Development of an Optogenetic Tool for Targeted Sensory Hair Cell Ablation in Zebrafish

By

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May, 2022

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Mechanosensory hair cells convert mechanical information into neuronal signals. Mutations in the genes required for hair cell function cause deafness and lead to the degeneration of sensory hair cells. Mammals are unable to regenerate these cells, leading to the permanent loss of hair cells. Research efforts are being made to stimulate hair cell regeneration in mammals and to repair genetic mutations via gene therapy to restore hearing. However, if sensory-deficient hair cells are unable to be regenerated, the effectiveness of gene therapy will be limited. To date, there are no studies examining whether mutant hair cells are able to regenerate after cell death. Other vertebrates, such as amphibians, birds, reptiles and fish, possess the ability to regenerate mechanosensory hair cells after cell death. Thus, it is of great value to investigate the genetic factors affecting the regeneration of the sensory hair cells in these species as a step towards initiating this ability in humans. This experiment uses the zebrafish model system to investigate the ability of sensory- deficient hair cells to resist ototoxic insults, and the proliferative and regenerative properties of the lateral line organ. To look at the regeneration capabilities of MET mutants, ablation methods were used to target sensory hair cells using the OptoBax system in zebrafish.

DEVELOPMENT OF AN OPTOGENETIC TOOL FOR TARGETED SENSORY HAIR CELL
ABLATION IN ZEBRAFISH

A Thesis

Presented to the Faculty of the Department of Biology

East Carolina University

A Partial Fulfillment of the Requirements for the Degree

Masters of Science in Biology

By

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May, 2022

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Chapter 1: Introduction

Mechanoelectrical Transduction of Sensory Hair Cells and the Interpretation of Sound

Mechanoelectrical transduction (MET) is the conversion of mechanical forces, such as osculating sound waves or vestibular stimuli, by sensory hair cells into electrical signals that can be interpreted by the brain (Goodyear et al., 2006). This perception and interpretation of sound waves is commonly known as hearing. Sensory hair cells are equipped with mechanosensory organelles, referred to as the hair bundle, consisting of rows of actin-filled stereocilia organized in a staircase fashion (Figure 1). To ensure that all components of the hair bundle move as a single unit during stimulation, stereocilia are tethered together by an interciliary structure called the tip link. This *trans*-heterometric protein complex consists of Cadherin23 (Cdh23) and Protocadherin15 (Pcdh15) at upper and lower ends of the tip-link, respectively (Kazmierczak et al., 2007). The tip link is critical to the function of the MET channel complex, which is associated with Pcdh15 at the lower end of the tip link and is critical to the function of the MET channel complex (Beurg et al., 2009). In addition to the Cdh23 and Pcdh15 proteins, the MET complex is also comprised of transmembrane proteins Lipoma HGMGIC fusion partner- like 5 (LHFPL5), Transmembrane inner ear (TMIE), and the pore-forming Transmembrane channel-like proteins (TMC1 and TMC2) (Kawashima et al., 2011; Kurima et al., 2015; Pan et al., 2013; Xiong et al., 2012; Zhao et al., 2014). The mechanical force is transferred to the MET channel through the tip link, allowing for the influx of K^+ ions and the depolarization of the hair cell (Figure 1). Depolarization opens the Cav1.3 L-type voltage-gated Ca^{2+} channels located at the base of the cell. The influx of Ca^{2+} ions into the cytoplasm triggers the release of the neurotransmitter glutamate onto the afferent nerve, transmitting the signal to the brain.

Sensory hair cells play the central role in mechanoreception and synaptic transmission. However, the development, function and maintenance of these cells is heavily reliant on the

nonsensory support cells (Wan et al., 2013). These cells have ridged cytoskeletons that aid in maintaining structural integrity of the sensory cells during stimulation and movements (Wan et al., 2013). These cells also help to maintain the environment surrounding the sensory hair cell to ensure optimal conditions (Figure 2B). Mutations in the MET complex cause disruptions in transduction as the MET channel cannot be opened. This maintenance consists of ion regulation, secreting the cupula, and the degradation of sensory hair cells in the event of cell death.

Apoptosis and Cellular Degeneration of Sensory Hair Cells

Apoptosis is a unique form of programmed cell death that is critical for the development and homeostasis of the neuromast. Much of the apoptotic process is regulated through the permeabilization of the outer and inner mitochondrial membranes (OMM and IMM). The permeabilization of the mitochondria can occur through extrinsic or intrinsic pathways. Extrinsic can be induced by cell surface receptors, such as FAS and tumor necrosis factor receptor-1 (TNFR1) (Youle & Strasser, 2008). Metabolic insults, transcriptional cues of various genotoxic agents will induce an intrinsic pathway. The intrinsic pathway begins with the inactivation of BH3- only protein induction or post translational activation which allows activation of BAX (Youle & Strasser, 2008). When activated, BAX promotes the release of mitochondrial contained proteins such as cytochrome C leading to mitochondrial fragmentation. Cytochrome C binds to the activated APAF1 creating an apoptosome that initiates the release of caspase-3 leading to cell death (Youle & Strasser, 2008). In mammalian mechanotransduction mutants, sensory hair cells will experience complex developmental failure and degenerate (Brandt et al., 2003; Platzer et al., 2000).

Although numerous studies have attempted to explain the process by which MET sensory hair cells degenerate, the majority of the process is unresolved. However, it is known

that once this degeneration has taken place, mammalian sensory hair cells are unable to regenerate and replace these cells. This permanent loss of sensory hair cells leads to a debilitating loss of function and deafness. In humans, mutations that disrupt sensory transduction in hair cells are a major cause of deafness and lead to sensory hair cell degeneration. Other causes of cell degeneration include age, injury, or ototoxic insults (waterborne copper, chemotherapy, aminoglycoside antibiotics). As humans are unable to regenerate these cells after experiencing cell death, sensory hair cells are permanently lost and lead to deafness.

Globally, 1.57 billion people had hearing loss in 2019 making up 1 out of every 5 people (Haile et al., 2021). This number is expected to rise to 2.45 billion by the year 2050 (Haile et al., 2021). Within the United States, 3 out of every 1000 children are born with a detectable level of hearing loss in one or both ears (WHO, 2017). With a growing number of people and aging populations, these populations of people with disabling or moderate hearing loss continues to grow. Early identification of hearing loss is met with invasive interventions to generate some ability to detect environmental cues. These interventions include hearing aids, cochlear implants, and other invasive devices. From a global economic standpoint, unaddressed hearing loss costs over 750 billion (USD) and is rising as the number of effected people increases (WHO, 2017).

As these interventions are expensive, painful, and are limited in their assistive capabilities, a vast amount of people in the deaf community must resort to educational and social support. Much of their communication is done through sign language, lip reading, and captioning. Even though the deaf community has found alternative methods to communicate, the inability to hear is still extremely limiting. Communication in the dark is nearly impossible,

and the reliance on other senses, such as sight and touch, is imperative. Although there is an immense amount of reliance on these other senses, the complex auditory and optic pathways are essential in order to interpret environmental mechanosensory stimuli (Cruz et al., 123 C.E.; Gupta et al., 2022). The restoration of sensory hair cells is critical to allow those affected by hearing loss to gain the ability to detect and interpret mechanosensory cues, communicate more effectively, and regain a valuable sense.

Numerous studies are currently investigating pharmacological, stem cell, and gene therapy strategies to repair deafness mutations in sensory hair cells. Recently, genetic therapies have shown promise with *in vivo* mice studies. Adeno-associated viral vectors carrying the correct coding sequence for *Tmc1* or *Tmc2* have been injected into the cells of *Tmc1* mutant mice leading to a partial recovery of hearing (Shibata et al., 2020). Other studies have investigated the regeneration of hair cells by converting support cells. Into sensory hair cells through the upregulation of bHLH transcription factors, such as Atoh1 or the silencing of Notch signaling to shift the balance of transcription regulation initiating the differentiation of supporting cells to sensory hair cells (Shibata et al., 2020). While these studies show some promise in sensory hair cell regeneration and repairing genetic mutations to restore hearing in wild type cells, it is unknown if these therapies will have an impact on MET mutant sensory hair cells.

Determining regeneration capabilities of wild type and MET mutant sensory hair cells is critical in determining the effectiveness of future genetic therapies and restorative efforts. Other species, such as avian, amphibians, and fish are able to regenerate sensory hair cells naturally. Investigating the regenerative processes in these species is the key to unlocking potential to regenerate sensory hair cells in humans.

Regeneration in Avian and Zebrafish Species.

Support cells, derived from mantel multipotent stem cells and located around and underneath the hair cells, are a source of progenitors that replace hair cells during homeostasis and regeneration in species that regenerate such as avian, reptiles and zebrafish(Lush & Piotrowski, 2014). During regeneration, support cells are integrated with sensory hair cells through two mechanisms; mitotic regeneration and direct phenotypic conversion (Atkinson et al., 2015). Mitotic regeneration is the process in which a support cells re-enter the mitotic cycle, divide to form daughter cells, and differentiate into sensory hair cells or support cells similar to development. During this process, one or both daughter cells undergo cell differentiation and become hair cells (Atkinson et al., 2015). Support cells that do not go through mitotic regeneration can also undergo direct transdifferentiation. Although this second process typically happens in avian species, support cells have the ability to alter their gene expression and convert into sensory hair cells without dividing. Both mechanisms have shown in chicken models that each mechanism is regulated differently as both show unique spatial and temporal patterns (Stone & Cotanche, 2007).

Zebrafish as a Model

Zebrafish make exemplary models in both sensory hair cell and regeneration studies. Zebrafish not only have the natural ability to regenerate sensory hair cells, but these cells are also homologous to the human sensory system. The lateral line sensory system of zebrafish is composed of sensory organs, referred to as neuromasts, made up of hair cells that are arranged along the body surface (Figure 2A). This arrangement makes sensory hair cells easily accessed and imaged *in vivo.* Cells can also be readily exposed to ototoxin treatments being used during regeneration studies. Once the hair cell has been treated with an ototoxin, the sensory hair cell

will degenerate and begin to regenerate less than 12 hours after loss. During this time, support cells will differentiate into sensory hair cells and move to the center of the neuromast to replace the sensory hair cell to restore function. Within 72 hours, neuromast will regain complete anatomical and functional properties (Pinto-Teixeira et al., 2013). Previous studies have demonstrated this full regeneration in wild type fish within 48 hours (Lush & Piotrowski, 2014).

Along with their regenerative capabilities, transgenic lines can also be created more simply than other comparative models. In zebrafish, eggs are excreted from the female, being fertilized, then develop outside the body. The microinjection of these eggs can then take place and places in a water filled dish for maturation. With the immense similarities to mouse and human sensory hair cells, and the natural ability to regenerate sensory hair cells, Zebrafish are a unique and exemplary model and are optimal for this study.

Intentions of the Study

To date, there are no studies examining whether mechanoelectrical transduction mutant support cells have the ability to regenerate sensory hair cells after cell death. Even though replacing genetic mutations in support and sensory cells is essential, it is extraneous without first knowing if mutant cells have the same regenerative properties. This study will address novel ways to ablate MET deficient sensory hair cells in order to determine if MET mutants are able to regenerate and if current genetic therapy have the potential to be effective in the restoration of functioning sensory hair cells in humans in the future.

Chapter 2: <u>Ototoxin Resistance in MET Deficient Zebrafish</u>

In the analysis of sensory hair cell regeneration in zebrafish, cell death is typically triggered using physical or ototoxic ablation using compounds such as heavy metals, chemotherapy drugs, or aminoglycoside antibiotics. Aminoglycosides enter the sensory hair cell through the mechanotransduction channel, and accumulate within the cell (Marcotti et al., 2005; Richardson et al., 1997a). This accumulation causes oxidative stress and initiates cell death (Hirose et al., 1997; Marcotti et al., 2005; Priuska & Schacht, 1995; Richardson et al., 1997b; Sha & Schacht, 2000). Due to the mechanoelectrical transduction channel being essential for toxins to enter the cell, it was hypothesized that MET deficient sensory hair cells would be resistant to aminoglycoside antibiotics, such as neomycin.

<u>**Ototoxin Resistance Materials and Methods**</u>

Husbandry of Zebrafish

Zebrafish used in following studies were maintained at 28°C and bred under standard conditions. All animal research was done in compliance with the guidelines set forth by the Institutional Animal Care and Use Committed at East Carolina University. All experiments on mutant and wild type larvae were done between 4-6 days post fertilization (dpf). Larvae in this stage of development are indeterminate of sex.

Experimental Design

This experiment was done using $cdh23^{tj264}$ mutants. These mutants were identified using their unique "circular" phenotype at 4 days post fertilization (dpf). Mutants that showed typical "circular" phenotype were separated from the Wild Type larvae. Wild Type larvae exhibit a normal startle response (moving straight forward). Concentrations of neomycin at 0, 50, 100, 200, and 400 µM were made using a 0.001 M Neomycin sulfate (Pharma-Tek, Huntington, NY)

stock solution. The stock solution was generated using the Neomycin sulfate powder (a mixture of 22.72 mg in 25 mL of Embryo Medium (E3). Stock solution was diluted into petri dishes in correspondence with the 0- 400 µM concentration levels. At 4 dpf, Wild type and mutant larvae were separated into 7 groups per concentration, anesthetized using 0.4% Tricaine mesylate (MS222) and exposed to the assigned neomycin treatment for 30 minutes. Standard controls were exposed to 0µM for Wild Type and mutant larvae exposed alongside neomycin experiments. After treatment, larvae were transferred to 0µM, in their respective groups, and allowed to recover for 1 hour. Larvae were anesthetized using 0.4% Tricaine mesylate (MS-222) mounted using E3 and. Z-stacks (1 µm optical sections) were collected with a Zeiss LSM700 microscope using Zeiss ZEN BLUE software. All images were taken using 488nm at a 2% intensity. Each image was analyzed, and minimally and equally enhanced using FIJI software.

Aminoglycoside Antibiotic Resistance Results

Neomycin experiments showed a decrease in initial number of hair cells per neuromast and that mutations disrupting mechanotransduction channel, such as $cdh23^{tj264}$, are resistant to aminoglycoside ablation (Figure 3). Wild Type larvae controls exposed to 0µM showed no sensory hair cell loss (Figure 3A). Using 400 µM of neomycin, there was successful ablation of sensory hair cells in wild type cells (Figure 3B). Wild Type neuromasts showed significant decreases in sensory hair cells per neuromast with increasing neomycin concentrations of 0-400 µM (p= 1.58E-12) (Figure 3E). In contrast, neomycin exposure to the $cdh23^{tj264}$ mutant showed no significant sensory hair cell death per neuromast using 400 µM (Figure 3C-D). There was also no significant difference between 0-400 µM neomycin exposure (p =0.062431) (Figure

3E). These results suggest that mechanotransduction channel deficient sensory hair cells are resistant to ototoxic ablation using neomycin.

<div align="center">**Aminoglycoside Antibiotic Resistance Discussion**</div>

Sensory hair cell regeneration in Wild Type larvae has been extensively explored. However, the ability for mechanoelectrical transduction (MET) deficient sensory hair cells to regenerate has yet to be investigated. It is commonly known that MET channel mutants are resistant to ototoxins, however, the concentration of neomycin at which these cells are capable of resisting was unknown. This experiment was designed to cover various concentrations known to effectively ablate wild type cells. As aminoglycoside antibiotics are widely and commonly used as an effective ablation method, Neomycin was chosen for this experiment.

A wide range of neomycin concentrations were used to demonstrate the effectiveness of ablation on wild type sensory hair cells, and to determine the specific concentration MET mutants might be affected. It was found that wild type sensory hair cells were significantly affected by neomycin ablation, whereas $cdh23^{tj264}$ mutants were not significantly affected (Figure 3E). This was consistent with the hypothesis that MET channel deficient cells, such as $cdh23^{tj264}$ mutants, are resistant to ototoxic insults.

