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Adult Zebrafish Lateral Line: A Well Supported System

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Abstract

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Biological Structure

Hair cells are required for hearing and balance. Hair cell death is caused by multiple environmental insults, such as prolong exposure to loud noises, aminoglycoside antibiotics, some chemotherapeutics, and heavy metals, and hair cell loss is irreversible in humans. However, many other vertebrates such as, birds, fish and amphibians have the ability to replaced lost hair cells.

Zebrafish has quickly become an excellent modeled to study hair cell biology. Aside from the hair cells found in the inner ear, zebrafish have externally located hair cells on the head and body that allow them to detect changes in water currents. Also, larval zebrafish can quickly regenerate lost hair cells after traumatic damage. How hair cell precursors are replenished or maintained throughout the animal's life is still unknown.

I investigated lateral line hair cell and support cell maintenance in adult zebrafish, in which growth is largely complete. I demonstrate that adult zebrafish not only replenish hair cells after a single instance of hair cell damage, but also maintain hair cells and support cells after multiple rounds of damage and regeneration. I observed that hair cells undergo continuous turnover in adult zebrafish in the absence of damage. Mitotically-distinct support cell populations were identified and show that hair cells regenerate from underlying support cells in a region-specific manner.

Using the transgenic *Zebrabow-M* fish line, I performed long-term multicolor clonal analysis to discover that lateral line neuromast drift towards clonality. However, I also observe that some neuromasts reach equilibrium and may indicate that multiple progenitors act to maintain the lateral line neuromast. Our results demonstrate that there are distinct support cell populations in the lateral line, which may help explain why zebrafish hair cell regeneration is extremely robust, retained throughout life, and potentially unlimited in regenerative capacity.

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TABLE OF CONTENTS

Chapter 1: Hair Cells in Vertebrates.....1

Hair Cell Anatomy and Disease.....1

Avian Hair Cell Regeneration.....2

Chapter 2: Zebrafish Lateral Line.....5

Lateral Line Patterning and Development.....5

Hair Cell and Support Cell Regeneration.....12

Cell Lineages in Regenerating Neuromasts.....15

Chapter 3: Robust Regeneration of Adult Lateral Line Hair Cells Reflects Continued Precursor Pool Maintenance..... 19

Introduction..... 19

Results..... 22

Discussion..... 30

Materials and Methods..... 37

Figures..... 42

Chapter 4: Delineate Support Cell Regeneration and Maintenance..... 51

Introduction..... 51

Results..... 54

Discussion..... 57

Materials and Methods..... 61

Figures..... 65

Chapter 5: Conclusions and Future Directions..... 68

References:..... 70

Chapter 1: Hair Cells in Vertebrates

Hair Cell Anatomy and Disease:

Hair cells translate mechanical stimuli into electrical signals that are transmitted through the auditory nerve to the brain. The hair cells derive their name from the apically positioned ciliary bundles that polarize the cell, forming a staircase-like structure with the tallest being a true kinocilia. Hair cells are located in both the auditory and vestibular tissues in vertebrates, with the vestibular system detecting balance and spatial orientation and the auditory system sensing sound. In the mammalian auditory system, hair cells are arranged in rows and are tonotopically positioned in the cochlea, a spiral-shaped sensory organ (Bekesy, 1960; Russell and Sellick, 1977). In the chick basilar papilla, hair cells detecting high-frequency sounds are located basally and low-frequency sensing hair cells are positioned apically (Ryals and Rubel, 1982). Channels located at the tips of the hair cell stereocilia are blocked or opened with the movement of the kinocilium, which causes a depolarization or hyperpolarization within the cell. A variety of structurally defined support cells surround the hair cells and provide structural support to the hair cells and neurons (Bohne and Rabbitt, 1983).

Hearing loss affects millions of individuals in the US, with the majority of cases are attributed to hair cell loss (Beisel et al., 2008). Multiples environmental insults can cause hair cell death, such as prolonged exposure to loud sounds, some chemotherapeutic drugs, and aminoglycoside antibiotics such as neomycin and gentamicin (Cruz, 1987;

Ryals and Rubel, 1988; Oesterle and Rubel, 1993; Baird et al., 1996; Baird et al., 2000; Taylor and Forge, 2005; Sone, 1933, Cotanche, 1987; Ma, 2008). Unfortunately, once hair cells are lost in mammals, they do not regenerate. However, non-mammalian vertebrates, such as salamanders (Stone, 1933), chickens (Cotanche, 1987), and fish (Lombarte, 1993), retain the potential to replenish hair cells. It is not known what molecular signals regulate regeneration, but by studying regeneration in other model organisms, we hope to uncover the differences and reestablish hair cell regeneration in mammals.

<u>Avian Hair Cell Regeneration:</u>

Experiments conducted in avian models have elucidated many of the cellular and molecular processes involved in development and regeneration of hair cells. In the avian vestibular epithelium, there is slow continuous hair cell addition (Jorgensen and Mathiesen, 1988; Roberson et al., 1992; Kil et al., 1997) that is coupled with a constant rate of hair cell death (Kil et al., 1997; Stone et al., 1999). In comparison, the avian auditory epithelium has no new hair cell addition post-hatching (Oesterle and Rubel, 1993). Nevertheless, both epithelia have the ability to spontaneously regenerate hair cells after hair cell ablation induced by acoustic trauma (Cotanche, 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988), or exposure to aminoglycoside antibiotics, with function fully restored by 2 months (Cruz, 1987; Tucci, 1990; Girod, 1991). Theses two regenerative tissues within the same organism provide a perfect opportunity to better understand and investigate the mechanism of regeneration. It is of particular interest to

better understand the mechanism(s) facilitating steady state proliferation in normally quiescent tissues. Given that the auditory sensory epithelia in mammals is quiescent and cannot regenerate following damage, understanding the signaling cascade that differs between cyclic and quiescent sensory epithelia will be crucial for hair cell restoration in mammals (Roberson and Rubel, 1994; Forge et al., 1998).

Hair cell regeneration in the basilar papilla, the auditory epithelium of chickens, is bi-phasic: the early phase is predominately non-mitotic followed by a mitotic wave of regeneration. Non-mitotic regeneration, transdifferentiation or phenotypic conversion, occurs when cells undergo gross morphological and molecular changes to assume characteristics of a distinct cell without cell division. The non-proliferative replacement of hair cells in chicken was shown by experiments in the basilar papilla using BrdU, a nucleotide analog that marks newly synthesized DNA in cells. In these experiments, chickens were treated with BrdU following hair cell loss; the initial wave of hair cell replenishment lacked BrdU incorporation, indicating that these hair cells regenerated in a non-proliferative manner (Roberson et al., 2004; Cafaro et al., 2007). These findings were further supported when mitotic inhibitors failed to prevent hair cell regeneration in the inner ear of chickens following hair cell loss (Adler and Raphael, 1996). These results are similar to what is observed in other hair cell regenerating organisms, such as frogs (Baird et al., 1996; Baird et al., 2000) and newts (Taylor and Forge, 2005). The second phase of regeneration occurs through cell division in the support cell population that produces both hair cells and support cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Hashino and Salvi, 1993; Stone et al., 1999). Studies suggest regeneration in the vestibular system is similar to the second wave of basilar papilla regeneration and occurs

predominately through the mitotic activity of support cells (Shang et al., 2010; Weisleder and Rubel, 1993). Identifying support cell markers will better help understand cellular and molecular events occurring during different environmental conditions.

In avian models, Prox1 and Gata3 expression can distinguish some auditory and vestibular support cells, respectively (Stone et al., 2004; Warchol and Speck, 2007). Prox1 expression is increased in support cells following damage and directs support cells to hair cell fate. In contrast, Gata3 expression is maintained following damage and is expressed in both regenerating and quiescent support cells. With various cell types in close proximity, molecules that signal through cell-to-cell contacts are logical candidates for regulating tissues maintenance and regeneration. It has been demonstrated that Notch signaling is important for cell fate choices in the nervous system through lateral inhibition (Lewis, 1991; Morrison, 1999; Haddon, 1998; Adam, 1998). The role of Notch signaling was investigated under damage and undamaged conditions. In undamaged basilar papilla, support cells express Notch receptor that inhibits hair cell fate promoting genes. Inhibiting Notch activity by blocking gamma secretase activity did not cause hair cell differentiation in undamaged tissue (Stone and Rubel, 1999). In contrast, hair cell overproduction through mitotic and non-mitotic activity of support cells is observed when Notch signaling is pharmacologically inhibited following tissue damage. When Notch signaling is constitutively activated during regeneration, support cells fail to replenish lost hair cells. These results indicate that Notch signaling is important for regulating the number of hair cells.

Chapter 2: Zebrafish Lateral Line System

Lateral Line Patterning and Development:

The development of the posterior lateral line (PLL) begins with the formation of the lateral line primordium. The primordium is a mass of (~100) cells that assemble just caudal to the otic placode and migrates down to the tip of the tail along the myoseptum (Metcalfe, 1985). The primordium begins to migrate at 18 hours post fertilization (hpf) and concludes its trip at 48 hpf, depositing five clusters of around 30 cells every 6-7 somites before breaking off ventrally, leaving 2-3 clusters that become the terminal neuromast (Kimmel et al. 1995). As the placode matures, the rostral portion of the placode begins expressing both *neurod* and *neurog1*, designating those cells to a proneural fate that will later become the posterior lateral line ganglion. The remaining caudal section of the placode expresses *six1b* and develops into the posterior lateral line primordium (pLLP) (Mizoguchi et al., 2011; Sarrazin et al., 2010). From here, the pLLP begins its long journey from the head, down to the tip of the tail (Metcalfe, 1985). Guiding the pLLP down the length of the fish is the carefully controlled interplay of the chemokine Sfd1a and receptors Cxcr4b and Cxcr7b (David, et al., 2002; Haas et al., 2006; Valentin et al., 2007).

As the pLLP travels down the trunk along the horizontal myoseptum, canonical Wnt and FGF signaling interaction begins patterning different regions of the pLLP (Aman et al., 2008; Lecaudey et al., 2008; Nechiporuk & Raible, 2008). Wnt expression found in the leading zone of the pLLP stimulates proliferation and restricts FGF expression to?? the trailing zone (Aman et al., 2008; Lecaudey et al., 2008; Nechiporuk et

al., 2008). In the trailing zone, FGF signaling initiates the formation of epithelial rosettes that will be deposited and become neuromasts. In the leading zone, Wnt signaling stimulates expression of the FGF inhibitor, *sef*, while FGF expression in the trailing zone drives the expression of Wnt inhibitor, *dkk1*, thus mutually excluding each other from their respective regions. Expression of each signaling pathway is crucial for proper lateral line formation, as altering either one results in defective migration or rosette formation (Aman et al., 2008; Lecaudey et al., 2008; Nechiporuk & Raible, 2008).

Neuromasts are derived from the epithelial rosettes that are formed in the traveling pLLP. Once cells leave the leading zone, FGF signaling directs the formation of the epithelial rosettes. *Fgfr1* expression is found throughout the trailing zone, except in a few cells that express *fgf10a* which will form the center of the rosettes by inducing apical constriction of the surrounding *fgfr1* expressing cells (Lecaudey et al., 2008; Nechiporuk & Raible, 2008). As the rosettes mature, these neuromast precursors move down the trailing edge, where they are deposited to make room for newly assembled rosettes. The rate of rosette assembly and deposition is closely regulated by the rate of cell production, as decreasing cell proliferation or increasing cell death causes a reduction in the number of neuromasts deposited by the pLLP (Aman et al., 2011). Because proliferation rates are higher in the leading zone, which is largely controlled by Wnt signaling, studies looked into the role of Wnt signaling on neuromast deposition. It was found that the Wnt inhibitor, Lef1*,* regulated the proliferation rates in the leading zone, and when over expressed lead to a premature halt of pLLP migration (Gamba et al., 2010; McGraw et al., 2011; Valdivia et al., 2011). *Lef1* controls proper neuromast deposition by stimulating the expression of FGF inhibitor, Dusp6*.* Thus, by appropriately maintaining Wnt and

FGF signaling domains within the pLLP, these two inhibitors control the rate of neuromast production and deposition (Matsuda et al., 2013).

FGF signaling is also important for establishing and differentiating neuromast cells. As the neuromast begins to mature, the central *fgf10a*-expressing cells begin a feedback interaction with Atoh1a, a basic Loop-Helix-Loop transcription factor required for hair cell development (Millimaki et al., 2007). Aside from promoting continued expression of *fgf10a*, *atoh1a* also begins establishing the Notch signaling pathway by driving the expression of *deltaD* (Matsuda et al., 2010). *fgf10a*-expressing cells continue to drive the expression of Fgfr1 in the surrounding cells, which then begin upregulating *notch3* expression. The expression of *atoh1a* and *deltaD* specifies cells to become hair cells and the deltaD ligand binding to Notch3 receptor in the surrounding cells represses *atoh1a* expression, relegating them to a support cell fate (Millimaki et al., 2007; Matsuda et al., 2010). In experiments where Notch signaling is abolished, *atoh1a* expression expands to the surrounding cells, committing them to a hair cell fate, which results in a reduction in the number of support cells (Millimaki et al., 2007; Matsuda et al., 2010). Thus, as in birds and mammals, Notch-mediated lateral inhibition is needed in order to establish the appropriate number of hair cells and support cells.

