proliferating cells, and Lplastin for visualization of inflammation.

Zebrafish Husbandry

An integrated Aquatics Habitats recirculating water system (Pentair; Apopka, FL) was used to raise, maintain, and house adult zebrafish in 1.5, 3.0, and 10 L tanks. All animals were raised and maintained on a 14:10 hr light-dark cycle with ambient room temperature at 28.5°C. Animal maintenance was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH; Bethesda, MD).

Ethics Statement

All animal procedures were done with approval by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic. Experiments were conducted according to relevant guidelines and regulations.

Light Damage

Light damage experiments were performed using a protocol adapted from Thomas and Thummel (2013). The adult zebrafish were dark adapted for 48 hours prior to beginning light treatment. After dark adaptation, 6 animals (the entire light damage test group) were placed in a 250 mL glass beaker with system water that was seated inside a 1L glass beaker with Milli-Q water (water purified by ion exchange) and exposed to high-intensity light from a 120W X-CITE series 120Q metal halide lamp (Excelitas, Waltham, MA) for 30 min and then exposed to 14,000 lux light from an illumination cage for 4 h (Song et al., 2020). Following light treatment, the animals were allowed to recover for 11 hours in system water (16 hours following the beginning of the treatment).

Metronidazole Treatment

Metronidazole treatments were performed on adult Tg(XOPS:ntr-YFP) YFP+ zebrafish. Treatment was conducted in 10 mM metronidazole (M3761, Sigma-Aldrich, St. Louis, MO) in system water for 24 hours at 28°C in the dark. Fish in this test group were then returned to system water and allowed to recover for 16 hours.

Collection, Embedding, Sectioning, and Antibody Labelling of Adult Zebrafish Eyes

All adult zebrafish were euthanized by immersion in ice-water. Eye collection was performed in 0.8X phosphate-buffered saline (PBS). From each animal, one eye was dissected, and the retina was suspended in trizol for RNA isolation. The other eye was removed and placed

in 4% paraformaldehyde/ 5% sucrose/ 0.8X PBS fixative overnight for preservation of the tissue. The eyes were removed from the fixative and the lenses were removed to ensure an even consistency in the block during sectioning. The eyes were then washed in 1X PBS for 20 minutes at room temperature, in 5% sucrose/ 0.8X PBS for 30 minutes at room temperature, in 5% sucrose/ 0.8X PBS for 2 hours at room temperature, and in 30% sucrose/ 0.8X PBS overnight at 4°C. The eyes were then washed in 15% sucrose/ 0.4X PBS/ 50% PolarStat Plus for 3 hours at 4°C. The eyes were removed from the wash. A cryomold was filled ¾ full with PolarStat Plus Embedding Media for Frozen Sections (StatLab, McKinney, TX) and the eyes were transferred to the mold using a plastic transfer pipette. The eyes were arranged with the pigmented dorsal eye to the left and facing the bottom of the cryomold. The cryomold was placed on dry ice to freeze until the block was opaque. The frozen block was removed from the mold and mounted in the -20°C cryostat using PolarStat Plus Embedding Media approximately 20 minutes prior to sectioning. The block was trimmed and oriented on the chuck with the dorsal side of the eyes to the left. The block was sectioned transversely at 10 uM through the entire eye and collected on permafrost plus slides in an alternating manner. Slides were allowed to dry for approximately two hours. The tissue sections were then outlined with a PAP pen. The slides were bleached in 1x saline sodium citrate (SSC), 5% peroxide, and 5% formamide to remove melanin until the tissue segments were pale brown/clear in color (approximately 5-10 minutes). The tissues were then rehydrated in 1X PBS in a Coplin jar for 20 minutes at room temperature. The slides were incubated at room temperature for 1 hour in blocking solution (0.1% tween, 0.1% DMSO, 5% normal goat serum in 1X PBS). Approximately 200 µL of primary antibodies were diluted into the blocking solution as follows: mouse monoclonal zpr1 (1:100, Zebrafish International Resource Center - Eugene, OR) and peanut agglutinin (PNA)-lectin conjugated to Alexa-568