MET mutants in previous studies to have significantly less sensory hair cells per neuromast during development such as *myo7aa (mariner)*, *pcdh15a (orbiter)*, *tomt (mercury)* and *lhfpl5b^{vo35}* (Seiler et al., 2005; Erickson et al., 2017; Erickson et al., 2020). This experiment demonstrated the same decrease in the quantity of initial hair cells per neuromast in $cdh23^{tj264}$ mutants using untreated, 0 µM, larval groups. Although the ultimate cause in this decrease is unresolved, it can be confirmed that there is a decrease in sensory hair cells per neuromast in $cdh23^{tj264}$ mutants, consistent with experimental MET channel mutant results from

other labs. This consistent decrease suggests that the regeneration of sensory hair cells would be affected in MET mutants and needs to be examined.

For nonfunctional or absent MET, it is unknown if these sensory hair cells are capable of regeneration or, if they are able to regenerate, how the regulation of hair cell per neuromast is affected. Because a there are no current studies that have examined MET ablation resistance, the confirmation that mechanoelectrical transduction (MET) channel deficient cells were resistant to ototoxic ablation was essential in determining the next course of action in the exploration of sensory hair cell regeneration.

Although physical ablation is an additional option for ablation, this method creates substantial confounding variables. Physical ablation would disrupt surrounding supporting cells that are essential in the regenerative process (Figure 2B-C). The development of a new ablation method was required to accurately determine if these cells can regenerate without these confounding variables.

Chapter 3: Successful Integration of OptoBAX System in Zebrafish

In the analysis of sensory hair cell regeneration in zebrafish, cell death is typically triggered using ototoxic compounds such as heavy metals or aminoglycoside antibiotics. The preliminary research using neomycin confirmed that mutations disrupting mechanotransduction prevent ototoxins from entering the cell and initiating cell death. For these nonfunctional or absent MET mutants, it is unknown if these sensory hair cells are capable of regeneration or how the regulation of sensory hair cell regeneration per neuromast is affected. Because current chemical and physical ablation methods would create confounding results, as they would disrupt surrounding supporting cells critical to regeneration, the development of a new ablation method is required to accurately determine if these cells can regenerate.

To study the regenerative properties of MET mutants, sensory hair cell ablation needs to be targeted, direct and effective. This targeted sensory hair cell death can be achieved using the OptoBAX system. In the engineered OptoBAX system, Cib is tethered to the mitochondrial outer membrane via fusion with a mitochondrial-targeting transmembrane domain. The pro-apoptotic protein BAX is expressed as a fusion with Cry2 where it resides harmlessly in the cytoplasm. When cells are exposed to 488 nm of blue light, Cry2 experiences a confirmational change and is rapidly recruited to Cib activating the OptoBAX system. Thus, BAX is rapidly recruited to the mitochondrial outer membrane where it induces the release of cytochrome C and the initiation of apoptosis (Figure 4) (Hughes et al., 2015). This system has previously been successfully implemented and studied in various cell types such as Cos7, HeLa, MtLn3 (Hughes et al., 2015). It was hypothesized that the integration and function of this system can be achieved in zebrafish to effectively study mechanotransduction mutant sensory hair cell regeneration.

Because this Optogenetic system has never been attempted in multicellular organisms, several variables need to be taken into consideration. To address these variables creating this new tool, it was decided to implement this system in two separate lines: Cry2-mCherry-BAX and Tom20-Cib-GFP. Initially integrating each construct into separate lines allowed for the determination of each construct's viability in the cell. The parameters of light exposure and frequency needed to activate the system in a multicellular organism is unknown as well. It is plausible that light from the environment (i.e. windows, fluorescent lighting, confocal imaging) would trigger activation before the analysis of these cells could be done. Overall, it was extremely advantageous and necessary to have both constructs integrated separately before the OptoBAX system was tested in a single, stable line. However, because previous studies have been successful in integrating and utilizing the OptoBAX in various cell types, it was hypothesized that the OptoBAX system could be integrated and used as an effective ablation tool.

Although this Optogenetic system has been used in other cells, much was unclear about how this system would function in zebrafish. Separate lines were made to ensure the constructs would not initiate cell death prematurely. It was also speculated that environmental light could trigger the light-initiated apoptosis. Separating these lines confirmed that both constructs could exist in the cell individually. The generation of both constructs was done simultaneously to ensure both lines would reach sexual maturity at the same time. As these fish were to be crossed and combine into one tool, it was imperative that the constructs be done at the same time.

Chapter 4: Cry2-mCherry-BAX

Cry2-mCherry-BAX Line Materials and Methods

Generation of Expression Constructs and Subcloning

A transgenic vector was created using the standard protocol of Tol2 gateway cloning system (Kwan et al., 2007) to develop the Cry2-mCherry-BAX expression construct (Table 1 OptoBAX1). Original DNA fragments were amplified using specifically designed primers (Supplemental PRIMERS 1). 200 µl Neb 5-Aplpha Competent E. coli cells were thawed on ice and divided into aliquots of 50 µl. 1-5 µl of 100pg diluted plasmid (Cry2-mChreey-Bax) was added to each cell mixture. Labeled mixtures were placed on ice for 30 minutes and then transferred to a bath to heat shock for 5 minutes. Pipette 500 µl of S.O.C. medium into each cell mixture. Cell mixtures were placed in a 37°C shaking incubator for 30 minutes. 200 µl-500 µl of each mixture was placed on a Kanamycin agar plate overnight in the incubator. Colonies were inoculated in LB media containing Kanamycin and left in the shaking incubator overnight. Inoculated colonies were purified using NEB Miniprep Kit protocol.

PCR purification and attB and attP (BP) Site Recombination

PCR purification was done using the Standard Monarch protocol with the exception of the final elution. Final PCR product elution was done using TE pH8. 5 µl reactions of BP Clonase (1 µl), TE pH 8 (2 µl), PCR purified Cry-mCherry- BAX product at 51.5 ng/µl (1 µl), as well as the PDONR-221(15 femtomoles) was allowed to sit at room temperature for 3-4 hours. 1 µl ProteaseK was added and allowed to sit at room temperature for 15 minutes. Standard transformation, inoculation, NEB Mini-prep Kit protocols (substituting TE pH 8 elution) were used to create Cry2-mCherry-BAX Entry clones. A confirmation digest was preformed using PVUII and a 1% electrophoresis gel.

attL and attR (LR) Site Recombination and Plasmid Isolation

5μl LR reaction preformed using 1 μl p5e- myo6b promoter (65.6 ng/μl), 1 Cry2mCherry-BAX, 1 μl Red Eye Destination Vector (560.3 ng/μl) (Supplemental 1), 1 μl p3e-pA (135.1 ng/μl), and 1 μl LR Clonase. Reaction was left overnight, digested with 1 μl ProteaseK at 37°C for 10 minutes and was transformed using High Efficiency E. coli cells and Ampicillin Agar plates. Inoculation of individual colonies was also preformed using Ampicillin LB. NEB Mini-prep Kit protocols were used to isolate plasmids. Confirmational digest was done to using PVUII and a 1% electrophoresis gel. The final construct can be seen in Table 1. Expression clone coinjected into Wild Type Larvae can be seen in Figure 6.

Microinjection of Expression Constructs

Standard Wild type lines were crossed, and larvae were microinjected 15 min post fertilization. Larvae were injected with Cry2-mCherry-BAX construct (diluted to10 ng/μl) and transposase RNA according to the Tol2 Multisite Gateway protocol. Larvae were left to develop for 5 days post fertilization (dpf) to ensure the proper development of the eye and allow for transgenic markers to be expressed.

Screening of Transgenic Expression

Stereoscope analysis was used to determine if the red transgenesis markers were expressed in the eye. Red eye expression indicated a successful transgenic integration, and lack of red eye expression indicated no integration. Larvae that displayed red eye expression were separated and kept to confirm cellular expression.

Confirmation of Cellular Expression using Confocal Microscopy

Larvae showing transgenic expression were anesthetized using 0.4% Tricaine mesylate (MS-222) and mounted using E3. Z-stacks (1 μm optical sections), of the lateral and posterior

cristae neuromasts, were collected with a Zeiss LSM700 microscope using Zeiss ZEN BLUE software. All images were taken using 561nm at a 2% intensity. Each image was analyzed, and minimally and equally enhanced using FIJI software. All new materials were used during the experimental and mounting process. Wildtype larvae were also imaged and compared to Cry2mCherry-BAX lines.

Screening for a Cry2-mCherry- BAX Stable line

Larvae that expressed mosaic patterns of integration were raised to sexual maturity (about 3 months). These mature fish were crossed to create a stable line. Larvae of these fish that were showing transgenic expression, were kept creating a stable line and were raised to sexual maturity as well. Larvae showing transgenic expression were also anesthetized using 0.4% Tricaine mesylate (MS-222) and mounted using E3. Z-stacks (1 µm optical sections), of the posterior and lateral cristae neuromasts, were collected with a Zeiss LSM700 microscope using Zeiss ZEN BLUE software. All images were taken using 561nm at a 2% intensity. Each image was analyzed, and minimally and equally enhanced using FIJI software.

<center>

Cry2-mCherry-BAX Integration Results

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Successful Generation of Cry2- mCherry- BAX Expression Clone

Original Cry2-mCherry-BAX DNA fragments, provided by Hughes Lab, were successfully amplified and subcloned into competent E. coli cells. Integration into the E. coli colonies were confirmed through the use of Kanamycin agar plates. NEB Mini Prep produced 246.7 ng /µl. PCR purification generated 51.5 ng/µl of each construct. Confirmation of the correct entry vectors, created by a BP recombination reaction, was established through the use of a PVUII (Cry2-mCherry-BAX digest and electrophoresis (1% gel). Using the predicted results generated using ApE (A plasmid Editor) application, the results suggested that the entry

<center>15</center>

clone for Cry2- mCherry-BAX was correct (Figure 5). Entry clones were sent to EuroFins and compared to predicted sequences. The Cry2-mCherry- BAX entry vector sequencing results were confirmed to be correct using ApE to compare the predicted and sequencing results (Sequences). LR recombination reaction results confirmed correct expression clone when compared to an ApE predicted digest (Figure 5B).

Microinjection of Expression Clone Analyses

Microinjected wild type lines were screened 5-6 dpf (days post fertilization) using a stereoscope. Larvae showed red eye transgenic expression and were separated (Figure 7A). Larvae expressed Cry2-mCherry-BAX construct in a mosaic pattern displaying both single and double red eye transgenic expression per clutch. Transgenic expression per clutch was dependent on the accuracy of the microinjection and embryo's ability to integrate the injected expression clone.

Screening for Mosaic Cellular Expression

Cellular expression in sensory hair cells were found to be irregular per neuromast in a mosaic pattern (Figure 7D-E). Larvae that possessed mosaic cellular expression were raised to sexual maturity (3 months old) and in-crossed for stable cellular expression. All larvae produced by mosaic in-crosses showing transgenic expression were separated and imaged for stable cellular expression.

Stable Cellular Expression of Cry2-mCherry-BAX Larvae Analyses

Fish produced from mosaic in-crosses were found to have transgenic expression using a stereoscope (Figure 8A). Confocal microscopy images of these larvae 5-6 dpf confirmed stable, cellular expression of Cry2-mCherry-BAX (Figure 8B-C).

Cry2-mCherry-BAX Integration Discussion

As transgenesis is an essential and necessary tool for assessing cell function and expression, traditional zebrafish transgenesis pose several obstacles. The most prominent obstacle is the low transgenesis efficiency and infrequent germline integration (Kwan et al., 2007). The Tol2 multisite gateway uses site- specific recombination- based cloning. This allows for the modular assembly of [promotor]- [coding sequence]- [3' tag] constructs in a Tol2 transposon backbone with a destination vector (Kwan et al., 2007). This Tol2 gateway cloning system was optimal for the study as it greatly facilitates zebrafish transgenesis, includes useful entry clones such as cytoplasmic and membrane- localized fluorescent proteins. It also enables the integration of larger sequences. The system's effectiveness in integration and the ability to integrate larger sequences, such as the Cry2-mCherry-BAX, also made this system ideal for the study. Entry clones were made simultaneously using a standard BP reaction including Gateway™ pDONR™221 Vector. Donor vector pDONR221, including Kanamycin resistance, was used because it has a pUC origin that produces high plasmid yields as well as a universal M13 sequencing site (ThermoFisher). After the BP reaction, bacteria were grown and inoculated using Kanamycin to confirm integration. ApE (A plasmid Editor) was used to predict the fragment sizes after digestion. Entry vector Cry2-mCherry-BAX was digested using PVUII and was confirmed (Figure 6B). Entry clones were sent to EuroFins to be sequenced. The Cry2mCherry- BAX entry clone sequencing results were confirmed to be correct using ApE when compared to the predicted results (Cry2-mCherry-BAX Entry Clone Sequence Comparison). To combine the entry clone and the destination vector, a standard LR reaction using ampicillin and High Efficiency E. coli was performed creating the Cry2-mCHerry-BAX Expression Vector. The expression vectors were also confirmed using a digest of PVUII

(Figure7C, 9C). Confirmed digest results were then used for microinjection into zebrafish larvae.

Microinjection using Coinjection Transposase RNA

Confirmed Cry2-mCherry-BAX expression vector was microinjected into 15-minute old embryos to integrate the OptoBAX system. Injecting a construct into a single cell of an embryo is essential for the integration of the construct. Transposase RNA was coinjected with the expression vector, according to the Tol2 Gateway protocol, to raise the integration efficiency. These embryos were screened at 5-6 days post fertilization on a Stereoscope for transgenic (red eye) expression (Figure 7C). Larvae that showed transgenic expression were separated and imaged using confocal microscopy to confirm cellular expression.

Screening for Mosaic and Stable Cellular Expression

Because dyes used in other experiments, such as FM dye easily contaminate specimen and look identical to mCherry fluorescence using confocal imaging, the posterior and lateral neuromasts were imaged as they cannot be contaminated by FM dye. This was to ensure that the construct was cellularly expressed. Confocal images confirmed cytoplasmic cellular expression in a mosaic pattern in both Cry2-mCherry-BAX injected larvae (Figure 7D-E). These larvae were raised to sexual maturity (3months) and in-crossed to create a stable line expressing Cry2-mCherry-Bax in every sensory hair cell per neuromast (Figure 8). Embryos from Cry2mCherry-BAX in-crosses exhibited transgenic and cellular expression (Figure 8A-C). Cellular expression in Cry2-mCherry-BAX sensory hair cells confirmed the constructs viability. Larvae with confirmed Cry2-mCherry-BAX expression were raised to sexual maturity.

Chapter 5: Tom20-Cib-GFP

Tom20-Cib-GFP Line Materials and Methods

Generation of Expression Constructs and Subcloning Competent E. coli Transformation

We generated transgenic vectors and using the standard protocol of Tol2 gateway cloning system (Kwan et al., 2007) to develop the Tom20-Cib- GFP expression construct (Table 1, OptoBAX2). Original DNA fragments were amplified using precisely designed primers (Sequences). 200 µl Neb 5-Aplpha Competent E. coli cells were thawed on ice and divided into aliquots of 50 µl. 1-5 µl of 100pg diluted plasmid (Tom20-Cib-GFP) was added to each cell mixture. Labeled mixtures were placed on ice for 30 minutes and then transferred to a bath to heat shock for 5 minutes. Pipette 500 µl of S.O.C. medium into each cell mixture. Cell mixtures were placed in a 37°C shaking incubator for 30 minutes. 200 µl-500 µl of each mixture was placed on a Kanamycin agar plate overnight in the incubator. Colonies were inoculated in LB-Kanamycin and left in the shaking incubator overnight. Inoculated colonies were purified using NEB Miniprep Kit protocol.

PCR Purification and attB and attP (BP) Site Recombination

PCR purification was done using the standard Monarch protocol with the exception of the final elution. Final PCR product elution was done using TE pH8. 5 µl reactions of BP Clonase (1 µl), TE pH 8 (2 µl), PCR purified Tom20-Cib product at 44.0 ng/µl (1 µl), as well as the PDONR-221(15 fmoles) sat at room temperature for 3-4 hours. 1 µl ProteaseK was added and allowed to sit at room temperature for 15 minutes. Standard transformation, inoculation, NEB Mini-prep Kit protocols (substituting TE pH 8 elution) were used to create Cry2-mCherry-BAX Entry clones. A confirmation digest was preformed using PVUII.

attL and attR (LR) Site Recombination and Plasmid Isolation

5μl LR reaction preformed using 1 μl p5e- myo6b promoter (65.6 ng/μl), 1 μl Tom-20-Cib (52.4 ng/μl), 1 μl Green Eye Destination Vector (259.5 ng/μl), 1 μl p3e- pA (135.1 ng/μl), and 1 μl LR Clonase. 1 μl of ProteaseK was added to the reaction and left in the thermocycle for 10 minutes at 37°C. Standard protocols for transformation (using High Efficiency E. coli) and inoculation were preformed using Ampicillin Agar plates and LB. NEB Mini-prep Kit protocols were used to isolate plasmids. Confirmational digest was done to using PVUII. CAP3 Sequence Assembly Program was used to create an expected sequence and compared with the sequencing results from Eurofins (Sequences).

