SOX2 IS NECESSARY AND SUFFICIENT TO INDUCE MÜLLER GLIA REPROGRAMMING AND PROLIFERATION IN THE REGENERATING ZEBRAFISH RETINA

A Dissertation

Submitted to the Graduate School of the University of Notre Dame in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy by

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April 2015
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Abstract

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In response to light-induced photoreceptor apoptosis, the zebrafish retina undergoes a robust regenerative response that results in recovery of lost or damaged rod and cone photoreceptors. This response initiates with a cell reprogramming event, in which a subset of Müller glia divide asymmetrically to produce a transiently amplifying neuronal progenitor cell (NPC) population. NPCs continue to proliferate and migrate from the inner nuclear layer to the outer nuclear layer, where they differentiate into new rod and cone photoreceptors. Müller glia reprogramming is an essential, rate limiting step in the regeneration program, but the molecular mechanisms that control this process are just starting to be elucidated. Induction of several pluripotency factors necessary for reprogramming of iPS cells, including lin28, sox2, klf4, myc, oct4 and nanog (Ramachandran et al., 2010), has been observed during the early stages of retina regeneration, but of these, only Lin28 function has been studied.
Sox2 is a well established neuronal stem cell-associated transcription factor that regulates neural development and adult neurogenesis in vertebrates. We found that Sox2 expression is maintained in the Müller glia and a subset of amacrine cells in the undamaged adult zebrafish retina, recapitulating that of other well-studied vertebrates. Expression of \textit{sox2} transcript increased significantly in retinas following 31 hours of light treatment, when Müller glia begin proliferating. This correlated with increased Sox2 protein expression in proliferating Müller glia. Knockdown of Sox2 expression decreased the number of proliferating Müller glia, demonstrating that Sox2 is required for Müller glia reprogramming and proliferation. In contrast, induced overexpression of Sox2 stimulated proliferation of Müller glia in the absence of retinal damage. We investigated the role of Wnt/\(\beta\)-catenin signaling, which is required for Müller glia reprogramming and is a known regulator of \textit{sox2} expression during vertebrate retinal development. Contrary to previous reports, we observed differential expression and non-redundant roles of the two zebrafish \(\beta\)-catenin paralogs. While \(\beta\)-catenin 2, but not \(\beta\)-catenin 1, was required for Müller glia proliferation, neither \(\beta\)-catenin paralog was required for \textit{sox2} expression following retinal damage. We also determined that Sox2 regulates expression of \textit{ascl1a} and \textit{lin28a}, but not \textit{stat3} expression following retinal damage. Additionally, \textit{sox2} expression was not dependent upon Ascl1a, suggesting Sox2 lies exclusively upstream of \textit{ascl1a}. Finally, we observed differential expression of Sox2 during later stages of regeneration. Specifically, all early NPCs express Sox2 at 51 hours, but later stage progenitors express Sox2 heterogenously. This study is the first to...
identify a functional role for Sox2 during Müller glial-based regeneration of the vertebrate retina.
This work is dedicated to my friends and family who have been a never-ending source of motivation and support. I truly could not have survived this journey without you.
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CHAPTER 1:  
INTRODUCTION  

1.1 History of retina regeneration in zebrafish 

In the early twentieth century, retinal regeneration had been well documented in urodeles (reviewed in Mitashov, 1996). Subsequently, Roger Sperry (1948) published the first set of experiments showing that several species of tropical marine teleosts were capable of fully regenerating optic nerves following transection. While these experiments revealed that optic nerve axons could be regenerated, it remained unclear whether retinal neurons were capable of cellular regeneration following neuronal cell death.

Nearly 20 years later, Lombardo (1968, 1972) observed that the goldfish retina was capable of complete epimorphic regeneration following partial surgical ablation. These results were confirmed using the Na$^+$/K$^+$-ATPase inhibitor ouabain to selectively ablate either the inner-most retinal neurons or all the retinal neurons (intravitreal injection of low and high concentrations of ouabain, respectively; Maier and Wolburg, 1979; Raymond 1988). Regeneration following both surgical excision and ouabain-induced toxicity revealed that the majority of cell proliferation did not originate from the stem cell population in the circumferential marginal zone (CMZ), which gives rise to
all neuronal classes except rod photoreceptors during normal persistent neurogenesis throughout the life of goldfish (reviewed in Otteson and Hitchcock, 2003). Rather, most of the cell proliferation in the damaged retina was found in a population of centrally-located inner nuclear layer (INL) cells, which were thought to be a second population of retinal stem cells that is restricted in its potential to produce only rod photoreceptors during persistent neurogenesis (Otteson and Hitchcock, 2003). Later, evidence emerged that these INL “stem cells” could be dedifferentiated Müller glia; however, goldfish lacked the genetic tools to confirm the source of the regenerated neurons in the damaged teleost retina.