(1:100, Thermo Fisher Scientific, Waltham, MA), mouse monoclonal zpr3 (1:100, ZIRC), mouse monoclonal PCNA (1:100, Sigma, St. Louis, MO) clone PC-10) and rabbit polyclonal L-plastin (1:1000, GeneTex, Irvine, CA, GTX124420) were added to each slide. Cover slips were added, and the slides were incubated overnight at 4°C in a humidified chamber. Three 10-minute washes with PBS-TD [1X PBS / 0.1% Tween-20 / 0.1% dimethyl sulfoxide (DMSO)] were then completed on each slide. The slides were incubated in Alexa-conjugated secondary antibodies (Invitrogen Life Technologies, Carlsbad, CA) diluted into blocking solution as follows: GAM647 (1:500) and PNA568 (1:100), GAM647 (1:500), GAM568 (1:500) and GAR647 (1:500), where GAM is goat anti-mouse and GAR is goat anti-rabbit. Incubation with secondary antibodies was conducted following the same protocol as that used with primary antibodies. After the three PBS-TD washes, the coverslips were mounted with fluoromount G and counterstained with 4,5-diamidino-2-phenylendole (DAPI) to stain the nuclei.

Immunohistochemical Fluorescence Imaging

A Zeiss Axio Imager.Z2 fluorescent microscope fitted with the Apotome.2 for structured illumination (Carl Zeiss Microscopy, Thornhill, NY) was used for optical sections, and ImageJ (U.S. National Institutes of Health, Bethesda, MD) and Adobe Photoshop (Adobe Photoshop, Berkeley, CA) were used to assemble image panels and figures. GraphPad Prism (v8) (GraphPad Software, La Jolla, CA) was used to analyze, graph, and determine significant differences in all data. Images were all taken at 20x of the central dorsal retina. Analysis was done only on retinal sections that were imaged in a position adjacent to the optic nerve. The density of cones in the outer segment was quantified by counting the number of PNA-positive outer segments in images of dorsal retina transverse sections. Counts were analyzed in Prism8 using one-way Brown-

Forsythe and Welch ANOVA tests with Dunnett T3 corrections. The density was calculated as the number of cone outer segments per 100 µm of retina distance.

RNA Isolation

One dorsal retina from each animal was dissected from an enucleated eye for RNA isolation. Isolation of total RNA was done using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA); the total RNA was then DNase treated with the Turbo DNA-free Kit (Invitrogen Life Technologies, Carlsbad, CA). The RNA isolation and DNase treatment protocol is subsequently described. Immediately upon dissection, the dorsal retina was placed in 250 µL of cold Trizol reagent. The sample was homogenized and an additional 250 µL of cold Trizol was added before placing the sample at -80°C. All samples remained frozen in Trizol until each study group was dissected to ensure parallel processing of RNA. Extraction was done using 100 µL of chloroform in each sample; the tube was then capped and inverted, then the solution was transferred to a phase-lock tube. Samples were centrifuged at 12,000 x *g* for 15 minutes at 4°C. The phase-lock gel created a physical separation of the Trizol reagent mix into an upper aqueous phase and lower organic phase to allow for simplified removal of the RNA-containing upper phase. The aqueous phase was transferred to a new sterile tube. For easier visualization of precipitated RNA, 1 µL of glycogen blue was added to the sample; glycogen blue co-precipitated with the RNA without affecting any subsequent part of the protocol. 250 µL of isopropanol was added to the sample and it was vortexed and incubated for 10 minutes at 4°C. The sample was then centrifuged at 12,000 x *g* for 10 minutes at 4°C, and a total RNA precipitate formed a blue gel-like pellet at the bottom of the tube. The supernatant was removed with a micropipettor and the pellet was resuspended in 500 µL of 75% ethanol. The sample was vortexed then centrifuged at 7500 x *g* for 5 minutes at 4°C. The supernatant was removed, and the RNA pellet was allowed