Microinjection of Tom-20-Cib-GFP Expression Constructs

Standard wild type lines were crossed, and larvae were microinjected 15 min post fertilization. Larvae were injected with the Tom-20-Cib-GFP construct (diluted to 10 ng/μl) and coinjected with transposase RNA according to the Tol2 Multisite Gateway protocol. Larvae were left to develop for 5 days post fertilization (dpf) to ensure the proper development of the eye and allow for transgenic markers to be expressed.

Screening of Transgenic Expression

Stereoscope analysis was used to determine if the green transgenesis markers were expressed in the eye. Green expression indicated a successful transgenic integration, and lack of green eye expression indicated no integration. Larvae that displayed green eye expression were separated and used to confirm cellular expression.

Confirmation of Cellular Expression using Confocal Microscopy

Larvae showing transgenic expression were anesthetized using 0.4% Tricaine mesylate (MS-222) and mounted using E3. Z-stacks (1 μm optical sections), of the lateral and posterior

cristae neuromasts, were collected with a Zeiss LSM700 microscope using Zeiss ZEN BLUE software. All images were taken using 488nm at a 2% intensity. Each image was analyzed, and minimally and equally enhanced using FIJI software. Wildtype larvae were also imaged under the same parameters.

Screening for Tom-20-Cib-GFP Stable line

Larvae that expressed mosaic patterns of Tom-20-Cib-GFP integration were raised to sexual maturity (about 3 months). These mature fish were crossed to create a stable line. Larvae of these fish that were showing transgenic expression, were kept creating a stable line and were raised to sexual maturity as well. Larvae showing transgenic expression were also imaged using Confocal microscopy to indicate the Tom20-Cib-GFP expression was taken at using 488nm at a 2% intensity. Each image was analyzed, and minimally and equally enhanced using FIJI software. Wildtype larvae were also imaged under the same parameters.

Tom20-Cib-GFP Integration Results

Successful Generation of Tom20- Cib-GFP Expression Clone

Original Tom-20- Cib-GFP DNA fragments, provided by Hughes Lab, were successfully amplified and subcloned into competent E. coli cells. Integration into the E. coli colonies were confirmed using Kanamycin agar plates. NEB Mini Prep produced 319.0 ng /μl. PCR purification generated 44.0 ng/μl of each construct. Confirmation of the correct entry vectors, created by a BP recombination reaction, was established through the use of an EcoRI Tom20Cib- GFP digest and electrophoresis (1% gel) (Figure 9). Using the predicted results generated using ApE (A plasmid Editor) application, the results suggested that the entry clone for Tom20Cib-GFP was correct (Figure 9A). LR recombination reaction results confirmed correct expression clone when compared to ApE predicted digest (9B).

Microinjection of Tom20-Cib-GFP Expression Clone Analysis

Microinjected Wild Type lines were screened 5-6 dpf (days post fertilization) using a stereoscope. Larvae showed green eye transgenic expression and were separated. Larvae expressed Tom20-Cib-GFP construct in a mosaic pattern displaying both single and double green eye transgenic expression per clutch (Figure 11C). Transgenic expression per clutch was dependent on the accuracy of the microinjection and embryo's ability to integrate the injected expression clone.

Screening for Mosaic Cellular Expression

Cellular expression in sensory hair cells were found to be irregular per neuromast in a mosaic pattern (Figure 11D-E). Larvae that possessed mosaic cellular expression were raised to sexual maturity (3 months old) to be in-crossed for stable cellular expression. All larvae produced by mosaic in-crosses showing transgenic expression were separated and imaged for stable cellular expression.

Stable Cellular Expression of Tom20-Cib-GFP Larvae Analyses

Larvae produced from mosaic in-crosses were found to have transgenic expression using a stereoscope (Figure 11). Confocal microscopy images of these larvae 5-6 dpf confirmed stable, cellular expression of Tom20-Cib-GFP (Figure 11D-E).

<u>**Tom20-Cib-GFP Integration Discussion**</u>

Using identical experimental methods, entry clones were made simultaneously using the Tom20-Cib-GFP construct and a standard BP reaction including Gateway™ pDONR™221 Vector. ApE (A plasmid Editor) was used to predict the fragment sizes after digestion and confirmed the correct entry clone for Tom20-Cib-GFP using PVUII (Figure 9A). Entry clones were sent to sequencing through EuroFins, compared to predicted sequences, and was

confirmed using ApE. (Tom20-Cib-GFP Matched Sequence). To combine the entry clone and destination vector, a standard LR reaction using ampicillin and High Efficiency E.coli, was performed creating the confirmed Tom20-Cib- GFP Expression Vector. The expression vectors were also confirmed using a digest of EcoRI (Figure 9B).

Microinjection using Coinjection Transposase RNA

Confirmed Tom20-Cib- GFP expression vector was microinjected into 15-minute old embryos to integrate the OptoBAX system. Transposase RNA was coinjected with the expression vector, according to the Tol2 Gateway protocol, to raise the integration efficiency. These embryos were screened at 5-6 days post fertilization on a Stereoscope for transgenic (green eye) expression (Figure 11). Larvae that showed green transgenic expression were separated and imaged using confocal microscopy to confirm cellular expression.

Screening for Mosaic and Stable Cellular Expression

Confocal images confirmed cytoplasmic cellular expression in a mosaic pattern in the Tom20-Cib- GFP injected larvae (Figure 11). Although there was no contamination concern, the Posterior and Lateral Cristae were imaged in consistency with the Cry2- mCherryBAX images. These larvae were raised to sexual maturity (3months) and in-crossed to create a stable line expressing Tom20-Cib- GFP in every sensory hair cell per neuromast (Figure 12). Embryos from Tom20-Cib- GFP in-crosses exhibited transgenic and cellular expression (Figure 12B-C). Mitochondrial Cellular expression in Tom20-Cib- GFP sensory hair cells confirmed the constructs viability. Larvae with confirmed Tom20-Cib- GFP expression were raised to sexual maturity.

Although both Constructs were developed into separate lines to ensure their individual viability, both were created simultaneously. This was to ensure each line of fish would reach

sexual maturity at the same time so that they could be crossed. This cross would then create the full OptoBAX system integration. When the individual Cry2-mCherry-BAX and Tom20-Cib-GFP reached sexual maturity, they were crossed and screened for transgenic and cellular expression.

Chapter 6: Cry2/Cib Stable Line

Cry2/Cib Stable Line Materials and Methods

Screening for Transgenic Cry2/Cib Expression

Larvae from Cry2-mCherry-BAX and Tom-20-Cib-GFP stable lines were crossed to create a singular stable Cry2-mCherry-BAX/Tom-20-Cib-GFP expressing both constructs. Stereoscope analysis was used to determine if the red and green transgenesis markers were expressed in the eye. Co-expression of a red and green eye indicated a successful transgenic integration, and lack of red, green, or red/green expression indicated no integration of the OptoBAX system. Larvae that displayed both red and green eye transgenic expression were separated and used to confirm cellular expression.

Confirmation and Analysis of Cellular Expression in Cry2/Cib line

Larvae showing dual transgenic expression at 5-6 dpf were anesthetized using 0.4% Tricaine mesylate (MS-222) and mounted using E3. Z-stacks (1 µm optical sections), of the lateral and posterior cristae neuromasts, were collected with a Zeiss LSM700 microscope using Zeiss ZEN BLUE software. All images were taken using 561nm, 488nm and DIC channels with a 2% intensity. Each image was analyzed, and minimally and equally enhanced using FIJI software. Wildtype larvae were also imaged under the same parameters for comparison.

Confirmation and Analysis of Cry2/Cib-GFP Stable Cellular Expression Results

Cry2-mCherry-BAX/Tom20-Cib-GFP larvae that expressed both red and green transgenic expression indicated a stable line with the full integration of the OptoBAX system (Figure 13). Duel expressing Cry2-mCherry-BAX/Tom-20-Cib-GFP larvae showed stable cellular expression of both constructs (Figure 13A-E). Stable cellular expression was verified by an

mCherry and GFP signal using 561nm and 488nm channels. This stable expression indicated that the OptoBAX system was fully integrated into the zebrafish.

Imaging also indicated that using alternating 561nm and 488nm frequencies triggered the initiation of the OptoBAX system. Limited exposure activated 94.01% of sensory hair cells per neuromast showed a sign of apoptotic initiation through the recruitment of Cry2-mCherry-BAX to the mitochondrially anchored Tom20-Cib-GFP (Figure 13)**.**

Discussion of Full OptoBAX System Integration

Current results suggest the successfully integration of the unique OptoBAX system into Zebrafish (Danio rerio) for the first time. All larvae that showed duel transgenic expression also showed the cellular expression of both Cry2-mCherry-BAX and Tom20-Cib-GFP constructs (Figure 13). Although it cannot be determined if this system is fully functional at this time without further experimentation, preliminary results that suggest the system is fully functional. During confocal imaging confirming the expression of the OptoBAX system, sensory hair cells were exposed to alternating 561nm and 488nm channels. As 488nm is the frequency of light that triggers the conformational change and recruitment of Cry2-mCherry-BAX to the Tom20-Cib-GFP, the exposure during the image collection triggered light-activated apoptosis. The migration of the Cry2-mCherry-BAX to the Tom20-Cib-GFP is seen as the red Cry2-mCherry-BAX forms an "oval ring" within the sensory hair cell, matching the green mitochondrial anchored Tom20-Cib-GFP (Figure 13 C-E). The preliminary study of 25 OptoBAX larvae showed that out of an average of 22 sensory hair cells per neuromast, 21 showed a sign of activation. Only an average of 1 sensory hair cell was inactive. This is a preliminary success rate of 94.01% activation. During analysis, the majority of the sensory hair cells that were not activated were deeper within the neuromast. As this part of the cell would have been imaged

last, as well as being the deepest within the cell, it is possible these cells did not have enough time to react to the light exposure before the optical section was taken. It is also possible that the hair cells deeper within the neuromast need to be exposed to a higher intensity or longer exposure of the 488nm light is needed to ensure the light reaches the cell. A larger, more intricate study needs to be performed to determine the parameters at which the OptoBAX is the most efficient in Zebrafish.

Even though the limited preliminary study did not reach a 100% ablation efficiency, this 94.01% ablation obtained was higher than the 85.71% ablation rate of neomycin used in the initial MET study (Figure 3). The preliminary results suggest that the OptoBAX system not only eliminates the confounding variables found in using traditional techniques but shows to be more efficient in sensory hair cell ablation.

Chapter 7: Limitations

Although this experiment shows great promise in the analysis of sensory hair cell regeneration in MET mutants, there are several limitations. The process of creating/screening a stable line of Cry2-mCherry-BAX-Tom20-Cib-GFP zebrafish was an extensive 2-year process. Due to this timeframe, there was a limited number of generations as well as a limited number of stable fish imaged used for this experiment. Further generations are required to determine the stability of the OptoBAX system. Higher numbers of larvae are needed to increase support of the hypothesis. Time restraints also limited the amount of time these fish were observed. There were intentions of continuing experiments and observing the fish after 488 nm of light exposure to see the death/regeneration of the sensory hair cell process. There was some evidence of cry2-BAX migration during imaging, but official experimentation and further investigation is needed to confirm.

Other limitations include the 488 nm light pulse exposure on the sensory hair cells. It was undetermined what intensity of light was needed to penetrate the sensory hair cell to initiate the light-initiated apoptosis. Knowing the parameters of light needed for sensory hair cell death is essential in confirming the functionality of the OptoBAX system.

This Cry2-mCherry-BAX-Tom20-Cib-GFP line was not introduced to any MET mutant lines. As there are many MET mutants, the Cry2-mCherry-BAX-Tom20-Cib-GFP would need to be added to various mutant lines in order to determine its compatibility and if the function of the OptoBAX system would be affected.

Chapter 8: Future Directions

Further experimentation is essential to support the effectiveness of the OptoBAX system, as well as the hypothesis that after targeted sensory hair cell ablation, MET deficient hair cells are able to regenerate. To do this, expanding the number of stable Cry2-mCherry-BAX-Tom20Cib-GFP zebrafish would allow for further confirmation of Cry2-mCherry-BAX-Tom20-Cib-GFP integration, function, and stability of the line. Larvae from the stable Cry2-mCherry-BAXTom20-Cib-GFP line will need to be introduced to 488 nm of light pulses at various time points and intensities to determine the parameters that initiate the light-triggered apoptosis. Once this has been established, the OptoBAX line will need to be integrated into various MET lines. Using the determined parameters of light exposure and intensity, larvae will need to be imaged after sensory hair cells have been successfully ablated to determine the effectiveness of this ablation method. Once confirmed, larvae will need to be monitored over a 72-hour recovery period to determine if sensory hair cells will regenerate after cell death (Figure 11). If larvae can regenerate these cells, then a second trial should be done to determine if the cells are able to regenerate a second time. It is only after these studies that the hypothesis can be confirmed or negated.

As the preliminary results suggest that the OptoBAX system is a fully functional ablation tool, there is hope that this system can be used to look at various MET mutants, their effects on sensory hair cell development, regeneration capabilities of MET sensory hair cells, and determine the potential effectiveness of genetic therapies in regenerative capabilities to restore hearing. As hearing loss and deafness effect so many people worldwide, the hope is that this tool will revolutionize how mechanoelectrical transduction sensory hair cells are studied while propelling deafness research forward into restoring hearing abilities in humans.

Chapter 9: Conclusion

Sensory hair cell regeneration has been the central focus of many labs internationally. Mechanoelectrical transduction (MET) deficient sensory hair cells are unique in that they cannot be ablated by traditional methods without creating confounding variables. This inability to ablate MET sensory hair cells effectively and accurately required the development of a new tool to determine if MET deficient hair cells are able to be regenerated after cell death.

The OptoBAX system in zebrafish still needs further examination to determine if the system is undoubtably fully functional in zebrafish. The timepoints and exposure at which the recruitment protein function is the most efficient needs to be explored, as well. Without further experimentation on the functionality and full effectiveness of this system in zebrafish, it is unable to be confirmed if the system effectively ablates sensory hair cells with light exposure. However, as the preliminary results show extreme promise, it is believed that this system will be able to effectively ablate sensory hair cells more efficiently than traditional ablation methods. This system will also be able to ablate sensory hair cells while eliminating the significant concern of confounding variables caused by physical and chemical ablation. Once the parameters of light exposure are determined, the investigation of if MET deficient sensory hair cells can regenerate from support cells after cell death can begin.

	Final Construct
OptoBAX1	pDESTtol2pACrymCherry; Myo6b; Cry2-mCherry-BAX-pA
OptoBAX2	pDESTtol2pACryGFP; Myo6b; Tom20-Cib-GFP-pA

Table 1: Transgenic constructs injected into Wild Type larvae to create transgenic lines.