Once hair cells and support cells are established within the neuromast, hair cells must begin their functional role: sensing water fluctuations in the surrounding environment. As the hair cells mature, hair cell bundles form on the apical surface of the cell, extending through the pore created by the mantle cells that form a protective layer surrounding the hair cells and support cells. These hair cell bundles are composed of short actin-based stereocilia and one long microtubule-based kinocilium. Within the

neuromast, there is an innate polarity that is established early in development by the migrating pLLP. Neuromasts that are deposited by the first migrating primordia, called primI, have anteroposterior polarity, while neuromasts deposited by second traveling primordia, primII, obtain a dorsoventral polarity (Lopez-Schier and Hudspeth, 2006; Lopez-Schier, 2004). The polarity of each respective neuromast is reflected in the position and orientation of the hair cell bundles. Half of the hair cells position their bundles towards one direction while the other hair cells establish their bundles in the opposite orientation. Hair cell depolarization is achieved when hair cell bundles are deflected towards the kinocilum, and hyperpolarization is the result of bundle deflection away from the kinocilum. This mirror symmetry ensures that neuromasts can detect water fluctuations from all directions.

It is important for neuromast function to have a stereotyped assembly and orientation of their resident hair cells. Hair cells must correctly sense the environment and appropriately relay that information to brain so that the fish can respond properly. In wild-type fish, live imaging of regenerating hair cells shows that underlying support cells divide symmetrically to give rise to two daughter hair cells. These two hair cells will orient their bundles with opposing polarity, and also rearrange their cell bodies to align with the established neuromast polarity. This mechanism ensures that the neuromasts maintain equal directional detection. Hair cell bundle organization is disrupted when planar cell polarity (PCP) molecules are altered. When the PCP genes are mutated, like in *vangl2* mutants, hair cell bundles are randomly oriented with respect to one another (Lopez-Schier and Hudspeth, 2006). Time-lapse imaging of *vangl2* mutants regenerating

hair cells, show that the hair cell precursors rearrange less frequently resulting in randomized polarity (Wibowo et al., 2011).

Neuromast hair cells are innervated by afferent neurons of the posterior lateral line ganglion (Metcalfe, 1985; Gompel et al., 2001; Raible & Kruse; 2001). The lateral line afferents project back to the hindbrain in a somatotopic manner, retaining the positional information of the neuromast they innervate (Alexandre and Ghysen, 1999; Liao et al., 2010). Each afferent neuron can innervate multiple hair cells of a single neuromast, and may also innervate multiple neuromasts. Afferent neurons are strict selectors of hair cell orientation/polarity, meaning that each afferent neuron will innervate hair cells oriented in one direction across multiple neuromasts (Faucherre et al., 2009; Nagiel et al., 2008). If an afferent neuron makes contact with a hair cell of an ‘inappropriate" polarity, it quickly severs the link to maintain the correct directional sensitivity. Neurons are also able to correctly detect hair cell polarity in newly regenerated hair cells following hair cell damage (Faucherre et al., 2009; Nagiel et al., 2008).

As the zebrafish grows, the posterior lateral line also expands and becomes more intricate. What started out as a single row of 7-9 neuromast along the trunk increases to four distinct posterior lines with an ever-increasing number of neuromasts (Lopez-Schier et al., 2004; Haehnel et al., 2012; Ledent et al., 2002; Sapede et al., 2002; Wada et al., 2010). These four posterior lines are derived from distinct traveling primordia. PrimI travels along the horizontal myoseptum, depositing the primary neuromast. Followed by primII, which travels along the same path as primI (Sapede et al., 2002). The last migrating primordium travels along the dorsal midline and is aptly name primD (Sarrazin

et al., 2010). Aside from depositing neuromasts, these migrating primordia deposit a single string of cells between neuromasts called interneuromast cells. During juvenile stages of development, the interneuromast cells begin to move ventrally, proliferating and coalescing to form a new neuromast while the primary neuromast remains in place. The ventral movement of these cells is necessary, as inhibitory cues created by glial cells associated with the lateral line nerve inhibit proliferation (Grant et al., 2005). When the glial cells are removed, either physically, or genetically, like in the *erbb2b* mutant, interneuromast cells do not move ventrally and develop neuromasts, because they develop prematurely. A study demonstrated that ErbB-expressing Schwann cells inhibit Wnt signaling, which then decreases proliferation and differentiation of precursor interneuromast cells (Lush and Piotrowksi, 2014).

Wnt signaling also plays an important role in a different mechanism of neuromast production: budding. Once the four posterior lines of single file neuromasts are established, the lateral line increases its complexity by budding new neuromasts from extant neuromasts to form collective neuromast clusters called stitches (Nunez, et al., 2009; Wada et al., 2010; Wada et al., 2008). Interplay between Wnt and the Wnt inhibitor, Dkk2, regulate the size and budding of accessory neuromasts. Wada et al., state that hair cells secrete Dkk2 protein to inhibit Wnt signaling in the surrounding support cells. When hair cells are ablated, using neomycin, *dkk2* expression is lost in mature neuromasts. Under undamaged conditions, the neuromast grows until hair cells are produced, after which proliferation occurs only at the periphery where Dkk2 concentrations are presumably low. Budding occurs in these peripheral support cells that still have Wnt activity that drives them to proliferate in order to bud off to become new

neuromasts. As new neuromast are made, the lateral line nerve innervating the founder neuromast innervates budding neuromasts. These innervating axons express R-spondin, a Wnt activator, which binds LGR receptors that stabilize Wnt signaling. In situ expression data show that neuromasts express *lgr6,* making it an ideal candidate for R-spondin-mediated Wnt maintenance (Wada et al., 2013). Not only is innervation important for the production of accessory neuromasts, it is also important for neuromast maintenance, since denervated neuromasts are lost over time (Wada et al., 2013). At the posterior end of the body, terminal neuromasts deposited early in development extend into the caudal fin as the fish grows. These eventually create four lines of neuromasts in between the caudal fin rays that collectively make up the caudal lateral line (cLL). The system is thought to be established by budding and creation of new migrating primordia (Dufourcq et al., 2006; Wada et al., 2008). Innervation is also required for their long-term maintenance of these neuromasts (Wada et al., 2013). All these studies indicate that lateral line maintenance and growth are dependent on innervation.

In addition to the ability to regenerate hair cells, the zebrafish can also regenerate entire lost neuromasts (Dufourcq, 2006). When a portion of the caudal fin is amputated, proliferation of support cells is induced in the neuromast nearest to the site of amputation. The support cells on the periphery of the neuromast form a primordium that migrates down the newly regenerated caudal fin. It should be noted that the newly generated primordium forms from cells that are separate from the blastema, a mass of undifferentiated cells that regenerates the rest of the amputated fin. Whether these cells are the same ones that replace hair cells during hair cell regeneration, or whether both forms of regeneration are governed by the same molecular signals, is unknown.

Hair Cell and Support Cell Regeneration:

Unlike the inner ear hair cells, lateral line hair cells are exposed to the environment. This exposure leaves hair cells vulnerable to physical and chemical assaults. Presumably because of this, zebrafish have the amazing ability to regenerate lateral line hair cells after hair cell loss (Lopez-Schier, 2006; Harris et al., 2003; Williams and Holder, 2000; Hernandez, 2006; Ma et al., 2008; Mackenzie and Raible, 2012; Wibowo et al., 2011) This section we will discuss the process of hair cell regeneration in more detail, including cellular and molecular mechanisms governing support cell division and hair cell differentiation. Zebrafish lateral line hair cells are structurally and functionally similar to the mammalian inner ear hair cells responsible for hearing and balance. For these reasons, zebrafish lateral line hair cells are being studied to better understand hair cell development, hair cell death and hair cell regeneration. Lateral line hair cells are susceptible to multiple ototoxins, including therapeutics such as aminoglycoside antibiotics, gentamicin and neomycin (Harris et al., 2003; Williams & Holder, 2000), heavy metal based chemotherapeautics (Ou et al., 2007; Ton and Parng, 2005), and free- floating metal ions (Fauchere et al., 2012; Hernandez, 2006; Linbo et al., 2006). In the absence of damage, lateral line hair cells are slowly lost and replaced by mitotic support cells.

Because zebrafish lateral line hair cells are on the surface of the fish, they are easily labeled using a number of different fluorescent vital dyes (DASPEI, FM-43, Acridine Orange, Cytogreen, Cytox, Yo-Pro-1). Using these dyes, researchers are able to visualize hair cells and study their behavior following various drug treatments. Some vital dyes are also useful because they are able to denote hair cell maturity, as these dyes

are only taken up when hair cells are mechanically transducing (Ma et al., 2008). Another measure of maturity is by aminoglycoside uptake. Treating zebrafish with the aminoglycoside antibiotic neomycin for one hour causes hair cell death in a dose-dependent manner. When zebrafish are placed into drug-free media, hair cell numbers are fully restored within 72 hours (Harris et al., 2003; Murakami et al., 2003; Lopez-Schier and Hudspeth, 2006; Santos et al., 2006; Ma et al., 2008). These hair cells are functional, not only by FM-43 uptake but also by their ability to orient with water flow called rheotaxis Coombs and Montgomery, 1999; Montgomery et al., 2003; Suli et al., 2012). However, not all aminoglyocoside antibiotics have the same effect on hair cell biology. Acute treatment with another aminoglycoside, gentamycin, does not cause complete hair cell death. When fish were incubated with each respective drug at the same concentration for the same period of time, the fish treated with neomycin always had significantly fewer hair cells (Owens et al., 2009; Montalbano et al., 2014). These results suggest that antibiotic-induced hair cell death is not universal and each antibiotic may be causing hair cell death via separate pathways.

Heavy metal-based chemotherapeutics and other metal ions such as copper and silver also cause lateral line hair cell death in a dose-dependent manner (Hernandez et al., 2006; Linbo et al., 2006; Olivari et al., 2008). When treated with low doses, ablated hair cells are replenished within 72 hours, similar to regeneration studies using neomycin (Mackenzie and Raible, 2012). However, when zebrafish are treated with high concentrations of copper, not only are lateral line hair cells killed, but zebrafish are no longer able to regenerate lost hair cells. The higher copper concentrations also seem to damage the underlying support cell population, which contains the hair cell precursor

population (Hernandez et al., 2007). Live imaging during regeneration (Lopez-Schier & Hudspeth, 2006; Wibowo et al., 2011; Mirkovic et al., 2012) and the use of proliferative markers such as BrdU and phospho-histone H3 have demonstrated that the majority of cell divisions occur within the first 24 hours after hair cell damage (Harris et al., 2003; Hernandez et al., 2007; Ma et al., 2008; Mackenzie and Raible, 2012; Wibowo et al., 2013).

Unlike the avian model, hair cell regeneration in zebrafish may be entirely dependent on the mitotic activity of underlying support cells. During early experiments, fish were incubated with BrdU during the varying periods of regeneration, but researchers were never able to confirm that all the new hair cells were derived from mitotic activity (Harris et al., 2003; Ma et al., 2008; Wibowo et al., 2013). As a result, this led researchers to hypothesize that some hair cells are produced through non-mitotic transdifferentiation. This regeneration occurs when a support cells goes through morphological and genetic changes to become lateral line hair cells. However, there is only one study that provides compelling evidence that hair cell addition after damage can occur by non-mitotic means (Hernandez et al., 2007). An alternative interpretation is that the observed hair cell addition might denote developmental hair cell addition as proposed by Mackenzie et al. To better understand hair cell regeneration, Wibowo et al. incubated fish in BrdU 24 hours before neomycin treatment and the following 48 hours during regeneration to label all proliferative hair cell precursors. They found that over 90% of regenerated hair cells were labeled with BrdU, further validating that hair cell regeneration is predominately through mitotic activity of support cells. To further confirm that hair cell regeneration occurs through proliferation, Wibowo et al. treated fish

with various cell cycle inhibitors following hair cell ablation and found that lateral line neuromasts were not able to replace lost hair cells. All these studies indicate that the hair cell regeneration is dependent on mitotic activity of underlying support cells.

Cell lineages in regenerating neuromasts:

So far, it is unknown whether hair cells and support cells are maintained through a specialized population of resident stem cells or all support cells have the ability to regenerate hair cells as well as self-renew. Currently, support cells are defined by a few generic characteristics: proximity to hair cells in the neuromast and gross morphology. Researchers are attempting to differentiate sub-support cell populations based on mitotic profiles during regeneration. Using BrdU as an indicator of mitotic activity, Ma et al. (2008) showed that during regeneration internal and peripheral support cells had distinct proliferation patterns, suggesting that they have different functions within the lateral line during regeneration. However, not all support cells divide and differentiate in response to hair cell loss, suggesting that some as of yet unidentified characteristic distinguishes sub-populations of support cells. The lack of markers to discriminate different support cell populations has hindered a more complete understanding of the mechanisms underlying hair cell regeneration. Of the approximately 50 support cells in a neuromast, which support cells are hair cell precursors? Where are they located? How are hair cell precursors replaced? Are they replaced symmetrically or asymmetrically?