to air dry at room temperature for 10 minutes. The pellet was then resuspended in 20 µL of RNase-free water, vortexed well, and placed on ice for 1 hour. The sample was removed from ice, vortexed again, and 1 µL of the sample was used to determine the RNA concentration in the sample with a Thermo Scientific NanoDrop 2000C (Thermo Fisher Scientific, Waltham, MA) in order to verify the success of total RNA isolation prior to DNase treatment. Then, a 15 µL aliquot of the RNA sample was transferred into sterile tubes. A master mix of 10X reaction buffer (2 µL), TURBO DNase (1 µL), and nuclease free water (2 µL) was created using the T7 kit (Thermo Fisher Scientific, Waltham, MA). The total volume of this master mix was created to accommodate for 5 µL of mix per sample according to the aforementioned ratios. 5 µL of the master mix solution was added to the sample for 20 µL total reaction. The sample was incubated at 37°C for 15 minutes, then 10 µL of nuclease free water, 0.5 µL of glycogen blue, and 13.5 µL of 8 M LiCl was added to the sample so that each tube contained 2.5 M LiCl. The sample was mixed well and precipitated for 1 hour at -20°C. Then, it was centrifuged at 12,000 x *g* for 30 minutes at 4°C. Following centrifugation, the supernatant was removed and the sample was washed in 500 µL of cold 70% ethanol, mixed thoroughly by inversion, and centrifuged again at 12,000 x *g* for 10 minutes at 4°C. The supernatant was then removed, and the pellet was allowed to air dry for 10 minutes at room temperature before being resuspended in 25 µL of nuclease free water. 1 µL of the sample was applied to the nanodrop as a quality control measure and to determine the RNA concentration in the sample. This procedure was conducted for each sample, and the same with the lowest RNA concentration was used to determine the maximum common amount of RNA that could be used for DNA synthesis.

cDNA Synthesis (RT-PCR)

A master mix of 5x iScript Reaction mix (4 µL) and iScript Reverse Transcriptase (1 µL) was created; the total volume of this master mix was created to accommodate for 5 µL of mix per sample according to the aforementioned ratios. Then, calculated amounts of RNA template and nuclease-free water were added to each sample for a 20 µL total reaction volume. The complete reaction mix was then incubated in a thermal cycler using the following protocol: priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C, reverse transcription inactivation for 1 minute at 95°C, and an optional hold at 4°C.

qPCR Reaction

A master mix was prepared for quantitative polymerase chain reaction (qPCR) using 10 µL TaqMan Universal Master Mix II, 1 µL Notch3 Expression Assay (20X), 1 µL 18S (reference gene) Gene Expression Assay (20X), and 7 µL of nuclease-free water. The total volume of master mix was prepared according to these ratios in order to have 20 µL total volume samples for qPCR, each containing 1 µL of cDNA. The samples were pipetted into PCR reaction plate wells and the PCR plate was heat sealed and centrifuged to spin down contents and eliminate air bubbles. The qPCR cycle protocol was then run as follows: uracil-DNA glycosylase incubating for 2 minutes at 50°C, polymerase activating for 10 minutes at 95°C, denaturing for 15 seconds at 95°C, annealing and extending for 1 minute at 60°C (PCR for 40 cycles).

GraphPad Prism (v8) (GraphPad Software, La Jolla, CA) was used to analyze, graph, and determine significant differences in all data. Significance was noted when $p < 0.05$.

RESULTS

Photoreceptors Degenerate in *eys*$^{-/-}$ zebrafish mutants, light-damaged wild-type zebrafish, and metronidazole-treated Tg(XOPS:ntr-YFP) YFP+ zebrafish