Zebrafish, a well established vertebrate system for addressing developmental genetics, was used to create retinal damage paradigms using surgical ablation and intense light treatment to study neuronal regeneration (Cameron et al., 2000; Vihtelic and Hyde, 2000). Fausett and Goldman (2006) showed that a transgenic zebrafish line expressing GFP from a fragment of the neuronal tuba1a tubulin promoter exhibited coexpression of GFP and Müller glial proteins in the BrdU-positive INL cells of the damaged retina. Moreover, these GFP-positive Müller glia-derived cells eventually coexpressed markers of ganglion and amacrine cells in the puncture-damaged retina, revealing they had the capacity to differentiate into retinal-specific neurons. The role of Müller glia as the source of neuronal progenitors in the damaged zebrafish retina was subsequently supported by lineage tracing experiments using transgenic lines that express GFP from the Müller glia-specific gfap (glial fibrillary acidic protein) promoter (Bernardos et al., 2007), as well as morpholino-mediated knockdown of PCNA.
expression in the light-damaged zebrafish retina, which prevented INL cell proliferation and resulted in apoptosis of Müller glia (Thummel et al., 2008a).

Several laboratories have defined specific events that occur within the adult zebrafish retina during damage and regeneration (Figure 1.1). These events include: 1) neuronal cell death, 2) dedifferentiation and proliferation of the primary (initial) Müller glia to produce neuronal progenitor cells (NPCs), 3) recruitment of additional (secondary) Müller glia to dedifferentiate and proliferate, 4) amplification of the number of NPCs, 5) migration of the NPCs to the damaged retinal layer(s), and 6) regeneration of the neuronal cell types that were lost. Depending upon the damage model that is utilized, some of these events are tightly associated with another step. For example, the dedifferentiation and proliferation of the primary and secondary Müller glia are usually not obvious unless the dedifferentiation and proliferation of the secondary Müller glia are blocked by the introduction of morpholinos (Nelson et al., 2012).

As efforts continued to characterize the cellular events associated with adult zebrafish retinal regeneration, the first analyses to identify gene candidates that regulate this response were performed. Initial microarray experiments focused on the gene expression changes in total retinas damaged by either surgical excision (Cameron et al., 2005) or constant intense light treatment (Kassen et al., 2007). Subsequently, Morris et al. (2011) performed microarrays on total adult Tg(XOPS:mCFP) retinas, which revealed gene expression changes specific to rod cell death and regeneration. Alternatively, microarray-based studies were performed on laser-captured ONL fragments from light-lesioned retinas (Craig et al., 2008) to specifically identify gene
Figure 1.1: Defining the cellular events during regeneration of the light-damaged zebrafish retina. Immunofluorescence of retinal cryosections (A-F) using antibodies to Rhodopsin (Rho; red), PCNA (green), TUNEL (orange) and nuclei (DAPI; blue). Time points correspond to the indicated cellular events, which are summarized in the schematic (G-L). Müller glia are coded as follows: PPMG, red; SPMG, yellow; QMG, green. Neuronal progenitor cells are represented as fusiform cells (cyan). Committed rod and cone progenitors in the ONL are represented as lighter shades of blue and magenta, respectively. Scale bar in panel A is 25 microns and is the same for panels B-F. GCL, Ganglion cell layer; INL, Inner nuclear layer; ONL, Outer nuclear layer; PCNA, Proliferating cell nuclear antigen; PPMG, primary proliferating Müller glia; QMG, quiescent Müller glia; SPMG, secondary proliferating Müller glia.
changes in dying and regenerating photoreceptors. A significant modification to these
approaches was the use of fluorescence-activated cell sorting (FACS) to isolate specific
cell populations from transgenic zebrafish expressing fluorescent proteins under the
control of cell-specific promoters. Qin et al. (2009) utilized the Tg(gfap:EGFP) zebrafish
line, which express GFP from a Müller glial-specific promoter, to successfully
characterize changes in gene expression during Müller glia dedifferentiation in the light-
damaged retina, while Ramachandran et al. (2012) performed a similar analysis with the
Tg(1016tub1a:GFP) transgenic line that expresses GFP in Müller glial-derived neuronal
progenitor cells.

The information generated from all of the microarray experiments has provided
a surplus of candidate genes that may regulate different steps during retinal
regeneration in teleosts. Numerous studies have tested the potential roles of these
candidates during retinal regeneration using a wide variety of techniques, including
mutant and transgenic zebrafish lines, application of small molecules, and introduction
of exogenous proteins and antisense-morpholinos to the adult retina. In the following
sections, I will focus on the genes and proteins that have been found to regulate
zebrafish retinal regeneration and will attempt to critically evaluate how these factors
may interact to orchestrate defined cellular events that occur during regeneration.