To better understand support cell lineages and cellular contributions, live imaging during regeneration using transgenic fish showed that hair cell precursors localize to dorsal and ventral poles within the neuromast. During time-lapse imaging, Wibowo et al.

2013, demonstrated that hair cell precursors migrated to the polar compartments from an unknown location. In situ expression data show that the polar compartments are regions of low Notch activity. The authors argue that the low Notch activity is required for support cells to become hair cell precursors. It has been previously shown that Notch signaling controls the extent of hair cell addition during regeneration. As discussed above, during development hair cell precursor expression of *atoh1a* promotes the expression of deltaA and deltaA, which bind the notch3 receptor and activate a signaling cascade that inhibits expression of hair cell fate-promoting genes. In situ expression data during regeneration support this idea. Under ambient conditions, both *atoh1a* and *deltaA* are expressed in a few cells in the center of the neuromast, while *notch3* is expressed more broadly across the neuromast. Twelve hours after neomycin treatment, most hair cells are gone, expression of all three genes is upregulated, and this expression level is maintained until 48 hours post-treatment, when they return back to baseline expression (Ma et al., 2008). Inhibition of Notch signaling with the gamma-secretase inhibitor DAPT has no effect on hair cell addition in the absence of damage. When zebrafish are treated with DAPT following neomycin-induced hair cell death, there is an increase in the number of proliferative support cells that is reflected in the number of regenerated hair cells (Ma et al., 2008; Wibowo et al., 2011). It seems then that lateral inhibition through Notch signaling is an important feedback mechanism that ensures the appropriate number of hair cells return but not triggering support cell proliferation in the absence of a damage signal.

As discussed above, Wnt signaling is an important regulator of proliferation, which is crucial for correct primordia migration, neuromast deposition, and neuromast

budding (Wada et al., 2013; Harris et al. 2003). Pharmacological treatment with the Wnt activator, 1-azakenpaullone, increases support cell proliferation in the absence of damage. When hair cells are ablated using neomycin, treatment with 1-azakenpaullone increases the total number of proliferative cells as well as the number of hair cells regenerated. Inhibition of Wnt signaling by overexpression of the Wnt inhibitor, *Dkk1b,* suppresses proliferation during hair cell regeneration. This is also supported by the negative feedback loop between Wnt and Dkk activity that regulates neuromast size and support cell proliferation (Wada et al., 2013).

Antibody staining is very inconsistent in zebrafish, making it difficult to reliably differentiate cell types using common immunohistochemistry techniques. Because of this, researchers have been forced to find other means of labeling different cell types. The continual improvement of transgenic efficiency has greatly improved our ability to visualize, differentiate, and manipulate lateral line cells. Early lateral line specific transgenics were discovered through simple GFP enhancer trap screens (Parinov et al., 2004; Scott et al., 2007). Transgenic lines that label subpopulations of neuromast cells have been used to perform gene expression analysis during different conditions to identify genes important for lateral line regeneration (Jiang et al., 2014; Steiner et al., 2014). A study investigating the gene expression profile of adult zebrafish inner ear hair cells following acoustic trauma discovered that the *stat3*/*socs3a* signaling pathway may play an important role during hair cell regeneration. The authors demonstrate that phosphorylated stat3 accumulates in lateral line support cell nuclei following hair cell death. Inhibition of stat3 using pharmacological inhibitors seemed to accelerate hair cell regeneration without invoking an overproduction of hair cells (Liang et al., 2012).

Genetic and pharmacological screens are underway to identify pathways involved in lateral line development, as well as hair cell death and regeneration (Granato et al., 1996; Malicki et al.,1996; Whitfield et al., 1996; Nicolson et al., 1998; Kappler et al., 2004; Obholzer et al., 2008; Owens et al., 2008; Namdaran et al., 2012). Only one hair cell regeneration mutant has been published, *phoenix*. The mutation, in the previously uncharacterized gene produces zebrafish that have normal lateral line development, however, hair cell regeneration is severely hindered following hair cell death. Although there have not been many mutants identified, studies like these illustrate the potential of forward genetic screens in discovering unlikely and undiscovered genetic pathways influencing lateral line hair cell regeneration.

Chapter 3: Robust regeneration of adult zebrafish lateral line hair cells reflects continued precursor pool maintenance

Introduction:

The phenomenon of regeneration allows multicellular organisms to restore lost or damaged structures. The robustness and degree of such processes depend on numerous factors, including tissue location, age and species (see Rando and Wyss-Coray (2014), Sousounis et al. (2014) for reviews). Humans and other mammals have restricted regenerative ability that becomes progressively more limited with age. A few tissues in mammals can regenerate robustly well into late age and after drastic trauma (e.g. blood, skin, and intestinal epithelia). These tissues are exposed to environmental insults that cause accumulation of acute damage over time, undergo continuous cellular loss and replacement, and have dedicated progenitor cells sequestered in distinct locations (reviewed in Barker et al. (2010); Tetteh et al. (2014)). However, some cell types, like mechanosensory hair cells located in the sensory epithelia of the adult mammalian ear, show little or no regeneration after age-related and/or trauma-induced hair cell death, which leads to permanent hearing and balance disorders (Hawkins and Atha, 1976, Raphael et al., 1991; Brigande and Heller, 2009). Non-mammalian vertebrates, such as salamanders and zebrafish, have the remarkable ability to regenerate a wide range of tissues, including limbs, heart, and spinal cord (see Gemberling et al. (2013), Simona and Tanaka (2013), Fior (2014) for reviews).

Zebrafish (Danio rerio) have hair cells that are structurally and functionally similar to mammalian hair cells (Whitfield, 2002; Nicolson, 2005). In addition to hair

cells within the inner ear, zebrafish have hair cells within the lateral line system, a sensory system that detects water fluctuations with externally-located sensory organs called neuromasts. Neuromasts are composed of centrally-positioned mechanosensory hair cells surrounded by non-sensory support cells (see Thomas et al. (2015) for review). Exposure to clinical therapeutic drugs such as aminoglycoside antibiotics that induce hair cell death within the mammalian inner ear also rapidly induce hair cell death in the lateral line system (Song et al., 1995; Harris et al., 2003; Ton and Parng, 2005 and Ou et al., 2007). However, unlike their mammalian counterparts, zebrafish have retained the ability to regenerate hair cells after damage (Williams and Holder, 2000; Harris et al., 2003; López-Schier and Hudspeth, 2006; Hernández et al., 2007; Ma et al., 2008; Wibowo et al., 2011 and Pisano et al., 2014).

It is unknown to what degree zebrafish neuromasts maintain hair cells and support cells under different environmental conditions throughout the animal's lifetime. It has been previously demonstrated in larval fish lateral line that terminally differentiated hair cells regenerate from symmetric divisions of underlying support cells (López-Schier and Hudspeth, 2006; Wibowo et al., 2011) and there is evidence to suggest that larval hair cells undergo turnover (Williams and Holder, 2000). However, continuous hair cell production from symmetrically dividing support cells would eventually deplete a hair cell precursor population. Given the relatively small number of larval support cells per neuromast, current models fail to account adequately for either turnover or neuromast growth through larval development into adulthood.

Using transgenic zebrafish, we analyzed the functions of different support cell populations within the zebrafish lateral line system during hair cell regeneration. Here we

demonstrate that not only adult zebrafish regenerate hair cells following ototoxin-induced damage, but also they can regenerate hair cells after multiple iterations of damage, well into old age. We show that under ambient conditions there is a constant loss and replacement of hair cells. Fate mapping studies reveal distinct precursors within neuromasts. Our results indicate that zebrafish lateral line hair cells have a largely unlimited regenerative ability that is retained into adulthood, and that at least two different support cell populations exist to maintain tissue integrity within the lateral line system.

Results:

Adult zebrafish regenerate posterior lateral line hair cells after neomycin-induced hair cell ablation

As previous studies have mostly focused on larval zebrafish, we wanted to investigate hair cell regeneration following damage in mature adults. We examined the regenerative ability of sexually mature adult zebrafish between 6-12 months using a transgenic line Ca-tuba1a:tdTomato, which labels all mature hair cells with the red fluorescent protein tdTomato, and sqet20Et, an enhancer trap line that labels a subset of support cells with GFP and therefore serves as a convenient landmark for neuromast position when hair cells are missing (Fig. 1A). Hair cell numbers were counted within all neuromasts of the most posterior stitches of the trunk peduncle, 1 h before neomycin treatment, and at 2 h and 72 h after neomycin treatment (Fig. 1A). Following neomycin exposure, the average number of hair cells per neuromast dropped by more than 75% after 2 h but then returned to pre-treatment numbers after 72 h of recovery in drug-free media (Fig. 1B). This rate of recovery is similar to previous reports examining hair cell regeneration and maturation in larval zebrafish (Ma et al., 2008; Mackenzie and Raible, 2012). These results indicate that lateral line hair cell regeneration is not developmentally limited and it is maintained in neuromasts of adult zebrafish.

Hair cell regeneration is complete in the lateral line system of aged zebrafish

We next determined whether regenerative capacity is lost with age. We compared hair cell replacement in adult 1-year-old zebrafish and 3-year-old zebrafish using the same damage method and transgenic fish as used for the previous experiment. We found that 3-year-old fish regenerated hair cells, completely restoring hair cell numbers (Fig. 2). In contrast to younger adults, hair cell replacement took longer and was not complete until 5 days after damage compared to 3 days after damage in younger animals. Nevertheless, these observations demonstrate that senescent zebrafish are able to fully replace hair cells lost after neomycin treatment.

Hair cells regenerate after multiple iterations of damage

We investigated the regenerative robustness and maintenance of hair cells and support cells after multiple rounds of neomycin damage. The same cohort of fish was followed through 10 sequential rounds of hair cell ablation and regeneration (Fig. 3A). Similar to the single regeneration paradigm, most hair cells were lost 2 h after neomycin exposure, and subsequently replaced during the recovery period following each treatment iteration. This experiment illustrates that lateral line hair cell regeneration is responsive and robust over many repeated insults.

Hair cells in larval zebrafish are generated from symmetrically dividing support cell precursors, suggesting that their differentiation would result in depletion of hair cell precursors. We therefore assessed whether there were changes in support cell numbers

with repeated rounds of hair cell loss in adults. Adult Tg(pou4f3:gap43-GFP) zebrafish, which express GFP in hair cells, were collected, fixed, and stained with pan-nuclear stain Sytox Green to identify support cells (Ma et al., 2008). Support cells were quantified and averaged after 1, 3, 6, and 10 sequential neomycin treatments (Fig. 3B). Only after 10 treatments did we observe a small but significant difference between the average numbers of support cells per neuromast in the treated group compared to the control. All other neomycin-treated groups showed no difference when compared to their respective controls (two way-ANOVA, p-value>0.05). The preservation of support cells and hair cells following 10 neomycin treatments suggests that support cell renewal is tightly regulated during hair cell regeneration.

Continuous hair cell loss and replacement occurs in the adult zebrafish lateral line

Regenerative tissues such as skin or intestine also exhibit cellular turnover to replace old cells that may have accumulated damage over time. We wanted to establish whether zebrafish lateral line hair cells are continuously lost and replaced in adult animals. We labeled a cohort of hair cells by first treating pou4f3:gap43-GFP transgenic fish and allowing regeneration to occur in the presence of BrdU to label newly regenerated hair cells. Retention of the BrdU label was then assessed over time under ambient conditions (Fig. 4A, diagram). GFP+ and BrdU+ hair cells were quantified for all the neuromasts in the posterior peduncle stitches at 2, 6, and 12 days after BrdU incorporation (Fig. 4B, left, center, right panels, respectively). Slightly, over half of the hair cells per neuromast were positive for both GFP and BrdU 2 days post-treatment. We

found that the percentage of BrdU+ hair cells decreased following 6 and 12 days post-BrdU exposure, while at the same time the total number of hair cells remained unchanged (Fig. 4C). These results suggest that adult lateral line hair cells regenerate through mitotic divisions and are lost and replaced over time under non-traumatic conditions.

We also monitored live turnover of lateral line hair cells using the transgenic line, myo6b:NLS-Eos, which labels lateral line hair cell nuclei with the photoactivatable fluorescent protein Eos. Under normal conditions Eos fluoresces green (Fig. 5A, left panel), but when briefly exposed to 405 nm UV light, the protein undergoes an irreversible conformational change to permanently fluoresce red (Fig. 5A center panel). Eos in hair cells of the peduncle stitches of adult Tg(myo6b:NLS-Eos) fish was photoconverted to the red conformation, and persistence of red-labeled cells was monitored over time. “Old” hair cells containing elevated levels of red fluorescence were present at the time of 405 nm laser photoactivation (Fig. 5A, arrowhead right panel), and “new” hair cells that did not have elevated levels of red fluorescence were hair cells generated after the photoactivation (Fig. 5A, arrow right panel). We note that both old and new hair cells express green Eos as photoconverted cells continue to make new protein. Immediately after UV exposure, all hair cells had elevated red fluorescence compared to non-converted controls. Over time, the average number of old hair cells slowly decreased, but the loss of old hair cells was complemented by the production of new hair cells (Fig. 5B, dotted lines). There was little loss of red fluorescence in cells that remain over the time course of the experiment. This is not surprising since we have observed retention of high fluorescence levels of red nuclear-Eos persisting for months following photoconversion. This experiment indicates that neuromasts continuously lose

and replace hair cells under ambient conditions and maintain a consistent number of hair cells over time.