Immunohistochemistry for rod and cone markers was used to confirm the effects of the acute injury incurred by two damage models, light-damaged *eys*$^{+/-}$ wild-type zebrafish and metronidazole-treated Tg(XOPS:ntr-YFP) YFP+ zebrafish, and zebrafish with inherited degeneration of photoreceptors (*eys*$^{-/-}$ mutants) compared to wild type fish. These are found in the supplemental figures as wild-type, *eys*$^{-/-}$ mutant (n = 4), light-damaged wild-type (n = 4), and metronidazole-treated Tg(XOPS:ntr-YFP) YFP+ (n = 6) zebrafish. Cryosections of the dorsal retina were stained with zpr1 and PNA to label cone outer and inner segments; the wild-type *eys*$^{+/-}$ zebrafish sections (Fig. 1A) were compared to those of *eys*$^{-/-}$ mutant (Fig. 1B), light-damaged wild-type (Fig. 1C), and metronidazole-treated Tg(XOPS:ntr-YFP) YFP+ (Fig. 1D) zebrafish. The same comparison was made using zpr3 stain to label rhodopsin in rod outer segments (Fig. 1E, 1F, 1G, 1H). In relation to the undamaged wild-type zebrafish, the *eys*$^{-/-}$ mutants exhibited cone disruption with shorter cones (PNA labeled). The rods in this group appeared to be unaffected compared to those in the undamaged wild-type zebrafish (Fig. 1B). The wild-type zebrafish with light damage experienced significant damage to both rods and cones; though staining was still prevalent, it appears that the staining located only dead cell debris, which was mis-located and in the process of being removed (Fig. 1C). The results with the light-damaged group were consistent with the expectation that extensive damage to rods and cones would occur following light damage (Thomas et al. 2012). The Tg(XOPS:ntr-YFP) YFP+ group treated with metronidazole also showed staining that was disorganized and appeared to be that of ablated, or eroded, cells (Fig. 1D) . This damage was only noticeable in rods, which was expected as nitroreductase is a rhodopsin promoter and thus only expressed in rods.

Notch3 Signaling is Downregulated in *bbs2*$^{+/+}$ WT; Tg(XOPS:ntr-YFP) YFP+ Zebrafish Treated With Metronidazole

Counts of PCNA-positive Müller glia cells in the inner nuclear layer and outer nuclear layer of the dorsal retinas of the *bbs2*$^{+/+}$ WT; Tg(XOPS:ntr-YFP) YFP+ zebrafish treated with metronidazole were greater than those in the *eys*$^{+/-}$ zebrafish and comparable to those in the light-damaged *eys*$^{+/-}$ zebrafish (Fig. 2). Though the difference between the counts in each of these groups and the *eys*$^{+/-}$ zebrafish is not significant, there is a correlation between a relatively greater number of PCNA-positive Müller cells and inflicted damage.

Notch signaling has previously been shown to regulate regeneration following retinal injury via light damage. It was shown that Notch3 expression is downregulated following the damage (Campbell et al. 2020). The research question was whether Notch3 signaling would be similarly downregulated in zebrafish expressing the nitroreductase gene following metronidazole treatment. To achieve cell ablation for this damage model, zebrafish expressing nitroreductase were placed in 10 mM metronidazole for 24 hours at 28°C in the dark. After 16 hours of recovery, retinas were collected and Notch3 expression was measured. Relative to *eys*$^{+/-}$ zebrafish, there was a small, insignificant downregulation of Notch3 expression in the *bbs2*$^{+/+}$ WT; Tg(XOPS:ntr-YFP) YFP+ zebrafish treated with metronidazole (Fig. 3).

Notch3 Signaling is Not Downregulated in *eys*$^{-/-}$ Zebrafish

Counts of PCNA-positive Müller glia cells in the inner nuclear layer and outer nuclear layer of the dorsal retinas of the *eys*$^{-/-}$ zebrafish treated were comparable to those in the *eys*$^{+/-}$ zebrafish and less than those in the *bbs2*$^{+/+}$ WT; Tg(XOPS:ntr-YFP) YFP+ and light-damaged *eys*$^{+/-}$ zebrafish (Fig. 2). Though the difference between the *eys*$^{-/-}$ zebrafish is not significant,

there is a trend that animals with inherited degeneration possess a relatively lower number of PCNA-positive Müller cells than those with inflicted damage.

Previous research has shown that Müller cells do not proliferate, and retinal regeneration does not occur in zebrafish mutants with cone photoreceptor degeneration and neuroinflammation. However, the Müller cells of these mutants do become proliferative as an injury response (Song et al. 2020). To determine whether Notch signaling plays a role in this phenomenon, Notch3 expression was measured in *eys*$^{-/-}$ zebrafish. In inflicted damage models, which experience photoreceptor regeneration following injury, Notch3 expression was downregulated (Campbell et al. 2020). Relative to *eys*$^{+/-}$ zebrafish, in the current research, there was no downregulation of Notch3 in the *eys*$^{-/-}$ mutants, and there was a small, insignificant upregulation (Fig. 3).