1.2 Dying retinal neurons stimulate regeneration

It was not initially appreciated that the extent of retinal damage/cell death is
directly correlated to the number of dedifferentiated/proliferating Müller glia
(Montgomery et al., 2010). This suggested that dying neurons stimulated Müller glia
dedifferentiation and/or proliferation. There are two possible mechanisms of action.
The negative-regulation model posits that an inhibitory factor, normally present in the
undamaged retina, is lost or downregulated in dying neurons, relieving the repression
on the regeneration response. The positive-regulation model, in contrast, relies on
inducing the expression of a regeneration signal in dying neurons that signal to Müller
glia to proliferate.

Several secreted factors were identified that could contribute to either of these
models. For example, Wnt signaling components, such as the wnt4a and wnt8b mRNAs
rapidly increased in expression following retinal stab lesions, suggesting Wnt ligands
could serve as a positive signal from dying neurons to Müller glia (Ramachandran et al.,
2011). However, the spatial expression of these genes was not analyzed until 4 days
post-injury, when they were enriched in expression in Müller glial-derived neuronal
progenitor cells (NPCs). Thus, the contribution of the NPCs in signaling from apoptotic
neurons to the Müller glia remains largely unexplored. In contrast, the Wnt pathway
inhibitor mRNAs (dkk1b, dkk2, dkk3 and dkk4) rapidly decreased in expression following
the stab lesion and overexpression of DKK1b using the Tg(hsp70l:dkk1b-GFP) transgenic
zebrafish line prevented Müller glia proliferation (Ramachandran et al., 2011). However,
expression of dkk1b decreased throughout the retina following the puncture lesion,
rather than specifically in the lesioned regions containing apoptotic neurons. While the
global downregulation could occur as a wave emanating from the injury site, this has
not yet been investigated. Thus, expression of Wnt pathway agonists and antagonists is
temporally consistent with the positive- and negative-signals for regeneration, respectively, however neither has been directly linked to dying neurons.

Nelson et al. (2013) directly tested the positive-regulation model by homogenizing light-damaged retinas at the peak of photoreceptor cell death and injecting the homogenate into healthy retinas. The light-damaged homogenate induced a significantly greater number of Müller glia to proliferate at 2 and 3 days post-injection, relative to the lysate from undamaged retinas. This demonstrated that the light-damaged retina produces factor(s) that can stimulate the regeneration process. Two-dimensional gel electrophoresis of undamaged and 16 hour light-treated retinal lysates followed by MALDI-TOF mass spectrometry identified several candidate proteins that significantly increased in expression following light-damage. This analysis ultimately revealed that TNFα was first expressed in dying rod and cone photoreceptors within 16 hours of constant light treatment, followed by increased expression in the Müller glia (Nelson et al., 2013). However, dying photoreceptors are not the only neurons that express TNFα, as ouabain-induced retinal damage resulted in increased TNFα expression in the dying INL and ganglion cell neurons (Nelson et al., 2013). Thus, TNFα appeared to be a general signal produced by all dying neurons in the zebrafish retina.

To determine if TNFα played a role in retinal regeneration, tnfa morpholinos were intravitreally injected and electroporated into the retina prior to the light damage (Nelson et al., 2013). Knockdown of TNFα expression significantly reduced Müller glia proliferation relative to the controls. Preliminary results also show that intravitreal injection of TNFα is sufficient to induce Müller glia proliferation in the undamaged
retina (Conner et al., 2014). These data provide compelling evidence for TNFα being the first identified factor produced by dying retinal neurons that directly signal Müller glia to dedifferentiate and proliferate in the regeneration program.