To investigate whether the continuous loss of hair cells reflects a cell intrinsic clock regulating lifespan, hair cells were temporally synchronized by first killing with neomycin. Newly regenerated hair cells were then marked by Eos photoconversion, and hair cell loss was then monitored over time (Fig. 5B, solid lines and dotted lines, respectively). There was no difference in the average number of old or new hair cells present 18 days post-photoactivation between synchronized and unsynchronized neuromasts, or in the rate of loss over the experimental period. These results suggest that hair cells do not have an internally calibrated schedule of senescence.

Label retaining support cells are localized at the anterior pole of neuromasts

A heterogeneous support cell population within zebrafish neuromasts could explain how hair cells are produced from symmetrically dividing support cells while retaining support cell numbers through multiple rounds of hair cell regeneration. Dedicated long-lived and self-replenishing support cell progenitors could replace and maintain hair cell precursors. A common method of differentiating mitotically distinct cell populations is through label-retention using a pulse-chase assay. We attempted to identify distinct support cell populations based on differential turnover using a transgenic fish line actb2:NLS-Eos, which expresses nuclear localized Eos in all support cells. Caudal fin neuromasts were used for this experiment, because phalloidin labeling showed that the polarities of the hair cells of these neuromasts are all oriented along the anterior–

posterior axis (IAC, unpublished results). We expected after multiple rounds of induced regeneration that multiple cell divisions would dilute the red Eos present in dividing support cells that give rise to hair cells, while support cells that did not divide or that divided less frequently would retain higher levels of the red Eos protein. To have a common frame of reference, individual neuromast shapes were warped and normalized in order to aggregate data of all neuromasts together to a single standardized model (see Supplemental text).

Eos was photoconverted in all support cells of neuromasts in the adult zebrafish caudal fin, and fish were then subjected to 5 sequential neomycin treatments separated by recovery periods to allow hair cells to regenerate. We found a large primary population of label-retaining support cells at the anterior end of the neuromast, and more variably a smaller secondary population was located at the posterior end of neuromasts. The dorsal and ventral regions of the neuromast had greatly reduced red Eos fluorescence (Fig. 6A and B). A kernel quantile regression model was performed on the aggregated set to test for significant differences in distribution (Fig. 6C; see Supplemental text for details). Using this analysis, we find distinct support cell populations within neuromasts are identified by label retention. More specifically, our analysis suggests that slowly dividing populations of support cells exist at the anterior–posterior poles of each neuromast, while support cells in other regions divide more rapidly.

Hair cell precursors are differentially distributed across the neuromast

Label retention studies suggest that support cells in different regions of the neuromast have distinct turnover characteristics following multiple rounds of regeneration. We next tested whether support cells in these regions had differential abilities to give rise to new hair cells after a single round of damage. Tg(actb2:NLS-Eos) fish were crossed with Tg(pou4f3:gap43-GFP) fish to make double transgenic animals we could use to label cell nuclei within neuromasts with Eos photoconversion and identify hair cells with membrane-bound GFP. We photoactivated Eos in approximately 6–8 support cells located in anterior, dorsal, posterior, and ventral quadrants of adult fin ray neuromasts following a single neomycin treatment, and then assessed regenerated hair cells for label after 72 h (Fig. 7A and B). As can be seen in Fig. 7B, we detected hair cells (denoted by GFP+ membrane) with photoconverted nuclei (arrowheads). We measured the red fluorescence of regenerated hair cells and compared it to red fluorescence in the surrounding activated support cells and found that it was significantly dimmer (Fig. 7C), consistent with the idea that hair cells are derived from dividing precursors and that other support cell divisions are less common. We found no significant difference in the ratio of fluorescence of hair cells to support cells when different quadrants were labeled.

We measured the fraction of total regenerated hair cells that were labeled with photoconverted Eos from each labeled quadrant (Table 1). We found that the chance that support cells produced new hairs cells was equivalent from dorsal, posterior, and ventrally positioned support cells but much less frequent from the anterior quadrant (chi-

square test, p=0.0002). We also found that cells photoconverted in the anterior quadrant were less likely to give rise to any hair cells (chi-square test, p=0.03). These results demonstrate that there are fewer dividing hair cell precursors in the anterior quadrant, a pattern complementary to the distribution of label-retaining compartments in the neuromast.

Discussion**:**

Regeneration of zebrafish lateral line hair cells in larval animals is well established, with dramatic hair cell loss after ototoxin exposure followed by complete hair cell regeneration from proliferating precursors (Williams and Holder, 2000 and Harris et al., 2003; Lopez-Schier and Hudspeth, 2006; Hernández et al., 2007; Ma et al., 2008; Wibowo et al., 2011; Mackenzie and Raible, 2012). At these stages, neuromasts have largely completed developmental addition of hair cells and are functionally mature. However, the zebrafish lateral line system undergoes considerable growth after larval stages, with addition of new neuromasts from multiple sources (Nuñez et al., 2009; Wada et al., 2013), and comparable dramatic changes occur throughout many tissues as fish reach adult stages (Parichy et al., 2009). We therefore tested whether hair cell regeneration was possible after growth of the fish was largely complete. We find that adult zebrafish lateral line neuromasts are highly responsive sensory organs that quickly and robustly replace hair cells following aminoglycoside-induced damage, as well as continuously replenishing hair cells undergoing turnover. These results show that adult regeneration is similar to regeneration of lateral line hair cells in larval zebrafish (Williams and Holder, 2000 and Harris et al., 2003; Lopez-Schier and Hudspeth, 2006; Hernández et al., 2007; Ma et al., 2008; Wibowo et al., 2011; Mackenzie and Raible, 2012). They complement and extend a recent report examining hair cell recovery in adult zebrafish after gentamicin treatment (Pisano et al., 2014).

Hair cell regeneration continues throughout the life of the zebrafish. In aged animals, we found that hair cell regeneration was complete although somewhat delayed.

Zebrafish have the capacity to regenerate many different adult tissues, including fins, heart, nerve, retina, spinal cord and brain (reviewed in Gemberling et al. (2013)). In some tissues regenerative capacity remains robust while in others it declines with age (Itou et al., 2012, Edelmann et al., 2013; Graciarena et al., 2014). Whether there are mechanistic differences in the replacement of lateral line hair cells at different developmental stages is unknown.

Tissues that show a robust regenerative response to damage often exhibit continuous turnover and replacement of cells. Previous reports showed expression of proliferative and cell death markers within larval neuromasts under ambient conditions, consistent with hair cell turnover (Williams and Holder, 2000). However dyes such as acridine orange used to detect cell death are actively taken up by living lateral line hair cells (Santos et al., 2006) making them an unreliable measure of turnover. Using a myo6b:NLS-Eos transgenic line, we observed that in adult zebrafish hair cells undergo continuous homeostatic hair cell death and replacement. This transgenic line allowed us to follow individual neuromasts longitudinally over time without perturbing normal hair cell function or sacrificing the animal for analysis. A potential caveat to this approach is interpreting the loss of photoconverted nuclei as hair cell turnover rather than asymmetric degradation of the red Eos protein within different hair cells of labeled neuromasts. This latter option appears unlikely, since we observed similar kinetics of hair cell turnover and replacement using a conventional BrdU pulse-chase paradigm. We and others detect little change in the high fluorescence levels of red Eos months after photoactivation in other cell types (our unpublished observations; McMenamin et al., 2014). It is possible that the

nuclear localization of the NLS-Eos protein protects it from degradation, which may make it amendable to other long-term cell labeling experiments.

The stability of photoconverted NLS-Eos allowed us to conduct a modified label-retention assay, often used to identify slow cycling progenitor populations. It shares some advantages with methods using labeled histones (Tumbar et al., 2004; Foudi et al., 2009) as it does not require BrdU incorporation during S-phase. We found that support cells in the anterior pole of caudal fin neuromasts, and to some degree in the posterior pole, retained higher levels of activated Eos after many rounds of induced proliferation from hair cell damage and regeneration. We note that we have identified these label-retaining compartments within the neuromasts of the caudal fin, where hair cells are polarized along the anterior–posterior axis. It would be interesting to know whether the location of the label-retaining compartments always corresponds with neuromast hair cell polarity, for example whether the compartment is rotated in neuromasts whose hair cells have dorsoventral polarity. Rapidly-dividing hair cell precursors have been described previously to divide in polarized compartments that are perpendicular with respect to hair cell polarization (Wibowo et al., 2011; Mirkovic et al., 2012), consistent with our observations that these compartments are less likely to retain label. We had previously noted that peripheral supports cells labeled with BrdU at a lower frequency and with different kinetics than more centrally-located cells but did not note orientation with respect to neuromast polarity (Ma et al., 2008). These results are consistent with our observations that label-retaining cells are more prevalent at peripheral locations.

Although Eos lineage tracing is a useful tool in marking and tracking cells, it is not without its limitations. First, the amount of Eos protein that can be photoconverted in

each cell at a given time is finite; this limits the number of cell divisions through which progeny can be tracked, given that each cell division reduces the fluorescence by at least half. We are not able to detect red fluorescence above background after any more than two cell divisions. Another problem arises with the density and sizes of cells within a given tissue. Eos is not photoconvertible with two-photon microscopy using current schemes. If the cells are small and tightly packed, such as it is within the neuromasts, labeling single cells is extremely difficult and usually is not achieved without inadvertently activating Eos in other cells due to light scatter.

Time-lapse studies of hair cell regeneration in larval zebrafish show that pairs of hair cells are produced from symmetric divisions of underlying support cells in a mechanism proposed to maintain hair cell polarity (López-Schier and Hudspeth, 2006; Wibowo et al., 2011;Mirkovic et al., 2012). These results suggest that continual hair cell production would eventually deplete hair cell precursors if no system were present to restore them. Both our Eos photoactivation cell-lineage assay and BrdU incorporation studies support the idea that regenerating adult lateral line hair cells are also derived from division of underlying support cells. We also did not observe an overall depletion of support cells after multiple hair cell regeneration cycles, indicating that support cells that act as hair cell precursors are replaced.

While the source of replacement hair cell precursors remains unresolved, we speculate that these cells may come from the slower-dividing label-retaining populations that predominate in the anterior quadrant of neuromasts. Preliminary Eos lineage tracing experiments suggest that some cells move from this zone towards the center where they can act as hair cell precursors (IAC, unpublished results). However, the limits of Eos

protein detection through multiple rounds of division (noted above) prevent a systematic analysis of cell lineage to the level needed to definitively identify the source(s) of progenitors. While previous analysis of regeneration in larval zebrafish demonstrates pairs of hair cells arise from symmetrically dividing precursors, it is possible that hair cells are also produced by asymmetric divisions in adults. Hair cell precursors themselves may also be generated by symmetric or asymmetric divisions. We cannot delineate whether support cells are dividing symmetrically or asymmetrically using the label-retaining assay; we can only conclude that label-retaining cells divide less frequently. Cell lineage and extended time-lapse studies, perhaps with indelible lineage tracing techniques using Cre-based methods, need to be conducted to distinguish amongst these possibilities.

Mammalian tissues, like the skin, blood, and intestine that have continuous cellular turnover (reviewed in Fliedner (1998), Fuchs (2009), Hsu et al. (2014), Tan and Barker (2014), and Clevers et al. (2014)) and high regenerative capability have been shown to have resident stem cell populations in distinct stem cell niches (see Barker et al. (2010), Tan and Barker (2014), Clevers et al. (2014), and Rezza et al. (2014) for reviews). It is thought that these secluded regions provide a regulated microenvironment that maintains stem cells in a more pluripotent or quiescent state. We speculate that the label-retaining population within the neuromast may reside within a distinct niche. Label-retaining support cells are found on the periphery and adjacent to interneuromast cell populations that act as a source of de novo neuromast generation (Grant et al., 2005). A possible function of these adult caudal fin interneuromast cells is to provide instructive cues to adjacent support cells to maintain some support cells in a more quiescent state, or

function as a hub to give support cells cellular/tissue polarity. Performing laser ablation experiments will help better understanding their role in neuromast maintenance.

During postembryonic stages, Wnt signaling instruct neuromasts to become organized into stitches with new neuromasts formed from cells migrating from the edge of existing neuromasts (Wada et al., 2013). In addition, during fin regeneration new fin ray lines form from a new primordium generated from the last remaining neuromast adjacent to the amputation site (Dufourcq et al., 2006). It is tempting to speculate that these new neuromasts arise from the same peripheral support cell pool that might repopulate hair cell precursors after turnover or regeneration. Besides Wnt, other regulators of larval neuromast maintenance have begun to be elucidated, including Notch and Jak-Stat signals (Ma et al., 2008, Wibowo et al., 2011, Liang et al., 2012, Head et al., 2013, Wada et al., 2013, Jacques et al., 2014, Jiang et al., 2014; Steiner et al., 2014). Whether these signals continue to be used during adult stages remains to be determined.