DISCUSSION

The results reported here are preliminary evidence for the role of the Notch signaling pathway as a critical step in the mechanism of regeneration of photoreceptors in zebrafish with inherited degeneration. This is consistent with what has been previously shown about the role of Notch in regeneration of photoreceptors in zebrafish following inflicted damage, specifically light damage (Song et al. 2020). Additionally, these results add to the body of data already in existence regarding regeneration in zebrafish models with inflicted damage by evaluating an additional damage model: the chemical ablation of photoreceptors by metronidazole in zebrafish expressing the nitroreductase gene. This study also produced results consistent with the available data, indicating that Notch3 expression is also downregulated in this damage model.

Though the trends in the presented data are consistent with the hypothesis that a compromised function of the Notch signaling pathway contributes to or is responsible for the

lack of regeneration in zebrafish models with inherited photoreceptor degeneration, the conclusions are indefinite because the upregulation, or lack of downregulation, of Notch3 expression in the mutant group was insignificant in comparison to the *eys*$^{+/-}$ heterozygous untreated group. Likewise, the quantification of PCNA-positive Müller glia cells was insignificant in its support of the hypothesis that inherited degeneration of photoreceptors is correlated with less proliferation of Müller cells than that of unaffected animals or animals with inflicted damage of photoreceptors. The lack of significant conclusions could be a result of the nature of the proposed mechanism which occurs in Müller cells. Müller cells do not constitute a majority of retinal cells and thus Notch3 expression in non-Müller cells in the retina could be obscuring any real differences in Müller cells, thereby masking the results of other cells that do not respond in the same way. This hypothesis could be explored by devising a method to investigate the parameters of this study with a focus on Müller cells.

There is potential for aforementioned methods to be developed and produce significant support of the Notch signaling pathway being a critical step in the mechanism of photoreceptor regeneration in zebrafish with mutations that lead to photoreceptor degeneration. In this case, additional work in the realm of the pathway is necessary for a full understanding of the relation between degeneration in the retina and the subsequent signals required to stimulate Müller glia regeneration. This work would include an exploration of the mechanism downstream of Notch that could be more directly tied to either Müller cell dedifferentiation or cell cycle control. Another course of action, given an assemblage of the expected results regarding regulation of Notch3 expression, would be an investigation of methods to experimentally modulate Notch activity as to induce cell proliferation and remedy the compromised function of the signaling pathway in zebrafish with inherited degeneration.

This study contributes to the body of work detailing the role of Notch3 regulation in photoreceptor regeneration across vertebrates. Though this regeneration is not possible in humans, advancements in this field of study could yield the potential to develop treatment options for humans inflicted with degenerative diseases of the retina that impact vision.