Sequences

Cry2-mCherry-BAX

Full Cry2-mCherry-BAX Sequence

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33

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Cry2-mCherry-BAX Entry Clone

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Cry2-mCherry-BAX Entry Clone Sequence Comparison

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8601 TTCAAGTTTGTACAAAAAAGCAGGCTccaccATGAAGATGGACAAAAAGACTATAGTTTGGTTTAGAAGAGACCTAAGGATTGAGGATAATCCTGCATTA 8700
     ......||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 643 GCCAACTTTGTACAAAAAAGCAGGCTccaccATGAAGATGGACAAAAAGACTATAGTTTGGTTTAGAAGAGACCTAAGGATTGAGGATAATCCTGCATTA 742
          *         *         *         *         *         *         *         *         *         *

              *         *         *         *         *         *         *         *         *         *
8701 GCAGCAGCTGCTCACGAAGGATCTGTTTTTCCTGTCTTCATTTGGTGTCCTGAAGAAGAAGGACAGTTTTATCCTGGAAGAGCTTCAAGATGGTGGATGA 8800
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 743 GCAGCAGCTGCTCACGAAGGATCTGTTTTTCCTGTCTTCATTTGGTGTCCTGAAGAAGAAGGACAGTTTTATCCTGGAAGAGCTTCAAGATGGTGGATGA 842
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              *         *         *         *         *         *         *         *         *         *
8801 AACAATCACTTGCTCACTTATCTCAATCCTTGAAGGCTCTTGGATCTGACCTCACTTTAATCAAAACCCACAACACGATTTCAGCGATCTTGGATTGTAT 8900
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
 843 AACAATCACTTGCTCACTTATCTCAATCCTTGAAGGCTCTTGGATCTGACCTCACTTTAATCAAAACCCACAACACGATTTCAGCGATCTTGGATTGTAT 942
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              *         *         *         *         *         *         *         *         *         *
8901 CCGCGTTACCGGTGCTACAAAAGTCGTCTTTAACCACCTCTATGATCCTGTTTCGTTAGTTCGGGACCATACCGTAAAGGAGAAGCTGGTGGAACGTGGG 9000
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 943 CCGCGTTACCGGTGCTACAAAAGTCGTCTTTAACCACCTCTATGATCCTGTTTCGTTAGTTCGGGACCATACCGTAAAGGAGAAGCTGGTGGAACGTGGG 1042
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              *         *         *         *         *         *         *         *         *         *
9001 ATCTCTGTGCAAAGCTACAATGGAGATCTATTGTATGAACCGTGGGAGATATACTGCGAAAAGGGCAAACCTTTTACGAGTTTCAATTCTTACTGGAAGA 9100
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              *         *         *         *         *         *         *         *         *         *
9101 AATGCTTAGATATGTCGATTGAATCCGTTATGCTTCCTCCTCCTTGGCGGTTGATGCCAATAACTGCAGCGGCTGAAGCGATTTGGGCGTGTTCGATTGA 9200
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9201 AGAACTAGGGCTGGAGAATGAGGCCGAGAAACCGAGCAATGCGTTGTTAACTAGAGCTTGGTCTCCAGGATGGAGCAATGCTGATAAGTTACTAAATGAG 9300
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              *         *         *         *         *         *         *         *         *         *
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 1743 GAATAAGAGTGATTGTTTCAAGCTTTGCTGTGAAGTTTCTTCTCCTTCCATGGAAATGGGGAATGAAGTATTTCTGGGATACACTTTTGGATGCTGATTT 1842

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 9901 GACCCAGAAGGTGAGTACATAAGGCAATGGCTTCCCGAGCTTGCGAGATTGCCAACTGAATGGATCCATCATCCATGGGACGCTCCTTTAACCGTACTCA 10000
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 2143 AGCACAGATCATGATCGGAGCAGCACCTGATGAGATTGTAGCAGATAGCTTCGAGGCCTTAGGGGCTAATACCATTAAAGAACCTGGTCTTTGCCCATCT 2242

10201 GTGTCTTCTAATGACCAACAAGTAGCCCGGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGC 10300
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 2243 GTGTCTTCTAATGACCAACAAGTAGCCCGGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGC 2342

10301 GCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCT 10400
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 3243 TGCAGAGGATGATTGCCGCCGTGGACACAGACTCCCCCCGAGAGGTCTTTTTCCGAGTGGCAGCTGACATGTTTTCTGACGGCAACTTCAACTGGGGCCG 3342
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             *         *         *         *         *         *         *         *
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Tom20-Cib-GFP

Full Tom20-Cib-GFP Sequence

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40

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Tom20-Cib-GFP Entry Clone

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Tom20-Cib-GFP Matched Sequence

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2235 AAGT---------------------------------------------------------------------------------------------- 2238
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44
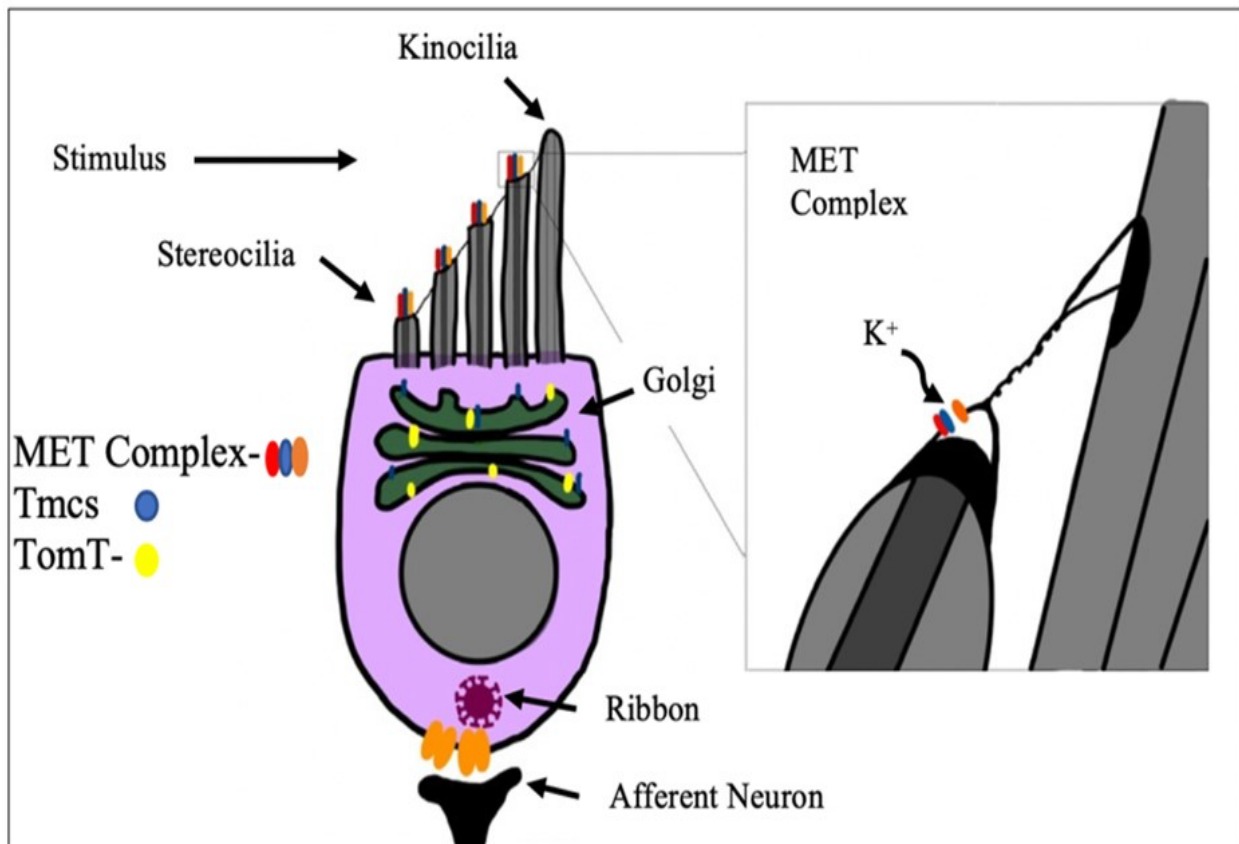
Figure 1: Individual sensory hair cell depicting MET complex. As the mechanical stimulus (the sound wave) pushes the stereocilia, the mechanical force is transferred through the tip link proteins. This force opens the MET complex to allow K^+ into the cell. This influx of K^+ depolarizes the cell.
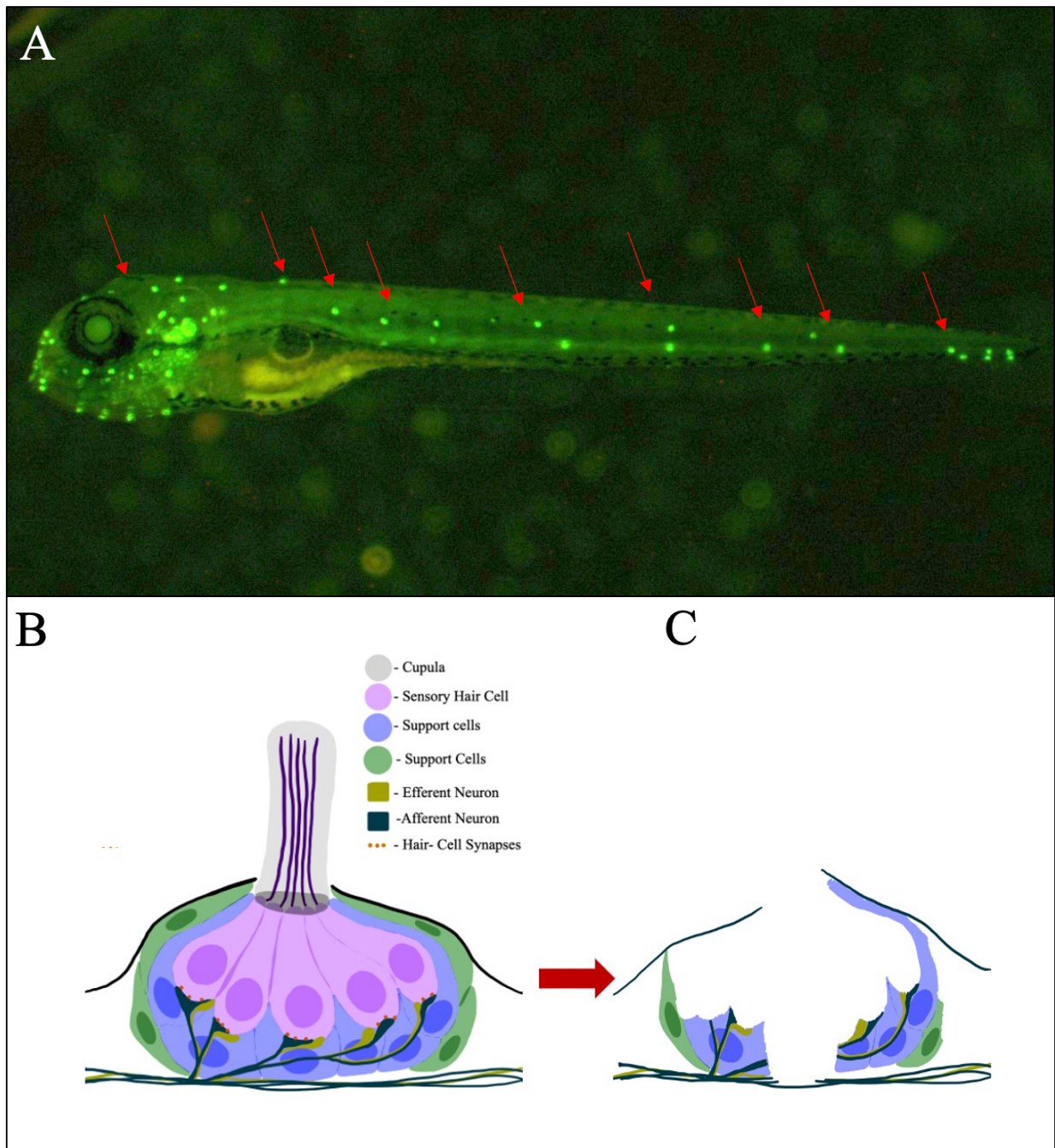
Figure 2: Sensory hair cell neuromast before and after physical ablation. A) Zebrafish under a stereoscope. The red arrows indicate the lateral line and where each neuromast is located. B) Cells that make up the neuromast. Light purple is a support cell. Green support cells are mantel pluripotent cells that differentiate into support cells. C) Neuromast after physical ablation. Physical ablation destroys surrounding support cells. This could affect regeneration as the support cells are essential to the regeneration process.

Figure 3: MET Complex Mutant *cdh23^tj264* and Wild type sensory hair cells treated with 400 μM Neomycin.
Larvae (4 dpf) were treated for 30 minutes with 400 μM neomycin. After treatment, larvae were placed in E3 for 1 hour for recovery and then imaged on the confocal. A-B) Ablation using 400 μM neomycin, were successful on Wild Type sensory hair cells. C-D) Mutant *cdh23^tj264* cells were unaffected by the neomycin treatment. E) Wild Type larvae showed a significant decrease in sensory hair cells per neuromast when exposed to various concentrations of neomycin. *cdh23^tj264* mutants showed no significant decrease in cell death when exposed to various concentrations of neomycin. (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Figure 4: OptoBAX System. In the engineered OptoBAX system, Cib is anchored to the mitochondrial outer membrane via fusion with a mitochondrial-targeting transmembrane domain Tom20. The pro-apoptotic protein BAX is expressed as a fusion with Cry2 where it resides harmlessly in the cytoplasm. When cells are exposed to blue light, Cry2 goes through a confirmational change and the Cib-Cry2 interaction is activated. BAX is rapidly recruited to the mitochondrial outer membrane where it induces morphological changes releasing cytochrome C and initiates apoptosis.

A

Size	site1		site2		Mass %
2120	PvuII	747	PvuII	2867	39
1942	PvuII	3702	PvuII	172	35
575	PvuII	172	PvuII	747	11
436	PvuII	2867	PvuII	3303	8
399	PvuII	3303	PvuII	3702	7

—172 PvuII
—747 PvuII
—2867 PvuII
—3303 PvuII
—3702 PvuII

M   1   2

B

Size	site1		site2		Mass %
4278	PvuII	4427	PvuII	8705	28
3412	PvuII	13416	PvuII	1430	22
2997	PvuII	1430	PvuII	4427	19
2120	PvuII	8705	PvuII	10825	14
1409	PvuII	11261	PvuII	12670	9
746	PvuII	12670	PvuII	13416	5
436	PvuII	10825	PvuII	11261	3

—1430 PvuII
—4427 PvuII
—8705 PvuII
—10825 PvuII
—11261 PvuII
—12670 PvuII
—13416 PvuII