Sensory hair cell regeneration occurs in all vertebrates examined except mammals (see Brignull et al. (2009) for review). In the inner ear, hair cells are regenerated from symmetric (Raphael, 1992, Tsue et al., 1994; Stone and Rubel, 2000) and asymmetric (Roberson et al., 1992; Stone et al., 1999; Stone and Rubel, 2000) support cell divisions, as well as through direct-phenotypic conversions (Jones and Corwin, 1996; Adler and Raphael, 1996; Baird et al., 1996 and Baird et al., 2000; Roberson et al., 2004; Taylor and Forge, 2005; Duncan et al., 2006; Cafaro et al., 2007). By contrast, regeneration in the lateral line of zebrafish larvae is largely through proliferation of precursors (Wibowo et al., 2011 and Mackenzie and Raible, 2012). Like the lateral line system, the avian vestibular epithelium also shows continuous hair cell turnover (Jørgensen and Mathiesen,

1988, Roberson et al., 1992 and Kil et al., 1997). It may be that zebrafish lateral line hair cell regeneration is simply redirecting mechanisms present for ongoing hair cell production that are greatly accelerated during massive hair cell loss due to trauma. Regardless of the mechanisms underlying replacement of lost hair cells, a system must exist to replenish support cells that are used to regenerate hair cells. To what degree mechanisms of hair cell regeneration are conserved across species remains to be determined.

Regeneration is a necessary and fundamental biological process that varies greatly amongst species. Mammals have a very restricted ability to regenerate when compared to cold-blooded vertebrates. The ability to restore damaged or lost tissue also decreases over the lifespan of most animals. It is encouraging to see that not only can zebrafish regenerate lateral line hair cells after multiple hair cell ablations, but also that they can regenerate hair cells as adults. Our results indicate that there are at least two distinct support cell populations within lateral line neuromasts. One population of support cells promptly gives rise to lateral line hair cells following hair cell ablation, while the other is a label retaining support cell population that is less mitotically active. Understanding the different environmental cues zebrafish support cells are exposed to during hair cell regeneration and hair cell maintenance may prove a crucial step in decoding and promoting hair cell and support cell regeneration in humans.

Materials and methods

Zebrafish strains and maintenance

Adult zebrafish were maintained at 28.5 °C. sqet20Et was a gift from Korzh (Parinov et al., 2004).Tg(pou4f3:gap43-GFP) was a gift from Baier and previously described in Xiao et al. (2005). To generate Tg(Ca-tuba1a:tdTomato), a Carassius auratus alpha-1-tubulin fragment containing 1696 bp of 5' flanking sequence (Goldman et al., 2001) was fused in frame to the tdTomato sequence, flanked by two I-SceI meganuclease recognition sites in the pBluescript vector. Linearized DNA containing this construct was co-injected with the I-SceI meganuclease enzyme into one-cell stage zebrafish embryos (Thermes et al., 2002). The myo6b promoter was previously described in Obholzer et al. (2008). Tg(actb2:NLS-Eos) fish were created using the Gateway system as previously described in (Kwan et al., 2007). All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Neomycin

Neomycin sulfate (Sigma, St. Louis, MO) was diluted in E3 embryo media (14.97 mM NaCl, 500 μM KCl, 42 μM Na2HPO4, 150 μM KH2PO4, 1 mM CaCl2 dehydrate, 1 mM MgSO4, and 0.714 mM NaHCO3) to a final concentration of 400 μM. All adult zebrafish hair cell ablations were carried out in 400 μM neomycin for 1 h followed by a 1 h recovery period in E3 media at 28.5 °C. Hair cells were scored before neomycin, 2 and

72 h after treatment, and then repeated as indicated in the experiment. Sibling fish were used as controls and were mock-treated by handling and relocating to drug-free media.

Adult zebrafish hair cell and support cell labeling

Adult zebrafish immunohistochemistry was performed as described in Ma et al. (2008).Tg(pou4f3:gap43-GFP) hair cells were stained using rabbit anti-GFP primary antibodies and Alexa 488-conjugated anti-rabbit secondary antibody (1:500; Molecular Probes). Support cells were visualized using the pan-nuclear stain SYTOX green (1:10,000; Molecular Probes).

Live hair cell regeneration counts were conducted in adult zebrafish submerged in E3 embryo media containing 0.2% buffered MESAB (MS-222; ethyl-m-aminobenzoate methanesulphonate) under epifluorescence using a 63× water objective on a Zeiss Axioplan 2 microscope. Support cell confocal images were taken on a Zeiss LSM 5 Pa confocal microscope. Hair cell and support cell counts were recorded from all the neuromasts of the caudal peduncle stitch, with a minimum of 9 neuromasts per fish and 4 fish per treatment. Whole images were processed using the Pascal software, ImageJ, and Adobe Photoshop CS4.

Adult zebrafish mitotic cell labeling

Adult zebrafish were incubated in 10 mM of bromodeoxyuridine (BrdU; Sigma) in E3 embryo media containing 1% dimethyl sulfoxide (DMSO) 12 h after neomycin treatment for 24 h and then fixed and stained 48 h, 6 days, and 12 days post-BrdU incubation. BrdU immunohistochemistry was performed as described in Ma et al. (2008): hair cells were labeled with mouse anti-parvalbumin primary antibodies and Alexa 488-conjugated anti-mouse secondary antibodies (1:500; Molecular Probes), and BrdU+ cells were labeled with rat anti-BrdU primary antibodies and Alexa 568-conjugated anti-rat secondary antibodies (1:200; Molecular Probes).

Graphs were generated and statistical analysis were conducted using Graphpad Prism.

Eos photoactivation and imaging

Adult zebrafish were anesthetized and placed into 6-mL chambers with the caudal fin stabilized with a sponge. Chambers were placed on an inverted Axio Observer D1 spinning disk system (Intelligent Imaging Innovations) equipped with an Evolve 10 MHz EMCCD camera (Photometrics) and a Zeiss C-Apochromat 63×/1.2 NA water objective. Camera intensification was set to 500, and 488 nm and 561 nm exposure times were adjusted to maintain maximum intensities below fluorescence saturation.

Photoactivation of green Eos protein was performed on Tg(myo6b:NLS-Eos) and double transgenic Tg(bactin2:NLS-Eos;pou4f3:gap43-GFP) fish by targeting a 405 nm laser through the 63× objective for three 10 ms pulse exposures. Nuclei in neuromasts are

easily distinguished from surrounding epidermal tissue by their smaller size and circumferential orientation.

Red Eos fluorescence analysis

For hair cell turnover, thresholding was established by creating Z-stack images of all photoconverted neuromasts and three non-photoconverted neuromasts for each fish. Maximum intensity projections were created for photoactivated and non-photoactivated neuromast Z-stacks. To determine the lower threshold for hair cell Eos activation, the basal red intensity levels and the standard deviations of the three non-photoconverted neuromasts were measured, averaged, and added with 2× the averaged standard deviations, as follows: (avg. red fluorescence from 3 non-activated neuromasts)+2(avg. s.d. red fluorescence from non-activated neuromasts)=lower threshold for Eos activation. Slidebook software was used for image analysis and Adobe Photoshop CS4 was used for figure production.

For label retention experiments, the position and red fluorescence of all nuclei of activated fin neuromasts were measured after 6 sequential neomycin treatments using ImageJ cell counter software. To compare all neuromasts to each other, neuromasts were aligned along the anterior–posterior axis and this diameter length was set to 1. All Cartesian coordinates were measured relative to this axis and then converted to polar coordinates. The red fluorescence was normalized to the cell with the highest red fluorescence intensity in each neuromast. 23 Neuromasts were aligned and stacked on top

of each other to get a distribution frequency. The median fluorescence regression diagram was created as described in the Supplemental text (Figs. S1–S4).

For the cell lineage tracing experiments, regions of interest were created using Slidebook software to selectively photoactivate Eos in subsets of support cells in predetermined regions of caudal fin neuromasts. Fish were treated with neomycin to ablate hair cells and induce support cell divisions. The red and green fluorescence levels were measured for all of the hair cells of activated neuromasts. The red-to-green fluorescence ratio was calculated for every hair cell and then plotted as a frequency histogram. Cells that had a ratio of 0.8 or higher were considered hair cells with activated Eos.

Figures:

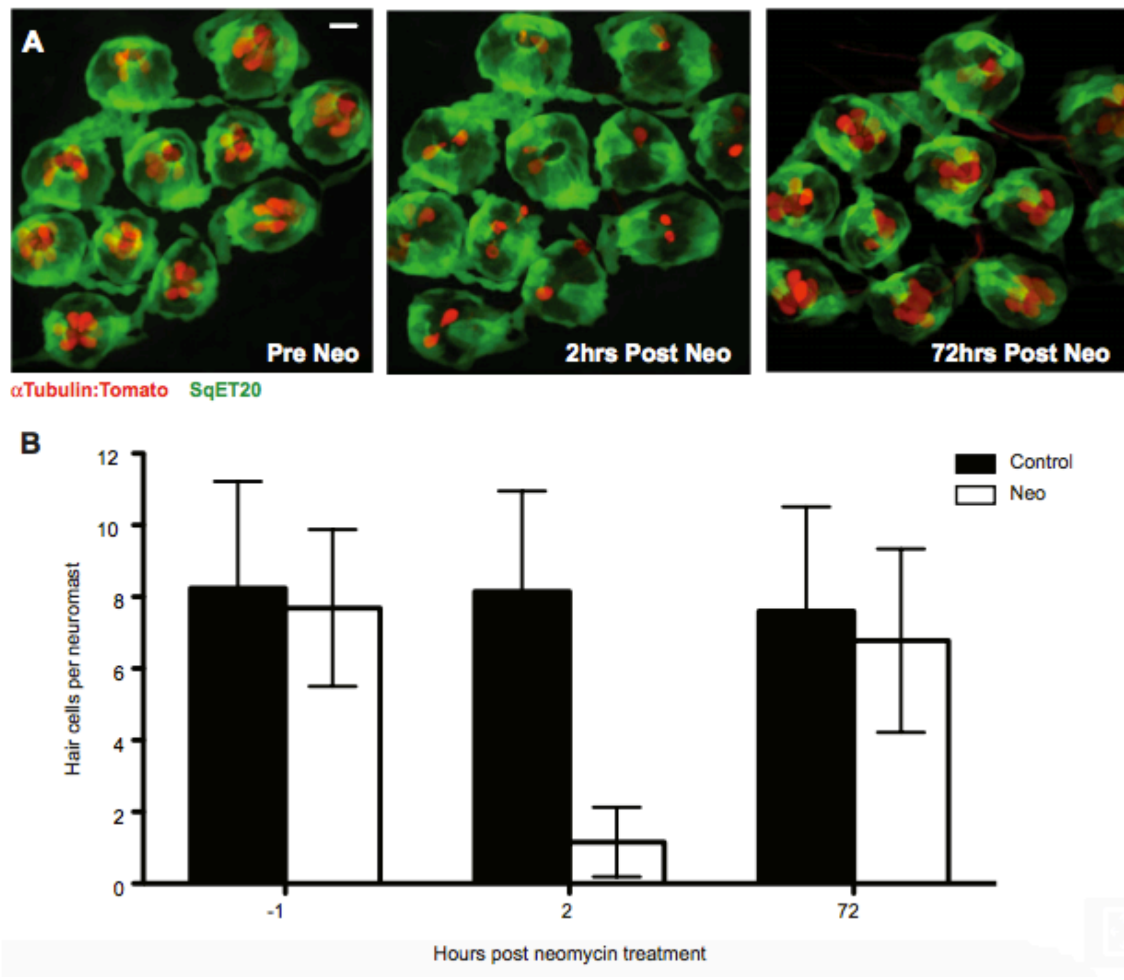

Figure 1. **Adult zebrafish lateral line hair cell regeneration after neomycin-induced ablation**. Confocal z-stack maximum projection images of a peduncle stitch (11 neuromasts) of an adult Tg(ET20;tuba:tdTomato) zebrafish with hair cells labeled with tdTomato (red) and a subset of support cells labeled with GFP (green). (**A**) Adult zebrafish peduncle neuromasts (left panel) were treated with 400 µM neomycin for 1 hour (middle panel) and then allowed to recover for 72 hours to assess hair cell regeneration (right panel) (**B**) Results are graphed as average number of hair cells per neuromast (± s.d.) for each treatment group. N= 4 adult zebrafish per group, 40+ neuromasts per group. Scale bar, 10 µM