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FIGURES

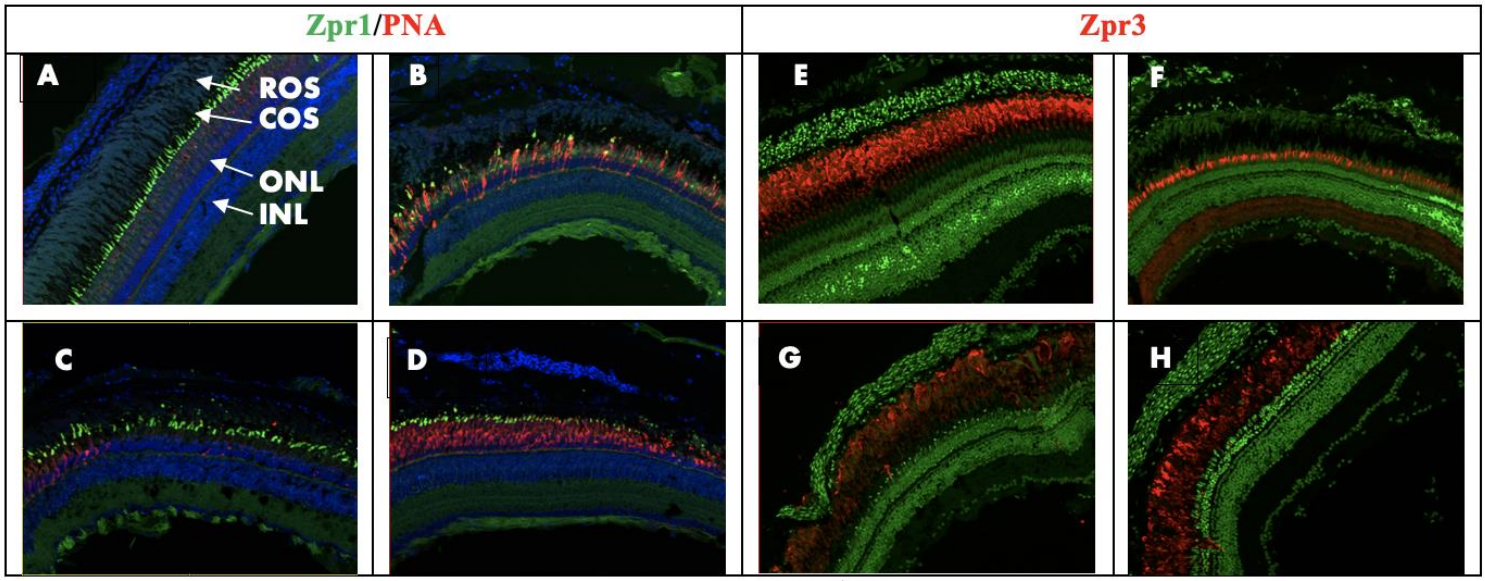

Figure 1. (A, B, C, D) Cryosections of wild-type, *eys*$^{-/-}$ mutant (n = 4), light-damaged wild-type (n = 4), and metronidazole-treated Tg(XOPS:ntr-YFP) YFP+ (n = 6) zebrafish stained with PNA (red) to label cone outer segments and zpr1 (green) to label cone inner segments. **(E, F, G, H)** Cryosections of wild-type (n = 5), *eys*$^{-/-}$ mutant (n = 4), light-damaged wild-type (n = 4), and metronidazole-treated Tg(XOPS:ntr-YFP) YFP+ (n = 6) zebrafish stained with zpr3 (red) to label rhodopsin in rod outer segments. Sections were counterstained with DAPI (blue in A-D, green in E-H). INL, inner nuclear layer; ONL, outer nuclear layer, COS, cone outer segment layer; ROS, rod outer segment layer.