M   1

Figure 5: BP and LR Digests Confirm Entry Cone and Expression Vector of Cry2mCherry-BAX. AttB and attP recombination reaction was used to integrate the DNA along with the pDONR221 donor vector. A) ApE predicted BP Entry Clone product digestion using PVUII (Left) and results confirming the correct entry clone (Right). M indicates the 1 KB Marker used to determine approximate size. Lanes 1 and 2 are both Cry2-mCherry-BAX-pDONR. The first two bands (2120 and 1942 KB) are hard to decipher and were sent to EuroFins for sequencing. Once sequencing results confirmed the Cry2-mCherry-BAX Entry clone (see sequence comparison), ApE predicted LR digestion using PVUII. B) Results confirming the correct expression clone. Wild type larvae were injected with the Cry2-mCherry-BAX expression clone and coinjected with transposase RNA according to the Tol2 Multisite Gateway protocol.
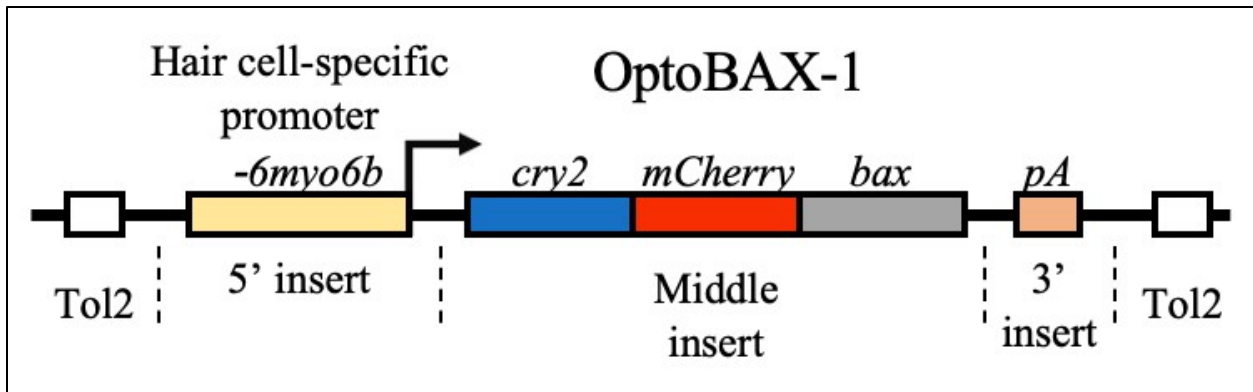
Figure 6: Expression Clone coinjected into the Wild Type Larvae and expressed in a mosaic pattern.
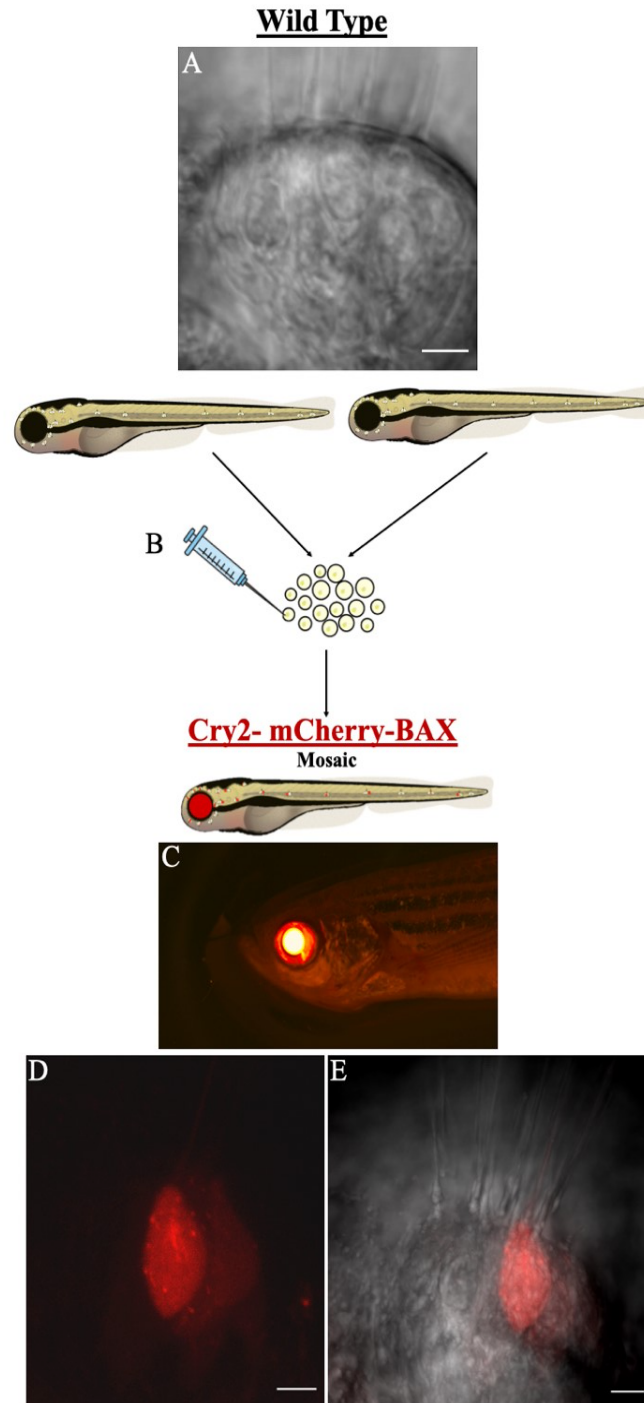
Figure 7: Microinjection and screening of Cry2-mCherry-BAX mosaic integration line 5-6 dpf. A) Wild Type sensory hair cell imaged at 561 nm to compare with mosaic larvae. B) Wild Type larvae were injected with the Cry2-mCherry-BAX expression clone and coinjected with transposase RNA according to the Tol2 Multisite Gateway protocol. C) Screening for transgenic expression was done using a stereoscope. Red eye indicated cellular expression. D) Cellular mosaic expression of Cry2-mCherry-BAX using confocal imaging. E) Cry2-mCherry-BAX merged with DIC.
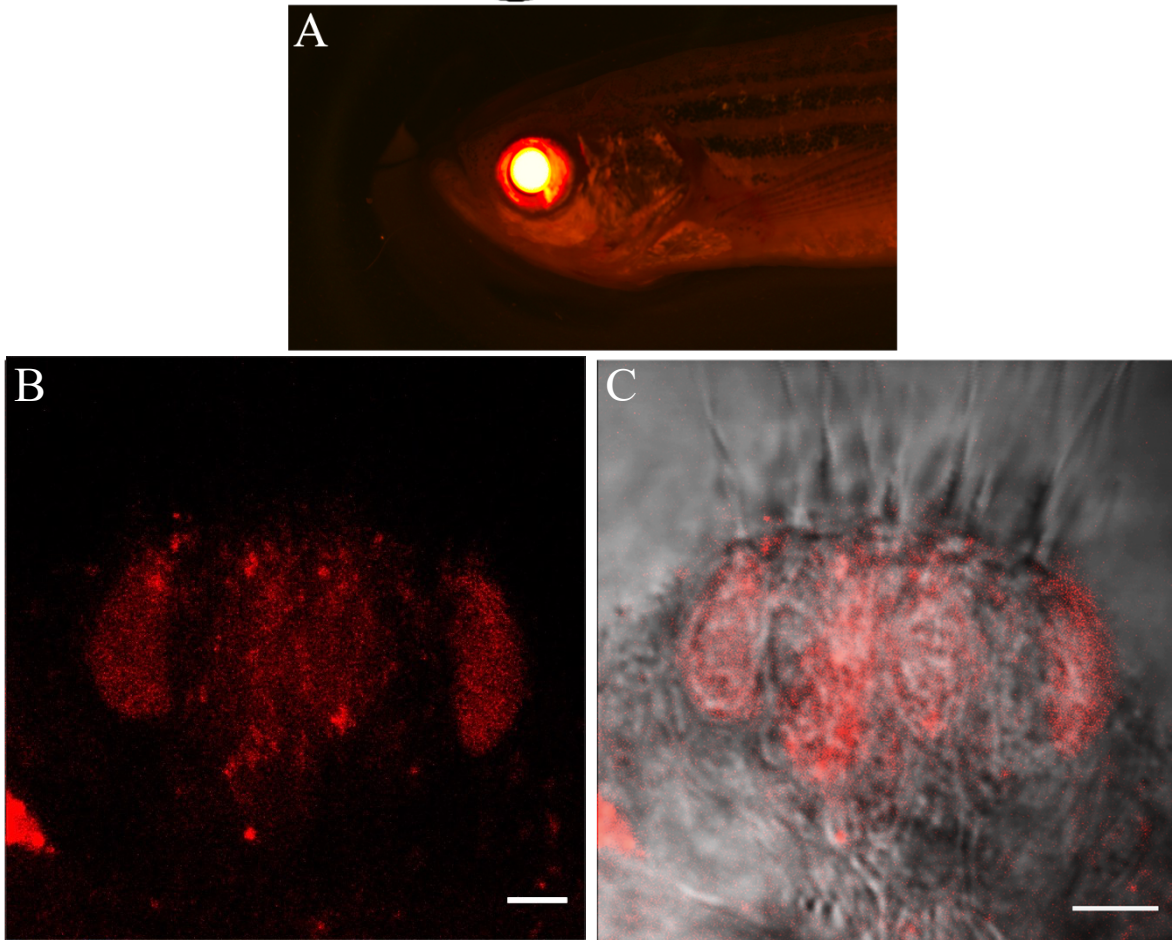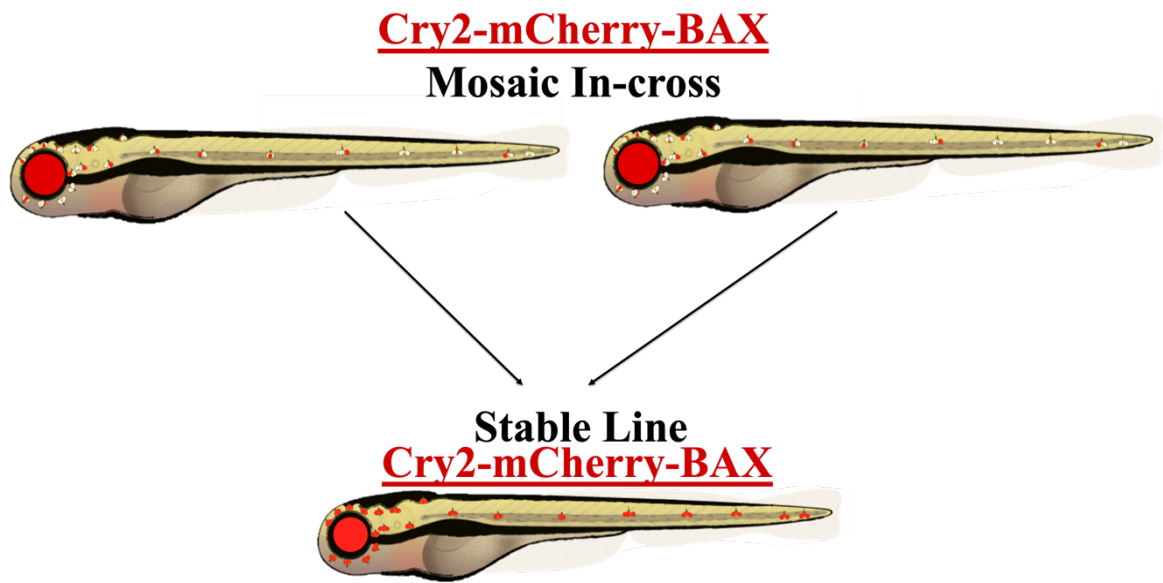
Figure 8: Stable Expression of Cry2-mCherry-BAX. A) Fish produced from mosaic in-crosses were found to have transgenic expression using the stereoscope. Red eyes indicate cellular expression. B-C) Confocal microscopy images of these larvae 5-6 dpf confirmed stable cellular expression of Cry2mCherry-BAX.
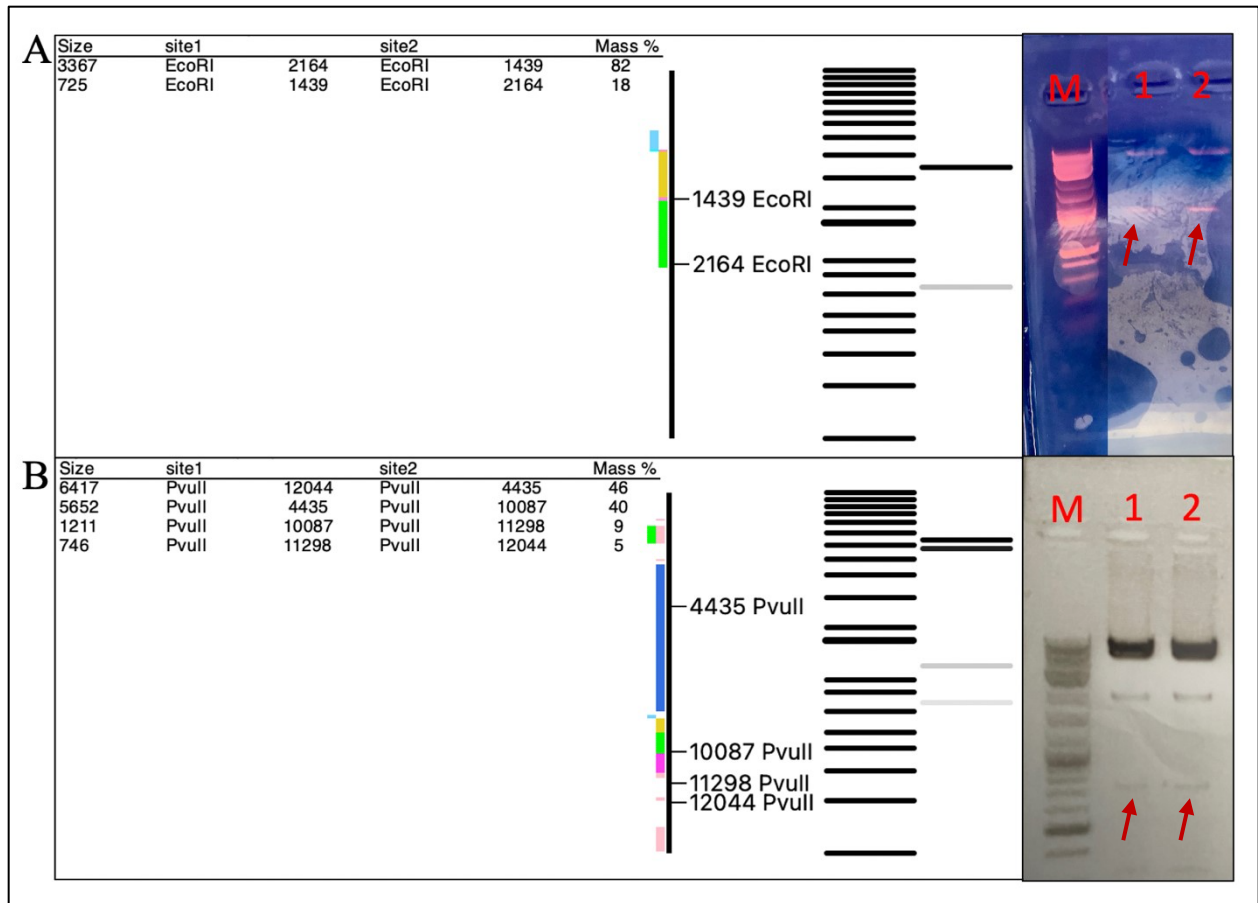
Figure 9: BP and LR Digests Confirm Entry Cone and Expression Vector Tom20- Cib-GFP. AttB and attP recombination reaction was used to integrate the DNA along with the pDONR221 donor vector. A) ApE predicted BP Entry Clone product digestion using ECORI (Left) and results confirming the correct entry clone (Right). M indicates the 1 KB Marker used to determine approximate size. Lanes 1 and 2 are both Tom20-Cib-GFP-pDONR. Both were sent to EuroFins for sequencing. Once sequencing results confirmed the Tom20-Cib-GFP Entry clone (see sequence comparison), ApE predicted LR digestion using PVUII. B) Results confirming the correct expression clone. Wild Type larvae were injected with the Tom20-Cib-GFP expression clone and coinjected with transposase RNA according to the Tol2 Multisite Gateway protocol.