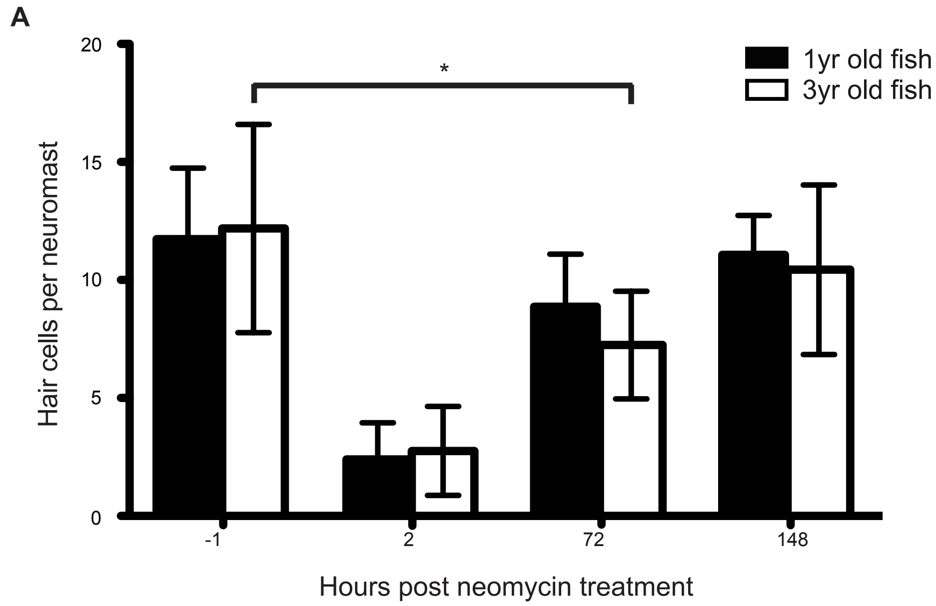

Figure 2. Lateral line hair cell regeneration of senescent adult zebrafish following hair cell death. 1-year-old and 3-year-old old zebrafish were treated with 400 µM neomycin for 1 hour and then allowed to recover for 72 hours. The results are graphed as average number of hair cells per neuromast (± s.d.) for each treatment group. N= 3 adult zebrafish per group, 20+ neuromasts per group; $p < 0.001$(ANOVA)

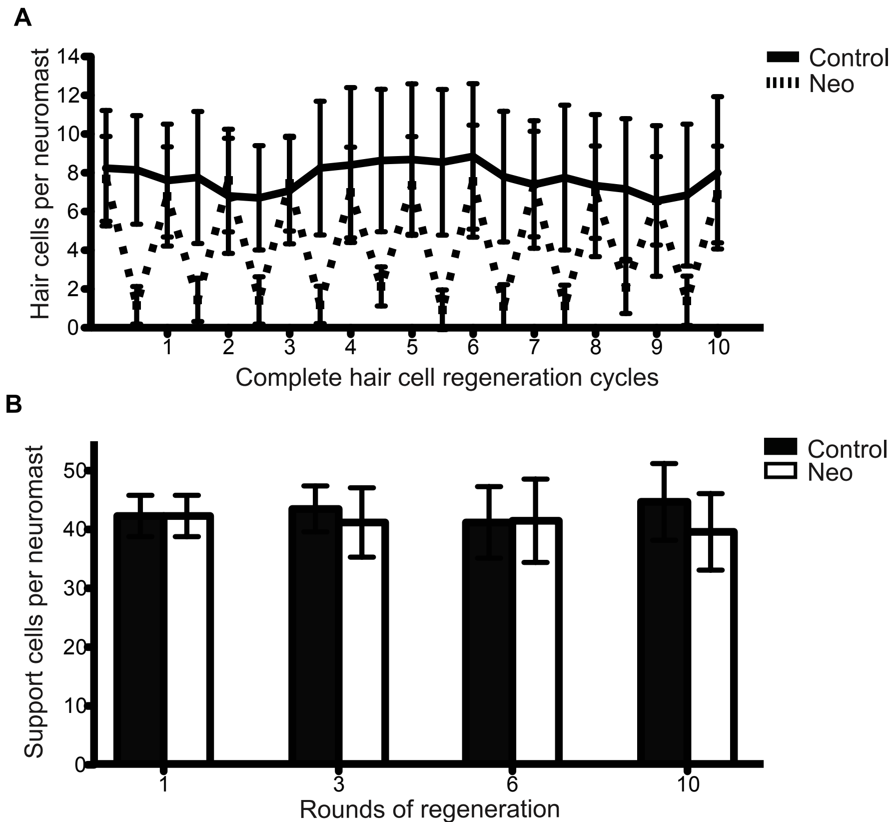

Figure 3. Hair cell and support cell regeneration in adult zebrafish after multiple sequential neomycin treatments. (**A**) Adult Tg(ET20;tuba:tdTomato) zebrafish were treated with 400 µM neomycin and allowed to recover for 10 sequential neomycin-induced hair cell destruction/regeneration cycles. Results are graphed as average number of hair cells per neuromast (± s.d.) for each treatment group. N= 4 adult zebrafish per group, 40+ neuromasts per group. (**B**) Support cell averages were quantified from adult Tg(*brn3c*:GFP) zebrafish that were neomycin treated 1, 3, 6, and 10 sequential times, euthanized, and stained with pan-nuclear stain Sytox (green) after each indicated neomycin treatment. Results were graphed as support cell averages per neuromast (±s.d.) for each treatment group. N= 3-4 adult zebrafish per group, 15+ neuromasts per group.

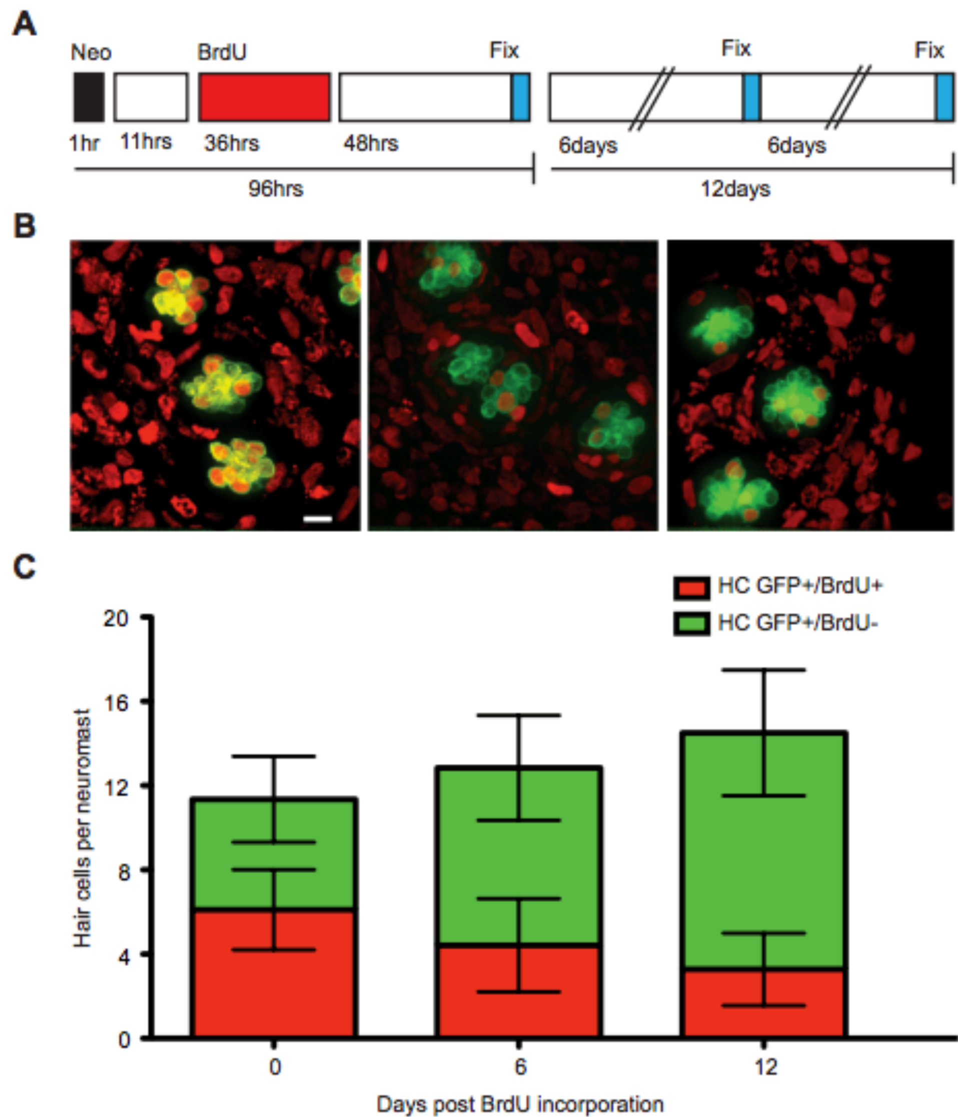

Figure 4. In adult zebrafish, BrdU labeled lateral line hair cells are lost over time under ambient conditions. (**A**) Schematic illustrating the experimental protocol: fish were treated with 400 µM neomycin for 1 hour, then allowed to recover for 11 hours, followed by a 36 hour 5 mM BrdU incubation period, and finally fixed and stained at the indicated time-points. (**B**)**.** Representative confocal z-stack maximum intensity projection images of neuromasts with hair cells labeled green with GFP and BrdU positive cell

nuclei marked red. BrdU+ hair cells 0 days (left panel), 6 days (middle panel), and 12 days (right panel) after hair cell regeneration. Scale bar, 10 μM (**C**) Graph illustrating stacked averages (±s.d.) of GFP+ only hair cells (green) and GFP+ hair cells colabeled with BrdU (red). N= 2-3 adult zebrafish per condition, 30+ neuromasts per group.

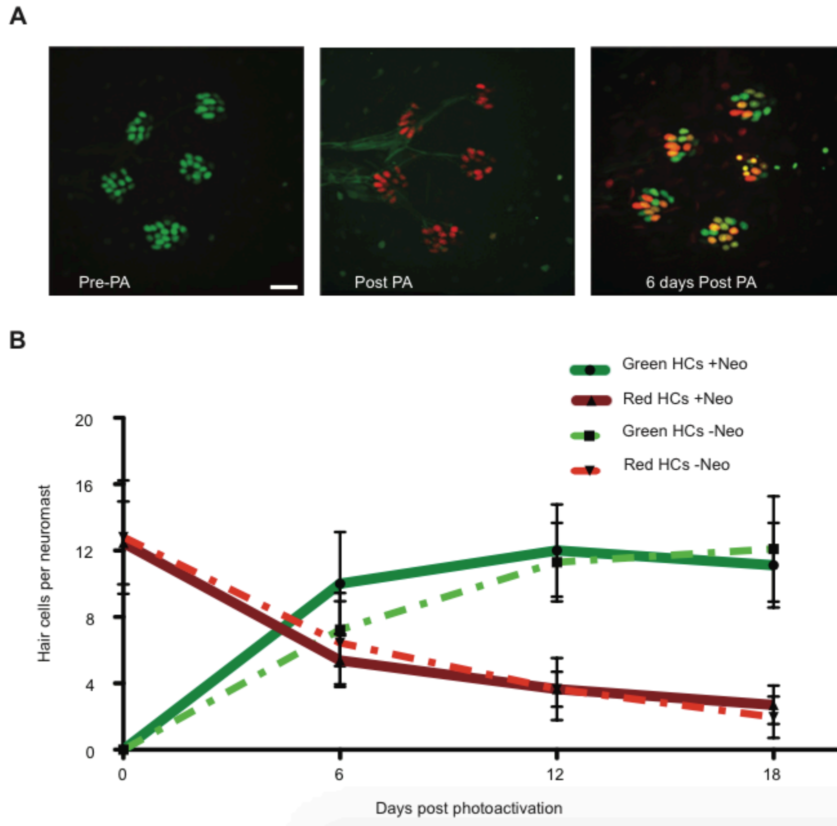

Figure 5. Hair cells are continuously lost and replaced in adult zebrafish lateral line. (**A**) Representative z-stack confocal maximum projection images of an adult Tg(*myosinVI*:nls-Eos) peduncle stitch (5 neuromasts shown) before photo-activation, or PA (left panel), immediately after PA (middle panel), and 6 days after PA (right panel). Because Eos is under the control of the MyosinVI promoter, green Eos is continuously produced therefore hair cells that were present at the time of photo-activation will have both green and red Eos present producing orange/yellow colored nuclei. Scale bar, 10 µM. (**B**) Fish were treated without or with neomycin to synchronize hair cell age through hair cell regeneration; then nuclear localized Eos was laser activated in all hair cells and followed over time. The results are graphed for the two pulse-chase hair cell turnover experiments, one without hair cell age synchronization and the other with. Dotted lines

represent averages per neuromast (±s.d.) of hair cells that were present at the time of Eos protein activation (red) or hair cells generated post-Eos laser activation (green) without neomycin treatment. N= 3 adult zebrafish, 20 neuromasts. Solid lines represent averages per neuromast (±s.d.) of hair cells that were present at the time of Eos protein activation (red) or hair cells generated post-Eos activation (green) with neomycin-induced hair cell age synchronization. N= 3 adult zebrafish, 17 neuromasts.