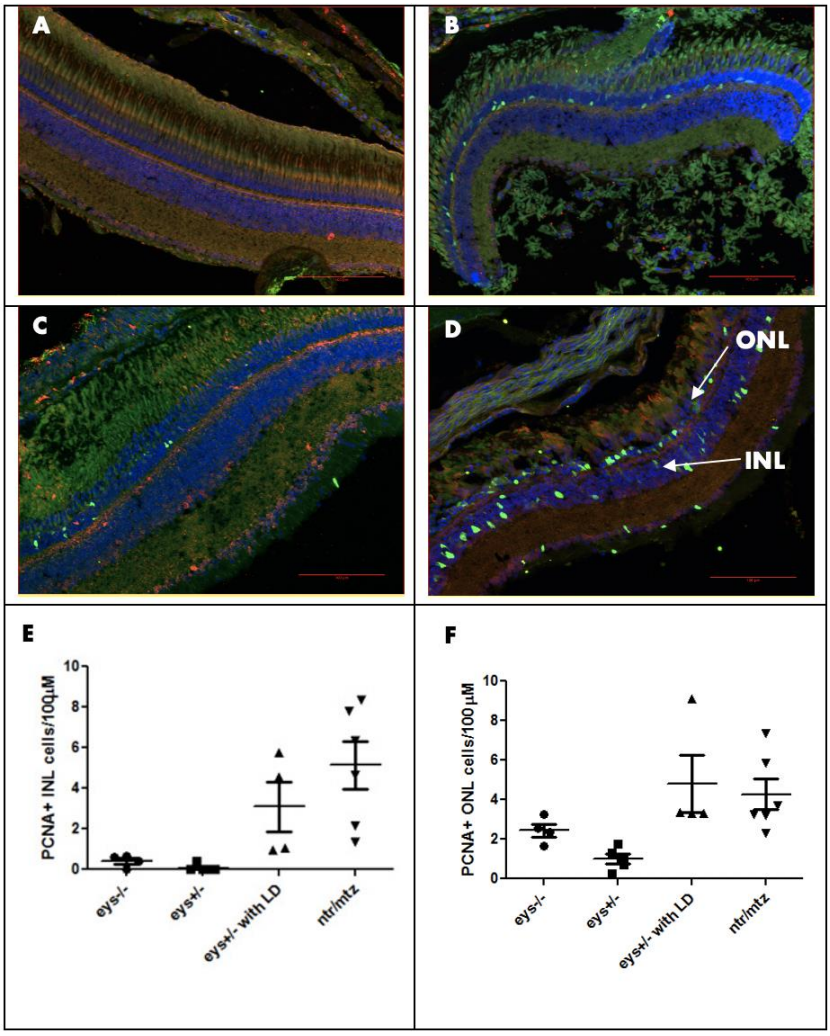

Figure 2. (A) Cryosection of wild-type (*eys*$^{+/-}$). **(B)** Cryosection of *eys*$^{-/-}$ mutants. **(C)** Cryosection of wild-type with inflicted light damage. **(D)** Cryosection of Tg(XOPS:ntr-YFP) YFP+ with metronidazole treatment. All sections were stained with PCNA (green) to label proliferating cells and counterstained with DAPI (blue). **(E)** Quantification of PCNA-labeled nuclei in the INL of the dorsal retina of wild-type, *eys*$^{-/-}$ mutant, light-damaged wild-type, and metronidazole-treated Tg(XOPS:ntr-YFP) YFP+ zebrafish. Each data point is representative of the quantified expression in an individual animal. **(F)** Quantification of PCNA-labeled nuclei in the ONL of th dorsal retina of wild-type, *eys*$^{-/-}$ mutant, light-damaged wild-type, and metronidazole-treated Tg(XOPS:ntr-YFP) YFP+ zebrafish. Each data point is representative of the quantified expression in an individual animal. Scale bar: 100 μm. INL, inner nuclear layer; ONL, outer nuclear layer.

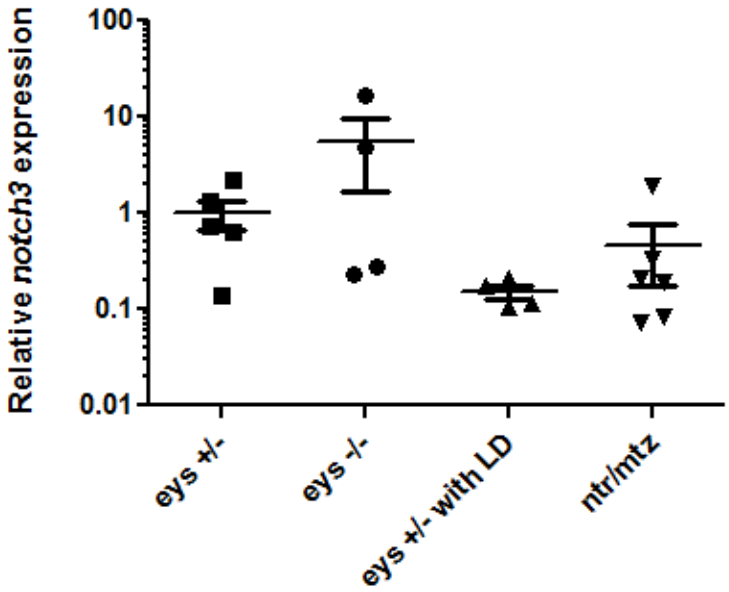

Figure 3. Quantification of relative *notch3* expression in wild-type (n = 5), *eys*$^{-/-}$ mutant (n = 4), light-damaged wild-type (n = 4), and metronidazole-treated Tg(XOPS:ntr-YFP) YFP+ (n = 6) zebrafish. Each data point is representative of the quantified expression in an individual animal.