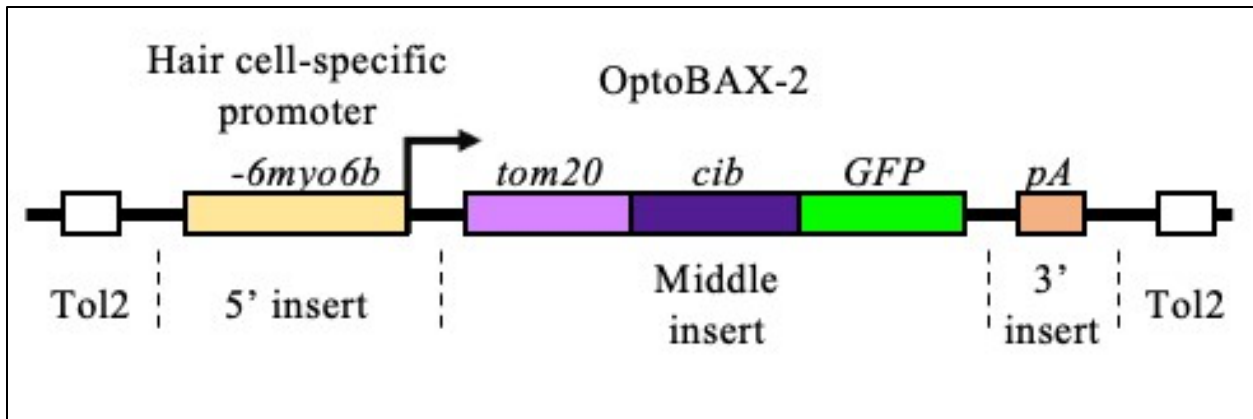
Figure 10: Expression Clone coinjected into the Wild Type Larvae and expressed in a mosaic pattern.
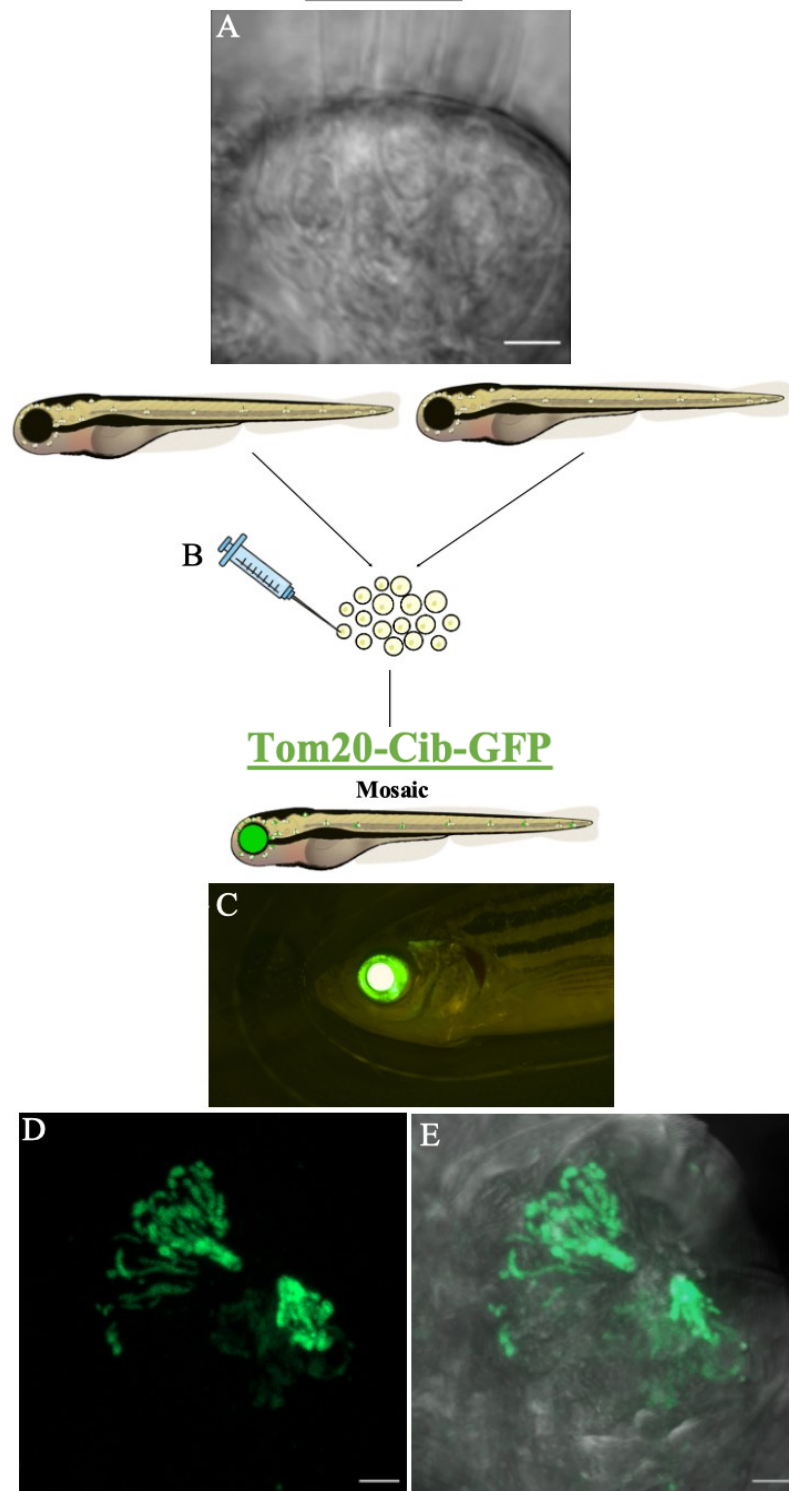
Figure 11: Microinjection and screening of Tom20-Cib- GFP mosaic integration line 5-6 dpf. A) Wild Type sensory hair cell imaged at 488 nm to compare with mosaic larvae. B) Expression Clone injected into the Wild Type Larvae and expressed in a mosaic pattern. C) Screening for transgenic expression was done using a stereoscope. Green eye indicated cellular expression. D) Cellular mosaic expression of Tom20-Cib- GFP in the Lateral Line (L1). E) Merged DIC and 488 nm channels.
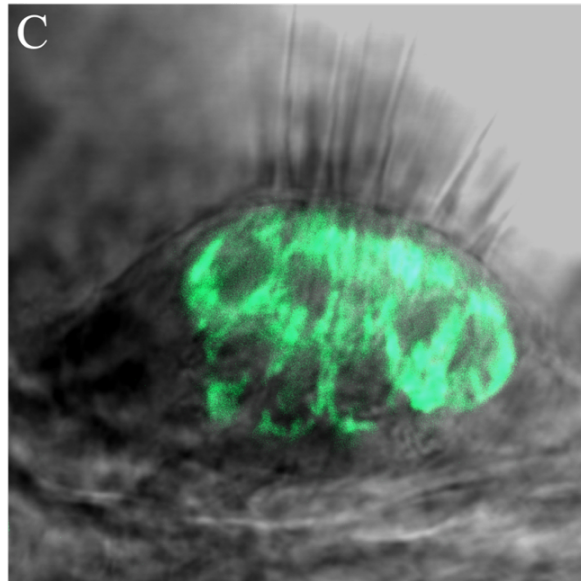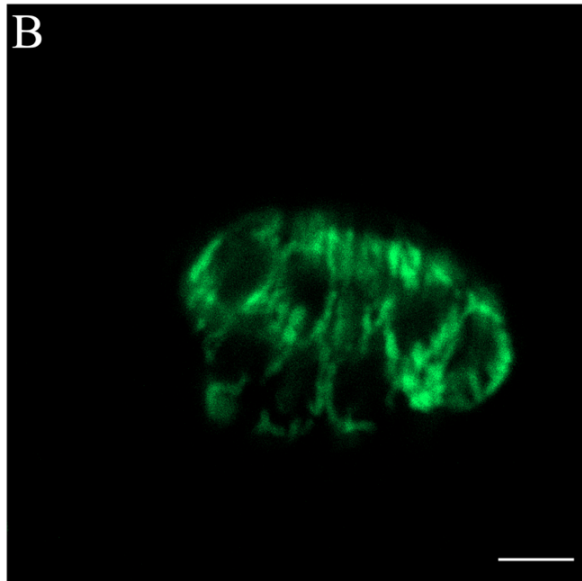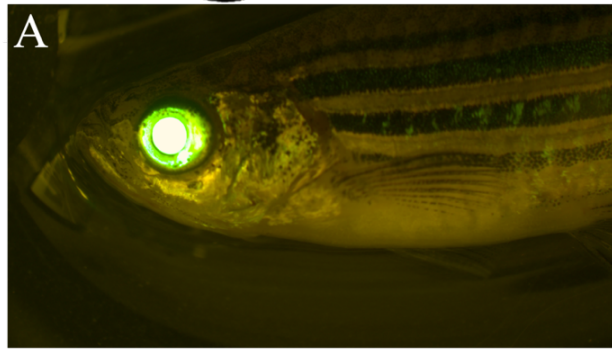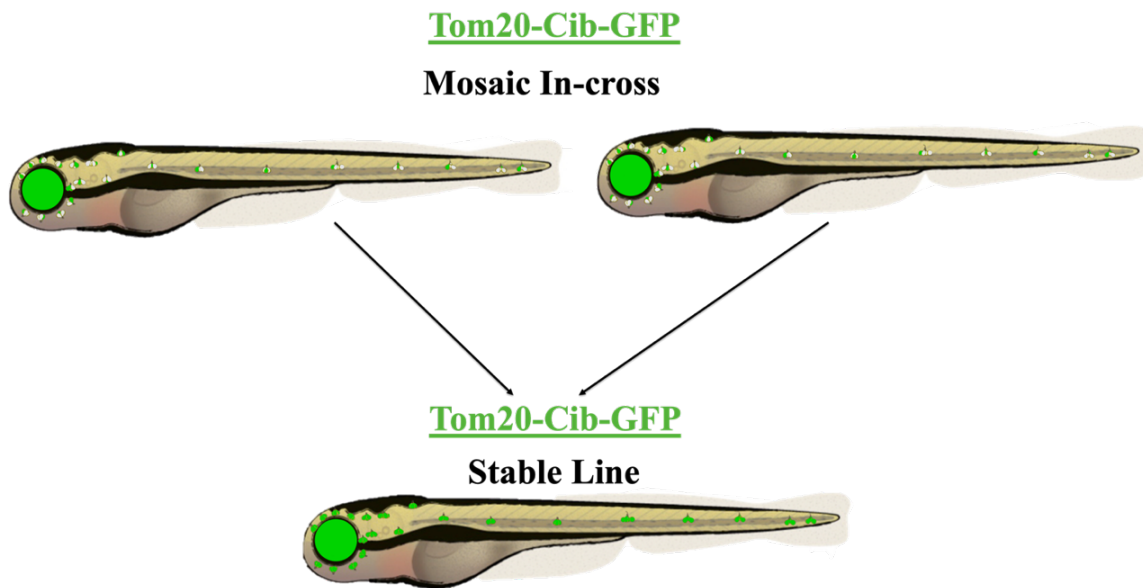
Figure 12: Stable Expression of Tom20-Cib-GFP. A) Larvae produced from mosaic in-crosses were found to have transgenic expression using the stereoscope. Green eyes indicate cellular expression. B) Confocal microscopy images of these larvae 5-6 dpf confirmed stable cellular expression of Tom20-Cib-GFP. C) DIC and 488nm merged image.
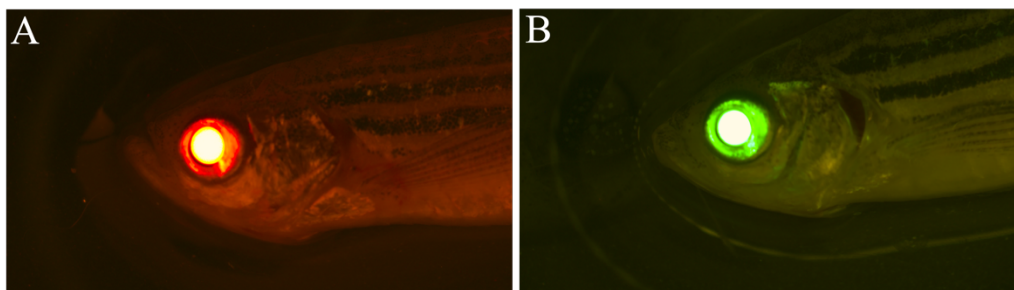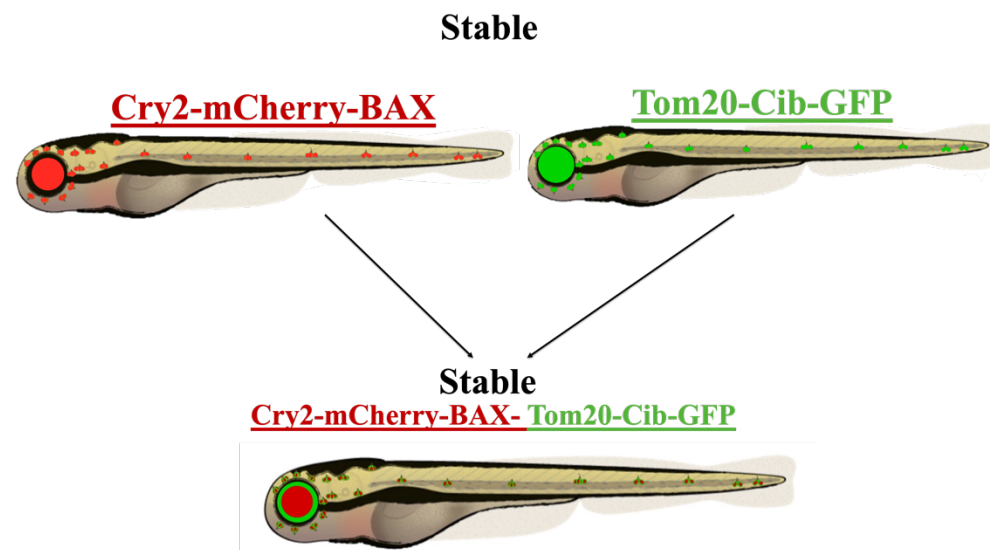
Figure 13: Successful integration of OptoBAX system in sensory hair cells of zebrafish.
Stable fish from Cry2mCherry-BAX and Tom20-Cib-GFP lines were crossed to create a single, stable line of zebrafish expressing both constructs. A) Transgenic expression of red eye indicating cellular expression of Cry2-mCherry-BAX. B) Transgenic expression of green eye indicating cellular Tom20-Cib-GFP expression. C) Confocal image of 561nm showing expression of Cry2-mCherry-BAX and migration/migration of Cry2- mCherry- BAX to the mitochondrial Tom20-Cib-GFP (migration indicated by white arrows). D) Confocal image of Tom20-Cib-GFP expression using 488nm. E) Merged image of 561nm, 488 nm, and DIC channels. Sensory hair cell images are of the posterior cristae within the inner ear of the zebrafish.
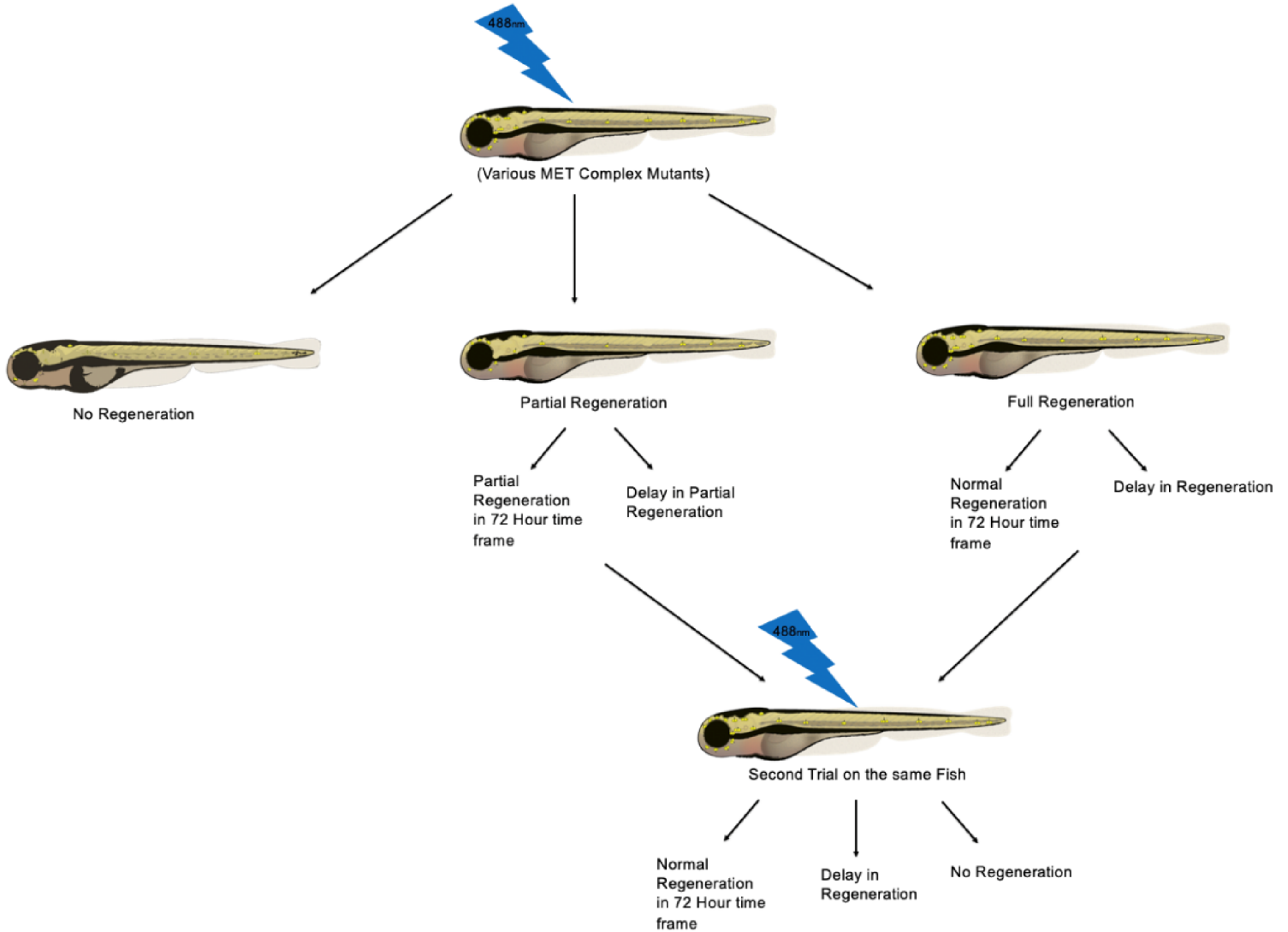
Figure 14: Future Directions and experimentation. As the system is designed to determine the effects of MET deficient hair cells on regeneration, this system will be integrated into various mutants, such as *cdh23^{tj264}* and run through experimentation on the ability of these cells to regenerate over a 72-hour period, as well as, regenerate a second time after ablation.