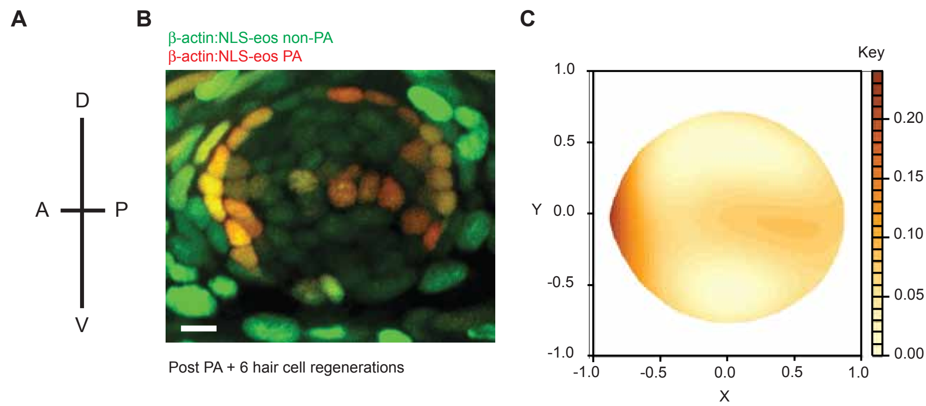

Figure 6. Label retaining support cells are localized to the anterior region of caudal fin neuromasts. (**A**) Schematic illustrating the orientation of neuromasts in the caudal fin that were analyzed; applies to panels B and C (**B**) Representative z-stack confocal maximum projection image of an adult Tg(*actb1*:nls-Eos) neuromast following entire neuromast Eos activation and 6 sequential hair cell regeneration cycles. Scale bar, 10 μM (**C**) Diagram illustrating the distribution of label retaining cells using the red fluorescence intensity smooth median regression of 23 overlaid neuromasts. N= 4 adult zebrafish.

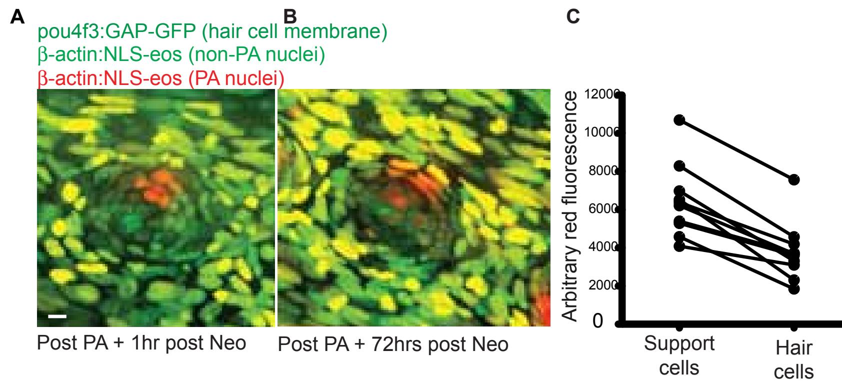

Figure 7. Selective support cell Eos activation shows non-equitable hair cell contribution during hair cell regeneration. (**A**) Selective support cells in adult *Tg(pou4f3:gap43-GFP;actb2:NLS-Eos)* caudal neuromasts were activated after neomycin treatment. Arrow indicates surviving hair cell without red nuclei. (**B**) Hair cell production was traced from labeled support cells during hair cell regeneration. Arrowhead illustrates hair cell with red nuclei produced from support cell with activated red Eos while the arrow illustrates a hair cell with no activated Eos protein. Scale bar, 10 µm. (**C**) The average red fluorescence of locally activated support cells and regenerated hair cells of 10 different neuromasts following hair cell regeneratio

Table 1.

Support cells in different quadrants have distinct capacity to generate new hair cells.					
Quadrant	Labeled HC	Total HC	Total HC (%)	N	With HC (%)
Anterior	15	139	10.7	15	53.3
Dorsal	35	123	28.5	14	85.7
Posterior	61	229	26.6	25	80
Ventral	68	225	30.2	24	91.7

N represents the total number of neuromasts photoconverted. Labeled and total hair cells (HC) are combined across all neuromasts analyzed

Chapter 4: Delineate Support Cell Regeneration and Maintenance.

Introduction:

Adult tissues exposed to the environment are constantly bombarded by external abuse. Accumulation of acute insults overtime can severely damage or destroy cells, leading to non-functional tissues and organs. In order to preserve healthy tissues, old and damaged cells are replenished through constant low-level proliferation and continuous programmed cell death. Aside from replacing lost cells with low levels of turnover, organisms must have ability to detect and appropriately respond to lost tissues/cells caused by more severe trauma.

Cyclic and regenerating tissues are usually maintained by a stem cell population that has the ability to self-renew as well as produce the various terminally differentiated cell types. As of yet, there is not a universal mechanism delineating how stem cells are maintained throughout an organism's life in their undifferentiated state. The local microenvironment, or stem cell niche where the stem cell resides is thought to produce signaling cues that interact with stem cells to regulate cell fate.

In some tissues, stem cells are thought to be long-lived and dedicated, remaining in an undifferentiated state by directing invariant asymmetric division that promotes self renewal as well as producing progenitor cells that differentiate to maintain tissue homeostasis (Cairns, 1975; Cotsarelis et al., 1990; Lajtha, 1979; Potten and Loeffler, 1990). Recent lineage tracing studies and clonal expansion analysis have revealed evidence that stem cells can be maintained through population stem cell renewal in a stochastic manner, wherein, on average half of the stem cell population differentiates to

perform tissue maintenance, while the other half of stem cells self-renews (Morrison and Kimble, 2006; Watt and Hogan, 2000; also for review Klein and Simons, 2011). Little is known about the mechanism that maintains stochastic stem cell fate in cyclic/proliferative tissues.

The zebrafish is an excellent model to study adult tissue homeostasis, as well as tissue regeneration following traumatic damage. The lateral line system is especially amenable to tissue maintenance and regeneration studies. As an externally located sensory system, the lateral line is easily visualized and imaged, and is accessible to drug/chemical treatment at all stages of development. The sensory units, called neuromasts, have centrally positioned mechanosensory hair cells that are surrounded by non-sensory support cells. Lateral line hair cells are structurally and functionally similar to the mammalian hair cells found in the inner ear, which are responsible for hearing and balance. However, unlike their mammalian counterparts, zebrafish have the amazing ability to replenish hair cells that are lost through naturally occurring turnover, or hair cells lost due to sudden trauma, as seen after exposure to ototoxins such as the aminoglycoside antibiotic neomycin (Williams and Holder, 2000; Harris et al., 2003; Lopez-Schier and Hudspeth, 2006; Hernandez et al., 2007; Ma et al., 2008, Wibowo et al., 2011; Mackenzie and Raible, 2012). Previous studies have demonstrated that hair cells are replenished by symmetric divisions of underlying support cells under ambient conditions and after trauma (Lopez-Schier and Hudspeth, 2006; Wibowo, 2011). Currently, it is unknown how support cells are maintained under different environmental conditions.

Recent technological advances have provided researchers with new tools to permanently and differentially label support cell populations of the zebrafish lateral line (Pan et al., 2013). The 'zebrabow' is a transgenic fish that uses Cre recombinase to randomly induce the expression of spectrally distinct fluorescent proteins in adjacent cells throughout the fish. Because this is achieved at the genomic level, each individual cell is permanently labeled, as well as all of its progeny. Using a heatshock inducible Cre transgenic line, *Tg(hsp:Cre),* crossed to *Tg(Zebrabow-M)*, we are able to temporally control the recombination event to perform lineage tracing and fate mapping during different stages of development. The additional ability to activate Cre-ER^{T2} in selective support cell populations using light-uncageable tamoxifen variants gives researchers more precision and control while studying tissue dynamics under various physiological conditions.

Using the zebrabow transgenic fish, we began to better differentiate the function and roles of different support cell populations in the zebrafish lateral line. We performed long term multicolor clonal analysis from larval to adult zebrafish and found that, under normal conditions, primary neuromasts as well as interneuromast cell-derived secondary neuromasts expand towards clonality, becoming mostly monochromatic 9 months after Cre induction. During repeated induced hair cell regenerations, support cell color clones fluctuate slightly in size but did not drastically expand as observed under ambient conditions. These results suggest that support cells have the ability to detect different environmental stimuli and respond appropriately to maintain proper hair cell and support cell numbers.

Results:

Multicolor neuromasts drift towards monoclonality under ambient conditions.

To better understand how neuromasts are maintained throughout the lifetime of zebrafish, we performed long-term cross-sectional clonal analysis using the double transgenic *Tg(ubi:Zebrabow-M;hsp:Cre)*. Neuromasts are structurally and functionally established by 5 days post fertilization (dpf), so we began our analysis from 5 dpf and continued to follow the neuromasts into adulthood. Fish were heat-shocked to induce Cre-recombinase activity 4 dpf, and then allowed a 24 hr maturation period to permit expression of new fluorescent proteins, which created spectrally distinct colors within the support cell population in larval zebrafish neuromasts (Fig. 1a). Neuromasts were imaged then analyzed using our semi-automated MATLAB algorithm to identify color and patterning 24 hours, 1-month, and 9-months post heat shock/Cre induction (Fig. 1a-c).

Spectral analysis of 5 dpf *Tg(ubi:Zebrabow-M;hsp:Cre)* zebrafish, 24 hours post heat shock, revealed that more than half of the neuromasts had 4 or more colors present in the support cell population, with 4 colors present in at least 50% of neuromasts (Fig. 1a, d). The color diversity was reduced by half 1 month following heat shock, with 50% of neuromasts analyzed containing only 2 colors (Fig. 1b, d). Finally, 9-months after cre-recombinase induction, 44% of neuromasts analyzed had 1 predominate color, while the other 25% and 20% of neuromasts still had 2 and 3 colors in their support cell population, respectively (Fig. 1c, d). Analysis of this data using one-way ANOVA showed that the

means are highly significantly different ($p<0.0001$). The authors note that not all of the neuromasts become monochromatic, with a substantial percentage (45%) of neuromasts at 9 months still having 2-3 colors within the support cell population. It is possible that neuromasts have multiple progenitor/stem cells maintaining tissue homeostasis and using the zebrabow we see this is reflected in the number of colors seen in mature neuromasts that have reached color equilibrium. We next analyzed whether this loss of color diversity was complemented by the expansion of one dominant color.

Using the same MATLAB algorithm, we quantified the total area that the most dominant color (i.e., the color occupying the greatest area) represented for each neuromast at each time point analyzed. The average area covered by the most dominant color 24 hours after heat shock was 47%, which expanded almost 2-fold (~%80) 9 months later (Fig. 1e). The area that the most dominant color covered at 9 months shows that over 70% of neuromasts had a dominant color represent over 75% of the support cell population. A one-way ANOVA analysis revealed a highly significant difference when comparing the mean area covered by the largest color overtime. These results further suggest that lateral line neuromasts progress towards clonality 9 months after the induction of color mosaicism.

Lateral line regeneration in adult Zebrabow fish does not drive clonal expansion in caudal fin neuromasts.

It is currently unknown whether hair cells and support cells are maintained by the same mechanism under all conditions. Our previous results indicate that neuromasts

eventually drift towards clonality under ambient conditions. However, neuromasts must also replace a more substantial amount of hair cells that are lost due to a sudden traumatic injury. Using the double transgenic *Tg(ubi:Zebrabow-M;hsp:Cre)* fish line, we characterized support cell dynamics following repeated neomycin-induced hair cell regeneration cycles. For these experiments, we followed adult zebrafish 9 months after heat shock and analyzed neuromasts located on the caudal fin, since they are distributed as single neuromasts that all have an established anterior-posterior hair cell polarity.

The 4 neuromasts that we followed had only two colors present in the support cell population. We measured the area each color clone covered of the entire neuromast, as previously described, following 6 regeneration cycles. The size of color clones remained relatively constant through 6 regeneration cycles. Three out of the 4 neuromasts had less than a 10% overall change in color clone expansion/contraction from beginning to end with the one outlier having a 22% coverage change (Fig. 2a). Clone sizes fluctuate between regeneration cycles with the average expansion/contraction being 6.3% (+/- 5.9). Twenty-one of the 24 regeneration cycles had an area fluctuation within one standard deviation, with the largest area change observed between one regeneration cycle being ~26% (Fig. 2b). Although there was constant expansion/contraction of color clones, the size of each respective clone was maintained at equilibrium. These two observations indicate that stimulation of hair cell regeneration in adult zebrafish three months post Cre induction does not direct or accelerate color clonal dominance, even after 6 regeneration cycles.

Discussion:

Many studies have investigated zebrafish lateral line hair cell regeneration following various ototoxin induced hair cell death. (Williams and Holder, 2000; Harris et al., 2003; Lopez-Schier and Hudspeth, 2006; Hernandez et al., 2007; Ma et al., 2008, Wibowo et al., 2011; Mackenzie and Raible, 2012). Lateral line hair cell regeneration is rapid and robust throughout the life of the fish (Williams and Holder, 2000; Harris, 2003; Ma, 2008; Cruz et al., 2015; see Chapter 3). Live imaging and lineage tracing experiments have demonstrated that hair cells are derived from underlying support cells (Lopez-Schier and Hudspeth, 2006; Wibowo et al., 2011; Cruz et al., 2015). However, little is known about how these symmetrically dividing hair cell precursors are replenished.

In Chapter 3, I described our examination of lateral line hair cell regeneration in adult zebrafish in which we determined that inducing multiple sequential rounds of hair cell regeneration in adult zebrafish did not deplete hair cells nor the support cell population. However, that study did not reveal the mechanisms by which this is regulated, including the manner by which support cells are renewed and the cell lineages involved in repetitive hair cell replacement support cells are renewed. Taking advantage of the ability of Tg(Zebrabow-M) to differentially and permanently label subpopulations of support cells, we found that initially spectrally mosaic neuromasts drift towards mono-chromaticity under ambient conditions. However, when mosaic neuromasts are subjected to repeated rounds of hair cell regeneration, the color clones do not progress towards

mono-chromaticity. Instead, color clones fluctuate between expansion and contraction to reasonably maintain their initial color clone size. The ability to continuously regenerate hair cells and support cells after multiple iterations of hair cell damage further previous adult zebrafish hair cell studies (Pisano et al., 2014, Cruz et al., 2015).