References

Atkinson, P. J., Najarro, E. H., Sayyid, Z. N., & Cheng, A. G. (2015). Sensory hair cell development and regeneration: Similarities and differences. In *Development (Cambridge)* (Vol. 142, Issue 9, pp. 1561–1571). Company of Biologists Ltd. https://doi.org/10.1242/dev.114926

Beurg, M., Fettiplace, R., Nam, J. H., & Ricci, A. J. (2009). Localization of inner hair cell mechanotransducer channels using high-speed calcium imaging. *Nature Neuroscience*, *12*(5), 553–558. https://doi.org/10.1038/nn.2295

Brandt, A., Striessnig, J., & Moser, T. (2003). Cav1.3 Channels Are Essential for Development and Presynaptic Activity of Cochlear Inner Hair Cells. *Journal of Neuroscience*, *23*(34), 10832–10840. https://doi.org/10.1523/jneurosci.23-34-10832.2003

Cruz, R. A., Varkey, T., Flavia, A., Paula, A., Samways, A., Garza, A., Greenlee, G., Friess, M., Sconzert, J., Afaf Aijaz, ·, Arruda, W., Khouri, J., Ellington, K., Frohman, T. C., Elliot, ·, & Frohman, M. (123 C.E.). Hearing abnormalities in multiple sclerosis: clinical semiology and pathophysiologic mechanisms. *Journal of Neurology*, *1*, 3. https://doi.org/10.1007/s00415-021-10915-w

Eckrich, S., Hecker, D., Sorg, K., Blum, K., Fischer, K., Münkner, S., Wenzel, G., Schick, B., & Engel, J. (2019). Cochlea-Specific Deletion of Cav1.3 Calcium Channels Arrests Inner Hair Cell Differentiation and Unravels Pitfalls of Conditional Mouse Models. *Frontiers in Cellular Neuroscience*, *13*, 225. https://doi.org/10.3389/fncel.2019.00225

Erickson, T., Pacentine, I. v., Venuto, A., Clemens, R., & Nicolson, T. (2020). The lhfpl5 Ohnologs lhfpl5a and lhfpl5b Are Required for Mechanotransduction in Distinct

Populations of Sensory Hair Cells in Zebrafish. *Frontiers in Molecular Neuroscience*, *12*, 320. https://doi.org/10.3389/fnmol.2019.00320

Goodyear, R. J., Kros, C. J., & Richardson, G. P. (2006). The Development of Hair Cells in the Inner Ear. In *Vertebrate Hair Cells* (pp. 20–94). Springer-Verlag. https://doi.org/10.1007/0-387-31706-6_2

Gupta, M., Ireland, A. C., & Bordoni, B. (2021). Neuroanatomy, Visual Pathway. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK553189/

Haile, L. M., Kamenov, K., Briant, P. S., Orji, A. U., Steinmetz, J. D., Abdoli, A., Abdollahi, M., Abu-Gharbieh, E., Afshin, A., Ahmed, H., Rashid, T. A., Akalu, Y., Alahdab, F., Alanezi, F. M., Alanzi, T. M., al Hamad, H., Ali, L., Alipour, V., Al-Raddadi, R. M., … Chadha, S. (2021). Hearing loss prevalence and years lived with disability, 1990-2019: Findings from the Global Burden of Disease Study 2019. *The Lancet*, *397*(10278). https://doi.org/10.1016/S0140-6736(21)00516-X

Hirose, K., Hockenbery, D. M., & Rubel, E. W. (1997). Reactive oxygen species in chick hair cells after gentamicin exposure in vitro. *Hearing Research*, *104*(1–2). https://doi.org/10.1016/S0378-5955(96)00169-4

Kawashima, Y., Géléoc, G. S. G., Kurima, K., Labay, V., Lelli, A., Asai, Y., Makishima, T., Wu, D. K., della Santina, C. C., Holt, J. R., & Griffith, A. J. (2011). Mechanotransduction in mouse inner ear hair cells requires transmembrane channel-like genes. *Journal of Clinical Investigation*, *121*(12), 4796–4809. https://doi.org/10.1172/JCI60405

Kazmierczak, P., Sakaguchi, H., Tokita, J., Wilson-Kubalek, E. M., Milligan, R. A., Müller, U., & Kachar, B. (2007). Cadherin 23 and protocadherin 15 interact to form tip-link filaments in sensory hair cells. *Nature*, *449*(7158), 87–91. https://doi.org/10.1038/nature06091

Kurima, K., Ebrahim, S., Pan, B., Sedlacek, M., Sengupta, P., Millis, B. A., Cui, R., Nakanishi, H., Fujikawa, T., Kawashima, Y., Choi, B. Y., Monahan, K., Holt, J. R., Griffith, A. J., & Kachar, B. (2015). TMC1 and TMC2 Localize at the Site of Mechanotransduction in Mammalian Inner Ear Hair Cell Stereocilia. *Cell Reports*, *12*(10), 1606–1617. https://doi.org/10.1016/j.celrep.2015.07.058

Lush, M., & Piotrowski, T. (2014). Sensory hair cell regeneration in the zebrafish lateral line: Zebrafish Sensory Hair Cell Regeneration. *Developmental Dynamics*, *243*(10), 1187–1202. https://doi.org/10.1002/dvdy.v243.10

Mackenzie, S. M., & Raible, D. W. (2012). Proliferative Regeneration of Zebrafish Lateral Line Hair Cells after Different Ototoxic Insults. *PLoS ONE*, *7*(10). https://doi.org/10.1371/journal.pone.0047257

Marcotti, W., van Netten, S. M., & Kros, C. J. (2005). The aminoglycoside antibiotic dihydrostreptomycin rapidly enters mouse outer hair cells through the mechano-electrical transducer channels. *Journal of Physiology*, *567*(2). https://doi.org/10.1113/jphysiol.2005.085951

Nemzou N., R. M., Bulankina, A. v., Khimich, D., Giese, A., & Moser, T. (2006). Synaptic organization in cochlear inner hair cells deficient for the CaV1.3 (α1D) subunit of L-type Ca2+ channels. *Neuroscience*, *141*(4), 1849–1860. https://doi.org/10.1016/j.neuroscience.2006.05.057

Pan, B., Géléoc, G. S., Asai, Y., Horwitz, G. C., Kurima, K., Ishikawa, K., Kawashima, Y., Griffith, A. J., & Holt, J. R. (2013). TMC1 and TMC2 are components of the mechanotransduction channel in hair cells of the mammalian inner ear. *Neuron*, *79*(3), 504–515. https://doi.org/10.1016/j.neuron.2013.06.019

Pinto-Teixeira, F., Muzzopappa, M., Swoger, J., Mineo, A., Sharpe, J., & López-Schier, H.

    (2013). Intravital imaging of hair-cell development and regeneration in the zebrafish.

    Frontiers in Neuroanatomy, *7*(OCT). https://doi.org/10.3389/fnana.2013.00033

Platzer, J., Engel, J., Schrott-Fischer, A., Stephan, K., Bova, S., Chen, H., Zheng, H., &

    Striessnig, J. (2000). Congenital deafness and sinoatrial node dysfunction in mice lacking

    class D L-type Ca2+ channels. *Cell*, *102*(1), 89–97. https://doi.org/10.1016/S0092-

    8674(00)00013-1

Priuska, E. M., & Schacht, J. (1995). Formation of free radicals by gentamicin and iron and

    evidence for an iron/gentamicin complex. *Biochemical Pharmacology*, *50*(11).

    https://doi.org/10.1016/0006-2952(95)02160-4

Richardson, G. P., Forge, A., Kros, C. J., Fleming, J., Brown, S. D. M., & Steel, K. P. (1997a).

    Myosin VIIA is required for aminoglycoside accumulation in cochlear hair cells. *Journal of*

    Neuroscience, *17*(24). https://doi.org/10.1523/jneurosci.17-24-09506.1997

Richardson, G. P., Forge, A., Kros, C. J., Fleming, J., Brown, S. D. M., & Steel, K. P. (1997b).

    Myosin VIIA is required for aminoglycoside accumulation in cochlear hair cells. *Journal of*

    Neuroscience, *17*(24). https://doi.org/10.1523/jneurosci.17-24-09506.1997

Seiler, C., Finger-Baier, K. C., Rinner, O., Makhankov, Y. v., Schwarz, H., Neuhauss, S. C. F.,

    & Nicolson, T. (2005). Duplicated genes with split functions: Independent roles of

    protocadherin 15 orthologues in zebrafish hearing and vision. *Development*, *132*(3), 615–

    623. https://doi.org/10.1242/dev.01591

Sha, S. H., & Schacht, J. (2000). Antioxidants attenuate gentamicin-induced free radical

    formation in vitro and ototoxicity in vivo: D-methionine is a potential protectant. *Hearing*

    Research, *142*(1–2). https://doi.org/10.1016/S0378-5955(00)00003-4

Shibata, S. B., West, M. B., Du, X., Iwasa, Y., Raphael, Y., & Kopke, R. D. (2020). Gene

therapy for hair cell regeneration: Review and new data. *Hearing Research*, 107981.

https://doi.org/10.1016/j.heares.2020.107981

Sidi, S., Busch-Nentwich, E., Friedrich, R., Schoenberger, U., & Nicolson, T. (2004). gemini

Encodes a Zebrafish L-Type Calcium Channel That Localizes at Sensory Hair Cell Ribbon

Synapses. *Journal of Neuroscience*, *24*(17), 4213–4223.

https://doi.org/10.1523/JNEUROSCI.0223-04.2004

Stone, J. S., & Cotanche, D. A. (2007). Hair cell regeneration in the avian auditory epithelium. In

International Journal of Developmental Biology (Vol. 51, Issues 6–7, pp. 633–647). Int J

Dev Biol. https://doi.org/10.1387/ijdb.072408js

Wan, G., Corfas, G., & Stone, J. S. (2013). Inner ear supporting cells: Rethinking the silent

majority. In *Seminars in Cell and Developmental Biology* (Vol. 24, Issue 5, pp. 448–459).

Elsevier Ltd. https://doi.org/10.1016/j.semcdb.2013.03.009

WHO. (2017). Global Cost of unaddressed hearing loss and cost-effectiveness of interventions.

In *Вестник Росздравнадзора* (Vol. 4).

Xiong, W., Grillet, N., Elledge, H. M., Wagner, T. F. J., Zhao, B., Johnson, K. R., Kazmierczak,

P., & Müller, U. (2012). TMHS is an integral component of the mechanotransduction

machinery of cochlear hair cells. *Cell*, *151*(6), 1283–1295.

https://doi.org/10.1016/j.cell.2012.10.041

Youle, R. J., & Strasser, A. (2008). The BCL-2 protein family: Opposing activities that mediate

cell death. In *Nature Reviews Molecular Cell Biology* (Vol. 9, Issue 1, pp. 47–59). Nature

Publishing Group. https://doi.org/10.1038/nrm2308

Zhao, B., Wu, Z., Grillet, N., Yan, L., Xiong, W., Harkins-Perry, S., & Müller, U. (2014). TMIE

    is an essential component of the mechanotransduction machinery of cochlear hair cells.

    Neuron, *84*(5), 954–967. https://doi.org/10.1016/j.neuron.2014.10.041

Appendix

Determination of the synaptic Ca²⁺ influx through the L-type calcium channels on Regulation

The development of mammalian sensory hair cells occurs in three different stages. These stages consist of the early embryonic, neonatal, and adult. The development of sensory hair cells depends on various signaling triggers and key factors that play a role in differentiating cell fate. Initial Notch, Wnt, and FGF signaling, along with the transcription factor Sox2, initiate the development of prosensory cells (Atkinson et al., 2015). With governing Shh signaling and the expression of the cell cycling inhibitor P27^{Kip1}, prosensory cells are differentiated into sensory and non-sensory cells (Atkinson et al., 2015). Prosensory cells will differentiate with Wnt and FGF, expressing the transcription factor Atoh1, inducing sensory hair cell fate. Nonsensory hair cell fates occur with the activation of Notch target genes (*Hes 1* and *Hes5*), promoting non-sensory (or supporting) cell fates (Atkinson et al., 2015).

Previous studies have indicated hair cell counts of MET mutant zebrafish show a decrease of sensory hair cells per neuromasts (Erickson et al., 2020; Mackenzie & Raible, 2012; Seiler et al., 2005). However, it remains unresolved if this decrease is directly caused by a lack of mechanotransduction or by a decrease in Ca²⁺ influx through the L-type calcium channels. The influx of Ca²⁺ through the Cav1.3 calcium channel is responsible for initiating the exocytosis of glutamate from the sensory hair cell ribbon synapse (Brandt et al., 2003; Platzer et al., 2000; Sidi et al., 2004). Previous studies also indicate that Ca²⁺ influx during development through Cav1.3 channel effect the size of the synaptic ribbons, the continued maintenance of the synapses within the cell (Sheets et al., 2012) and the maturation of cochlear hair cells (Brandt et al., 2003;

Eckrich et al., 2019; Nemzou N. et al., 2006). As the Ca^{2+} influx has such an influence on cellular activities, there is potential for the regeneration of these cav1.3a mutants to be affected in a negative way. This experiment was run under the hypothesis that the Ca^{2+} influx through Cav1.3a promotes developmental function in sensory hair cells during the regulation of hair cell number in neuromasts and influences the regeneration of these hair cells.

To determine if synaptic Ca^{2+} influx regulates hair cell number in neuromasts during development, cells will be quantified and compared hair cell abundance between *cacna1da* (Voltage-dependent L-type calcium channel subunit alpha) mutants to Wild Type siblings. After the initial sensory hair cell quantification, the maturation was analyzed, and observed the regeneration capabilities of the cell after antibiotic ablation.

Cav1.3 Calcium Channel Quantification Methods

cav1.3a mutant hair cells lines were crossed to include *Tg(myo6b:egfp-pa)vo68Tg*. Green fluorescent protein (GFP) is localized to the sensory hair cell using the myosin6b promotor. This is expressed in every sensory hair cell at every stage of hair cell development. This made the identification of sensory hair cells simpler and counts more accurate. Heterozygous *cacna1da* mutants were identified through fin- clip sequencing techniques. Larvae that expressed GFP were separated into 2 groups of 33: mutant and Wild Type. Cav1.3 mutants can be separated from Wild Type siblings as they will not react to water stimulation while wild type larvae exhibit a normal startle response. Larvae were anesthetized using 0.4% Tricaine mesylate (MS-222) and mounted live using E3. Z-stacks (1 µm optical sections), of the lateral and posterior cristae neuromasts, were collected with a Zeiss LSM700 microscope using Zeiss ZEN BLUE software.

Cav1.3 mutants, as well as wild type siblings, were imaged at 5 dpf using the 488nm

channel at a 2% intensity. Each image was analyzed, and minimally and equally

enhanced using FIJI software.

Results

Wild Type larvae presented an average of 18 (18.43) sensory hair cells per neuromast

(Appendix Figure 1). *cav1.3a* mutant hair cells indicated a significant decrease ($p \leq 4.27E-44$) in

the initial sensory hair cell count with an average of 7 cells per neuromast (Appendix Figure 1).

Discussion

cav1.3a mutants exhibiting a decrease in initial cell count indicates that Ca^{2+} influx

influences the generation of sensory hair cells. The *cav1.3a* mutants block Ca^{2+} influx through

the Cav1.3 L-type voltage- gated Ca^{2+} channels while leaving the MET of the hair cells intact

and functional. However, because Ca^{2+} signaling plays a role in regulating gene expression and

cellular maturation, *cacna1da* mutant cells not only show a delay in initial development of

sensory hair cells but may experience delays in maturation during early development.

Proportional analysis of sensory hair cell Ontogeny of Cav1.3a-deficient Hair Cells

Methods

Using the tg(myo6b: cav1.3a-GFP)^{tc323d} mutant lines, the proportion of

cellular maturation can be determined through FM 4-64 screening. Mutant and

wildtype larvae were separated into 2 groups of 33 and exposed to FM- 464 larvae for

30 seconds. Larvae were anesthetized using 0.4% Tricaine mesylate (MS-222) and

mounted live using E3. Z-stacks (1 µm optical sections), of the lateral and posterior

cristae neuromasts, were collected at 5 days post fertilization with a Zeiss LSM700

microscope using Zeiss ZEN BLUE software. All images were taken using 488nm

and 561nm at a 2% intensity. Each image was analyzed, and minimally and equally enhanced using FIJI software.

Results

Wild Type sensory hair cells showed no significant decrease in the number of sensory hair cells per neuromast and the maturation of each sensory hair cell. Within an average of 18 sensory hair cells, and average of 1 sensory hair cell did not reach sensory hair cell maturation (Appendix Figure 2). In contrast, mutants showed that out of an average of 7 sensory hair cells per neuromast, only an average of 2 sensory hair cells reached maturation and were able to transduce
(Appendix Figure 2).

Discussion

Hair cells that have the ability to transduce will allow the FM-464 dye to enter the hair cell. Transduction of the dye indicated that the sensory hair cell is mature. Absence of FM dye would indicate that the sensory hair cell is not yet mature and unable to transduce. Wild Type larvae showed that 94.5% of sensory hair cells reach maturation by 5 dpf (Appendix Figure 2). Cav1.3 mutant larvae, however, showed that only 71.43% of sensory hair cells were able to reach maturation. Along with the initial decrease in sensory hair cells per neuromast, these indicate that sensory hair cell development is affected by *cav1.3a* mutations. To determine the effect of the Ca^{2+} influx on regeneration activity, *cav1.3a* mutant hair cells must be ablated and analyzed to determine if their regeneration is affected.

Effect of cacna1da Mutant Hair Cells on the Regeneration of Hair Cells Methods

Tg(Myo6b: cav1.3a- EGFP)^{tc323d} mutant lines were used in order to make sensory hair cell identification and counts manageable. As the MET channel was functional, the aminoglycoside antibiotic neomycin was used to chemically ablate the sensory hair cells of mutant and Wild Type siblings. Wild Type and mutant larvae were separated and placed into 5 groups according to various time points (averaging 2 fish per group totaling 8 larvae). Larvae were exposed to 400 µM of neomycin for 30 minutes. All larvae were also allowed to recover for 1 hour in E3. Larvae were anesthetized using 0.4% Tricaine mesylate (MS-222) and mounted live using E3. Z-stacks (1 µm optical sections), of the lateral and posterior cristae neuromasts, were collected at 5 dpf with a Zeiss LSM700 microscope using Zeiss ZEN BLUE software. All images were taken using 488nm at a 2% intensity. Each image was analyzed, and minimally and equally enhanced using FIJI software Images were collected at various time points (12, 24, 48, 72 hours) within a 72-hour period post treatment.

Results

After sensory hair cell ablation using 400 µM neomycin, Wild Type larvae showed a significant decrease in sensory hair cells. Mutant larvae also showed a decrease in sensory hair cells per neuromast. Over a 48-hour period, Wild Type larvae showed significant sensory hair cell regeneration. Sensory hair cell regeneration was not significant in *cav1.3a* mutants (Appendix Figure 3).

Discussion

To study the regenerative capabilities of *cav1.3a* mutants, the sensory hair cells of 5 dpf larvae needed to be ablated. Once the sensory hair cells were ablated using 400

µM neomycin, regeneration was observed over the course of 72-hour period. Wild type and mutant larvae were observed after a 1-hour recovery period to ensure sensory cell death occurred. Sensory hair cell death was observed in both Wild Type and Cav1.3 mutants. Wild Type larvae showed significant increase in sensory hair cells after 12, 24, and 48 hours (p ≤7.0989E-06) (Appendix Figure 3). Cav1.3 mutant larvae showed a significant increase in sensory hair cells from 12, and 24 hours (p ≤ 0.12825393). Results indicated that Cav1.3 mutants showed an increase of sensory hair cells through regeneration from 1- 48 hours (Appendix Figure 3). The experimental method called for the Larvae from both groups to be imaged at 72-hours, however, larvae in both Wild Type and mutant groups died during the recovery period. Because of this loss, there were limited regeneration experimental results to 48-hours post treatment.

Results suggest that L-type calcium channels and the influx of Ca^{+2} is essential to sensory hair cell development, maturation, and regeneration. The increase of sensory hair cells at 48 hours for the *cav1.3a* mutants indicates that the sensory hair cells are able to regenerate. However, the regeneration rate is delayed compared to Wild Type cells. Although final regeneration results show an increase in sensory hair cells during the 48 hours post treatment, this experiment would need to be replicated with a larger experimental group to confirm the effect of L-type calcium channels and the influx of Ca^{+2} on regeneration.

Limitations

The most considerable limitation to this experiment was the number of viable larvae and sensory hair cells observed. Although the limited number of larvae show some

support of the hypothesis that the synaptic Ca^{2+} influx through the L-type calcium channel has a negative effect on sensory hair cell regeneration, the number of larvae used was not sufficient. The modest support of the hypothesis is likely a type two error. Limited time constraints prevented the generation of more mutant larvae to increase experimental observation amounts.

Future Directions

This experiment was designed to include a broad number of genetic variants as well as a vast number of larvae over a long period of time. This experiment will need to be repeated with a more extensive time frame, a vast number of mutant variations, and a large amount of larvae to determine if the synaptic Ca^{2+} influx through the L-type calcium channel has an effect on sensory hair cell regeneration. Although this experiment needs to be repeated, the overall experimental design has the potential to uncover the influence of synaptic Ca^{2+} influx through the L-type calcium channel on regeneration.
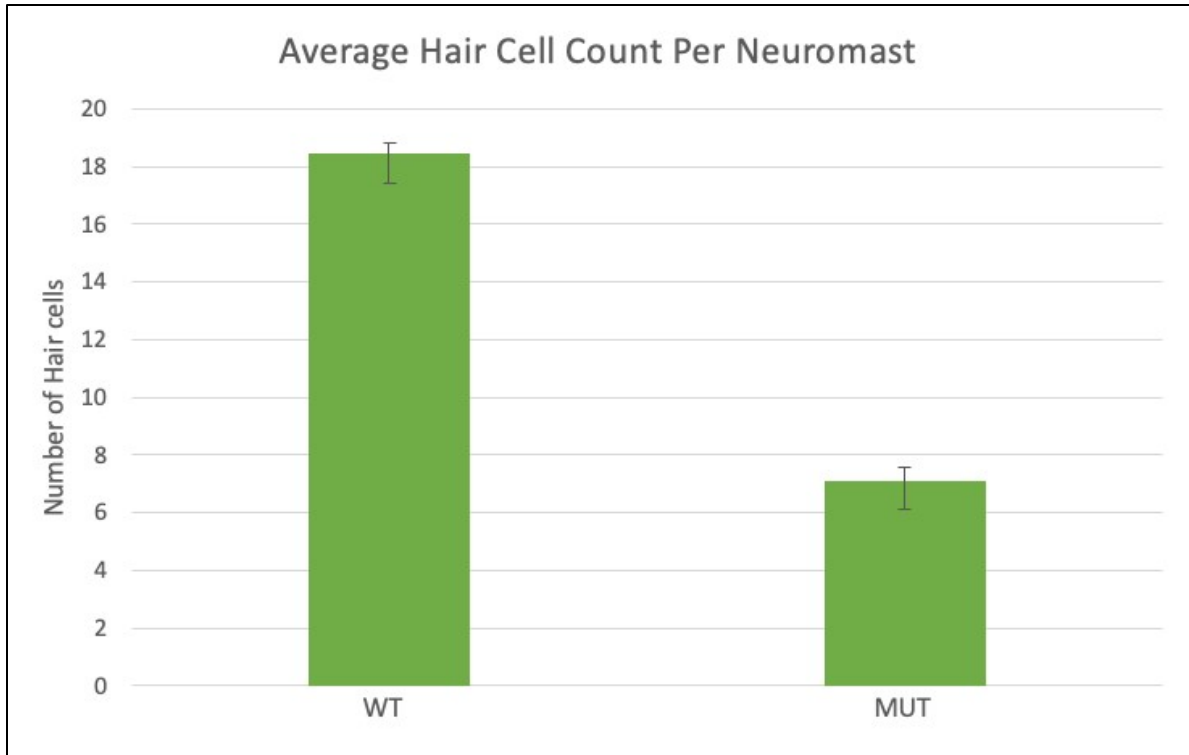
Appendix Figure 1: Average sensory hair cell count per neuromast of Wild Type and Ca^{+2} channel mutant larvae. Images of initial sensory hair cell quantity were collected 5 dfp. Wild Type larvae had an average of 18 sensory hair cells per neuromast. Cav1.3 mutant larvae neuromasts averaged 3.5 sensory hair cells. There is a significant difference between the initial number if sensory hair cells of wildtype larvae and Cav1.3 mutants (**** p ≤ 0.0001).
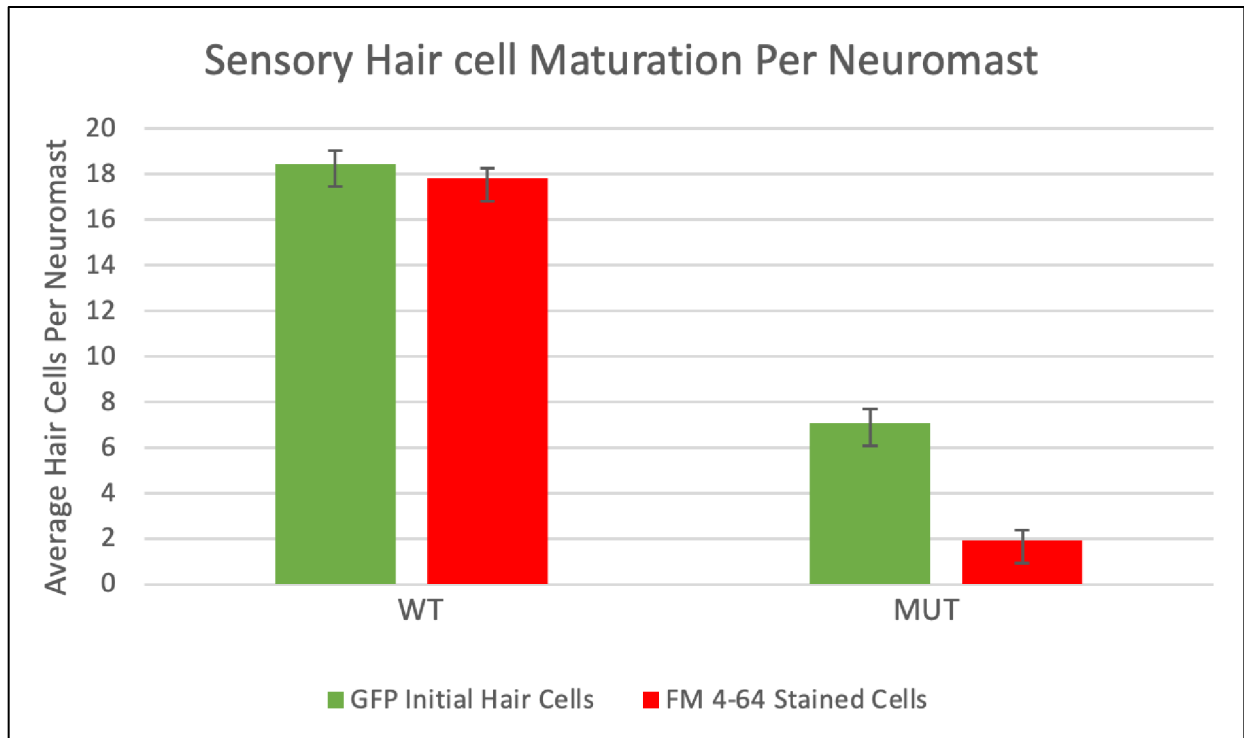
Appendix Figure 2: Sensory hair cell maturation in *cav1.3a* mutant larvae is significantly delayed. Wild Type Larvae showed an average of 18 sensory hair cells, and an average of 1 sensory hair cell did not reach sensory hair cell maturation. Cav1.3 mutants showed an average of 7 sensory hair cells per neuromast. An average of 2 sensory hair cells reached maturation and were able to transduce.
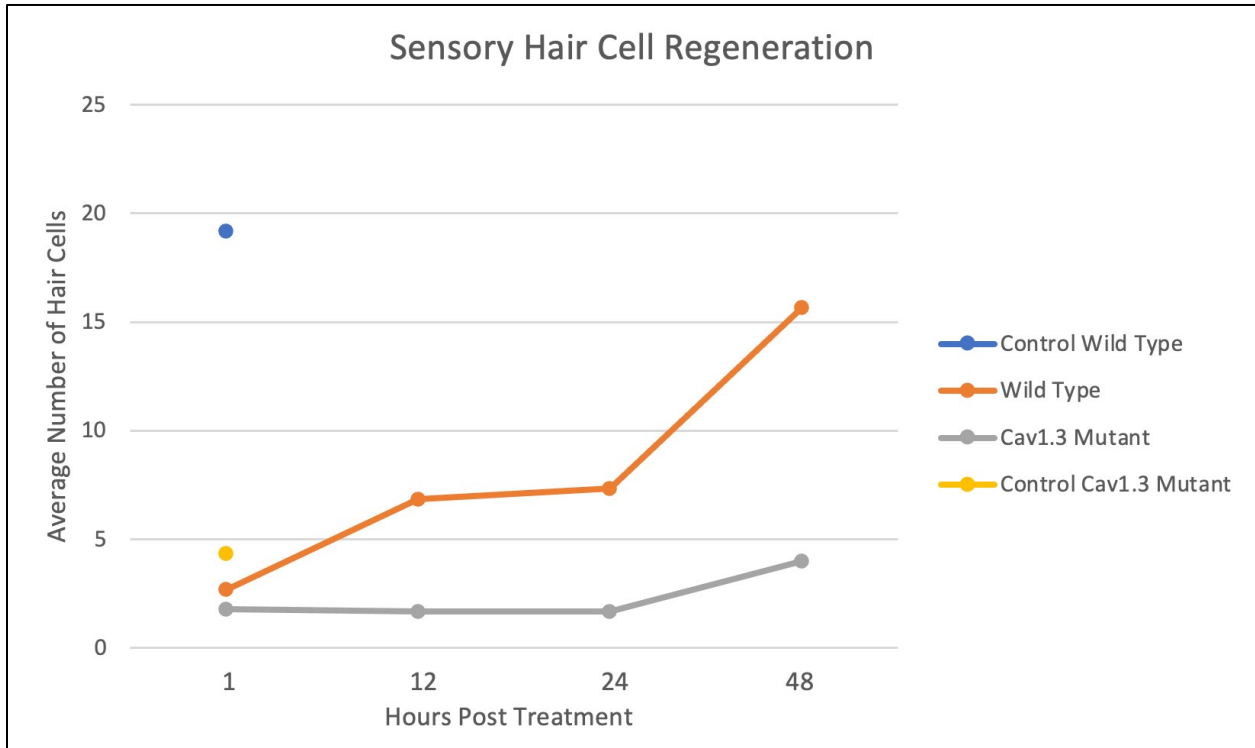
Appendix Figure 3: Significant regeneration of *cav1.3a* mutant sensory hair cells.
Initial sensory hair cell counts were taken of Wild Type (Blue) and Cav1.3 mutant
larvae. After sensory hair cell ablation using neomycin, Cav1.3 mutants show a
significant sensory hair cell regeneration within a 48-hour recovery period ($p \leq$
0.12825393).