Many exposed epithelia have continuous replenishment of damaged or lost cells to ensure proper tissue function (see Barker et al., 2010; Clevers et al., 2014). The external location of neuromasts and hair cells leave them vulnerable to environmental insults. Death and proliferative markers in larval fish show that hair cells undergo continuous turnover under ambient conditions (Williams and Holder, 2000), and our study using photoconvertible proteins in adult zebrafish (Cruz et al., 2015; Chapter 3) supports this finding. Although cellular turnover was not continuously followed in the study described in this chapter (Chapter 4), the analysis of mosaic neuromasts at discrete developmental stages following induced spectral diversity revealed a reduction of color diversity within the support cell population over time. A hierarchical clonal expansion model may explain the reduction of color diversity within neuromasts overtime. The continual turnover of hair cells induces the depletion of a dedicated hair cell precursor population color. Depletion of these hair cell precursors is accompanied by the loss of the associated color that is then replaced by the color of a progenitor population. This continues until the stem cell population color that maintains and gives rise to all the cells in the neuromast has replaced all the transient cell population colors. Although these results indicate that all hair cells and support cells can be derived from a common progenitor, the location of this progenitor pool has yet to be identified.

Adult stem cells in cyclic and regenerative tissues are commonly located in stem cell niches, a specialized microenvironment that maintains stem cell plasticity and quiescence. To determine if there is a stem cell niche within lateral line neuromasts, we followed individual mosaic neuromast in adult zebrabow-M fish. To expedite clonal expansion, we promoted multiple rounds of hair cell and support cell regeneration by treating with ototoxin neomycin. To our surprise, we did not see a directed expansion or contraction of support cell clones over 6 regeneration cycles. Rather, support cell clones expanded or contracted in between regenerations but generally returned to the pre-regeneration size. This seems to contradict the results of the long-term clonal analysis where we see eventual clonal dominance.

A possible explanation may be that 3 months after heat shock, the caudal fin neuromasts support cell progenitor clones have reached equilibrium. The caudal fin neuromasts have an inherit AP polarity for their hair cells, as they form a single string on neuromasts connected my interneuromast support cells along 4 rays on the fin. Potentially, there are two stem cell populations in caudal fin neuromasts at the anterior and posterior ends of neuromasts. These stem cells would equally contribute to produce support cells and hair cells throughout the organism's lifetime under ambient and stress conditions. During multiple regenerations, you would observe that the area change of a clone fluctuates but never is depleted. This is idea is supported by the presence of 2-color neuromasts observed in adult zebrafish maintained in ambient conditions 9 months post heat shock. Hopefully, by inducing the color mosaic expression of different fluorophores in adult Zebrabow, we will get better single cell color diversity in order to get the resolution to identify individual stem cells and their niche.

Although regeneration is a vitally important trait, the ability to replace lost tissue varies greatly across the metazoans. The regeneration gamut spans from the incredible regeneration observed in some invertebrates, which have the amazing ability to completely reconstitute an entire individual from tiny body portions; to organisms with more limited regenerative capacity, such as mammals and birds, who can only replace a subset of tissues and cells. How stem cells are maintained to ensure tissue homeostasis is an important question that is yet to be answered. The zebrafish lateral line gives researchers a unique system to study tissue maintenance under various environmental conditions. Although it is important to examine the mechanism of tissue maintenance during regeneration, investigation of undisrupted tissue is essential for understanding stem cell biology. Our results highlight that idea; under ambient conditions spectrally diverse neuromasts progress towards clonality, while support cell clones remain in a steady-state under stress. The second result suggests that two distinct stem cell populations maintain caudal fin neuromasts. Whether these stem cell populations have distinct stem cell niches within the neuromast is still unknown. The identification of stem cells and their niche is necessary to re-establish hair cell and support cell regeneration in humans.

Materials and Methods:

Zebrafish strains and maintenance

Adult and larval zebrafish were maintained at 28.5°C as described in Westerfield (2000). Double transgenic *Tg(Hsp:Cre;ubi:Zebrabow-M)* fish were made by crossing *Tg(Hsp:Cre)* and *Tg(ubi:Zebrabow-M*), which were gifts from the Schier lab (Pan et al., 2010 and Pan et al., 2013, respectively). Double transgenic *Tg(ubi:Cre-ER^{T2};ubi:Zebrabow-M)* was a gift from the Zon lab, described in Pan et al., 2013. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Neomycin treatments

Neomycin sulfate (Sigma, St. Louis, MO) was diluted in E3 embryo media (14.97 mM NaCl, 500 µM KCl, 42 µM Na_2HPO_4, 150 µM KH_2PO_4, 1 mM $CaCl_2$ dehydrate, 1 mM $MgSO_4$, 0.714 mM $NaHCO_3$) to a final concentration of 400 µM. All adult zebrafish hair cell ablations were carried out in 400 µM neomycin for 1 hour followed by a 1 hour recovery period in E3 media at 28.5 °C. Hair cells were scored before neomycin, 2 and 72 hours after treatment, and then repeated as indicated in the experiment. Sibling fish were used as controls and were mock-treated by handling and relocating to drug-free media.

Cre Expression

hsp:Cre transgenic line

4 dpf larval zebrafish were placed into 1.5ml tubes filled with 1ml of pre-heated 37°C E3 embryo media that were then transferred to a 37°C water bath for 10-20 minutes. Fish were then returned to a 28.5°C incubator to recover before short-term induction imaging or placed on the zebrafish aquatic system until 4 and 36 weeks have elapsed to perform long-term clonal analysis. Adult zebrafish were place into 500ml beakers that were filled with 100ml of 28.5°C E3 embryo media and placed into 37°C water baths for 20-30 minutes, and then returned to their tanks in the fish facility and maintained as 28.5°C until imaging and neomycin treatment.

CreER^{T2} transgenic line

Double transgenic *Tg(ubi:CreER*^{T2}*;ubi:Zebrabow-M)* were treated with caged cyclofen-OH (5-15 µM) for 15-60 minutes at different developmental stages as described in the results. After treatment, fish were then washed 3 times in fresh E3 media.

Image Acquisition and Image Analysis:

Adult and larval fish were anesthetized in E3 media containing 0.2% buffered MESAB (MS-222; ethyl-m-aminobenzoate methanesulphonate) and fish were

individually placed into 8-milliliter glass bottom chambers for imaging. Larval fish were stabilized using a small net and weighted harp. Adult zebrafish were stabilized by placing a fitted sponge across the trunk and caudal fin of fish. Chambers were placed on an inverted Axio Observer D1 spinning disk system (Intelligent Imaging Innovations) equipped with an Evolve 10 MHz EMCCD camera (Photometrics) and a Zeiss C-Apochromat 63×/1.2 NA water objective. Camera intensification was set between 100-200, and 445 nm, 488 nm and 561 nm exposure times were adjusted to maintain maximum intensities below fluorescence saturation.

MatLab color and area acquisition algorithm:

The MatLab code takes RGB (Red/Green/Blue) images and changes them to Hue grayscale images (mapping the colors in the original image onto a range of integer values that represent those colors from 0 to 256). It then creates a histogram from this grayscale Hue image. The shape of this histogram is determined by the frequency of the different colors in the original image. For instance, if purple (a mix of red and blue) was common in the original image, the histogram would have a large group of values (a peak) at around 200, which is the purple range). The code then uses a peak finding algorithm on this histogram to identify clusters of values (peaks in the histogram) by identifying minimums and maximums in the Hue histogram. Color groupings in the image are then just clusters between minimums (e.g., purple cells might be all values between 185 (the left minimum) and 210 (the right minimum). Because the histogram represents the number of pixels present in this range occupied by a peak, the code then simply adds up

the total number of pixels in each peak. This is the area in the original image occupied by a particular color.

Figures:

Figure 1. **Under normal conditions, support cell color clones expand over time towards monochromaticity**

Single support cell layer slices of confocal z-stacks were analyzed 24 hours (**A**), 1 month (**B**), and 9 months (**C**) post heat shock in double transgenic *Zebrabow-M;hsp:Cre* fish. Representative single image slices of the support cell population of neuromasts 24 hours, 1 month, and 9 months post cre recombinase (color diversity) induction. (**D**) Quantification of the color diversity found in neuromasts' support cell populations at varying times post cre recombinase induction. (**E**) Results are graphed as the average

percent area (± s.d.) covered by the largest color clone at varying times post heat shock. (N=5 fish per group, 23+ neuromast per time point.

Figure 2.

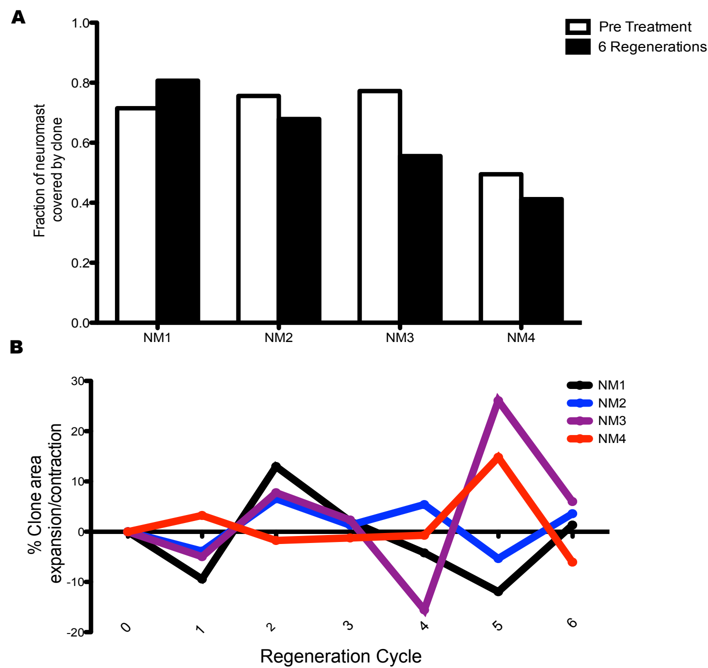

Figure 2. **During repeated regeneration cycles, support cell color clones reach equilibrium rather than monochromaticity .**

Tg(*Zebrabow-M;hsp:Cre)* fish were heat shocked at 5 dpf and allowed to grow up to 3 months post fertilization when neuromasts on the caudal fin were imaged after each of 6 neomycin-induced hair cell regeneration cycles. (**A**) Comparison of color clone sizes before neomycin treatments and after 6 sequential hair cell regeneration cycles. (**B**) Percent changes in color clone sizes between regeneration cycles for 4 neuromasts.

Chapter 5: Conclusions and Future Directions

Hair cell development and regeneration are ever-growing fields of research. There are collective efforts being made to better understand the genetic networks that regulate development, maintenance, and regeneration of hair cells. Avian and zebrafish gene expression profiles in both regenerating and non-regenerating sensory epithelia are important to identifying key genetic differences and similarities in hair cell and support cell biology. The recent technological advances in gene editing using TALENs and CRISPRs make reverse genetic screens easier, faster and cheaper (see Shalem et al., 2015 and Maggio and Goncalves, 2015 for reviews). This technology will allow researchers to selectively disrupt genetic pathways to ascertain their role in hair cell development and regeneration. Although TALENs and CRISPRs will allow researchers to better investigate developmental and regeneration genes *a priori,* it would serve the scientific community well to continue forward genetic screens to discover new and mysterious genes that no one would associate with hair cell biology.

Zebrafish transgenics allow researchers to observe how these genes function in undisrupted conditions which gives us a better understanding of their spatial and temporal role during development and regeneration. Identifying genetic markers to create transgenic lines to distinguish sub-support cell populations will be vital for these experiments. Transgenesis using *Tol2* and *Sleeping Beauty* transposons has made creating fluorescently labeled transgenics easier and more efficient (see Kawakami, 2005 for review). The addition of fluorescent proteins and the Cre-Lox system to zebrafish

research is enabling researchers to more effectively identify and follow the development of stem/progenitor cells and their progeny (Jopling, 2010; Matsuda, 2013; Huang, 2014).

The stem cell population(s?) that maintains the lateral line system, under normal conditions and after hair cell damage, has yet to be identified. It is my hope that with the new tools available that these cells will be quickly identified and the genes that govern them. Experiments using the Zebrabow transgenic fish in this study take us one step closer to this goal, and experiments that are currently underway will identify the mechanism maintaining lateral line homeostasis.

It is evident that, to better understand lateral line development, maintenance, and regeneration, further studies must be performed on zebrafish during different developmental stages. These studies will help us give us better insight on the different cellular and molecular mechanisms regulating hair cell and support cell maintenance under an array of developmental and environment pressures. Larval lateral line hair cell regeneration and developmental studies have enlightened us on early developmental signals, hair cell death and cellular dynamics during hair cell regeneration. However, as organisms grow and change, so must the cellular and molecular architecture that governs organ and tissue maintenance to correctly adjust for the ever-changing size and environment stimuli experienced. Hopefully, these studies have helped better understand the cellular composition of the lateral line and guide researchers in the future.

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