1.3 Primary Müller glia reprogramming and proliferation

Müller glia respond to TNFα and potentially other signals from dying photoreceptors by initiating gene programs that are characteristic of both dedifferentiation and proliferation (Figure 1.2). These gene programs include increased expression of cell cycle genes, such as pcna and cyclin b1 (Kassen et al., 2007; Kassen et al., 2008); cytoskeletal components tuba1a (Fausett and Goldman, 2006) and nestin (Gorsuch and Hyde, unpublished data); the proneural bHLH transcription factor gene ascl1a (Fausett et al., 2008); the cell adhesion protein N-cadherin (Raymond et al., 2006); and the pluripotency factor genes lin28, oct4, nanog, klf4, myca, mycb, and sox2 (Ramachandran et al., 2010). The expression of Ascl1a and Sox2 proteins was also confirmed in the PCNA-positive Müller glia of the light-damaged retina (Nelson et al., 2012; Gorsuch and Hyde, unpublished data). Many investigations have been, and continue to be, aimed at understanding how these factors regulate Müller glia dedifferentiation and promote the initial mitotic event that gives rise to the transient NPC population (Figure 1.2). The first gene identified to be essential for zebrafish Müller glia dedifferentiation was ascl1a. Expression of the ascl1a transcript increased within 6 hours of the puncture damage in the proliferating Müller glia near the wound (Fausett
Figure 1.2: Signaling events necessary for Müller glial dedifferentiation and proliferation. Dying retinal neurons (e.g. rod and cone photoreceptors) secrete TNFα (green triangles), which activates a dedifferentiation program in primary proliferating Müller glia (PPMG). Black lines represent active signaling and grey lines indicate repressed signaling. Dashed objects represent inactive components. Question marks indicate uncertainty between the indicated signaling interactions. The identity of the Notch ligand and its location, either in the photoreceptors or an INL cell type, remain unknown. Ascl1a, Achaete-scute homolog 1a; PCNA, Proliferating cell nuclear antigen: Stat3, Signal transducer and activation of transcription 3; TNFα, Tumor necrosis factor-alpha; TNFSR, TNF superfamily receptor.
et al., 2008). Similarly, expression of the Ascl1a protein was restricted to the PCNA-positive Müller glia within 31 hours of constant light treatment (Nelson et al., 2012). Morpholino-mediated knockdown of Ascl1a expression in the regenerating adult retina revealed that it is essential for both Müller glia proliferation (Fausett et al., 2008; Nelson et al., 2012) and transcriptional activation of the tuba1a promoter’s E-box domain in the -1016-a1T:GFP transgene reporter (Fausett et al., 2008).

In contrast to the increased ascl1a expression, a broad decrease in dkk1b expression was described in the punctured retina (Ramachandran et al., 2011). The dkk1b gene, which encodes a member of the Dickkopf family of secreted Wnt inhibitors, exhibits a transient expression pattern that was complementary to the ascl1a expression pattern. By 2 days after the stab wound, dkk1b expression is restricted to retinal neurons and non-proliferating Müller glia, while ascl1a expression is limited to the subset of dedifferentiated Müller glia (Ramachandran et al., 2011). Because dkk1b expression was dependent on ascl1a expression and overexpression of Dkk1b from the Tg(hsp70l:dkk1b-GFP) heat shock-inducible transgene prevented damage-induced Müller glia proliferation (Ramachandran et al., 2011), Wnt signaling may be necessary for Müller glia dedifferentiation and proliferation through an Ascl1a-dependent mechanism.

RT-PCR analysis of Wnt ligands and receptors revealed increased injury-dependent expression of several genes, including wnt4a, wnt8b and fzd2 (Ramachandran et al., 2011). Of these, only wnt4a expression was reported in ascl1a-positive Müller glia, and this expression was dependent on ascl1a at 2 days post-injury.
In canonical Wnt signaling, β-catenin is the terminal effector molecule, functioning as a transcriptional coactivator with Tcf/Lef transcription factors. Thus, translocation of β-catenin to the nucleus indicates active Wnt signaling. Ramachandran et al. (2011) showed that decreased dkk1b expression drove nuclear β-catenin accumulation in dedifferentiating Müller glia at 2 days post-injury, consistent with the expression of the Wnt reporter Tg(TOP:dGFP) in NPC clusters at 4 days post-injury. Pharmacological stabilization of the β-catenin destruction complex also inhibited Müller glia proliferation. Perhaps the most interesting finding from these experiments was that pharmacological inhibition of GSK3β was sufficient to activate the expression of several genes associated with the normal Müller glia dedifferentiation program (ascl1a, lin28, mycb, wnt4a and fzd2) and stimulate Müller glia proliferation in the absence of damage (Ramchandran et al., 2011). The increase in ascl1a expression following GSK3β inhibition is curious, given that ascl1a is required for dkk1b suppression and activation of wnt4a, which would logically occur prior to β-catenin stabilization (Ramchandran et al., 2011). Because the GSK3β inhibitor-injected eyes were not analyzed until 4 days post-injection, it is possible that exogenous Wnt activation is sufficient to stimulate Müller glia dedifferentiation and proliferation in the absence of ascl1a, and the increased ascl1a expression is due to the Müller glial-derived NPCs. Alternatively, ascl1a and β-catenin could function in a regulatory feedback loop during the early stages of Müller glia dedifferentiation.

As a whole, these experiments provided strong evidence for a Müller glia dedifferentiation program in which Ascl1a inhibits dkk1b expression (Figure 1.2). This
reduction in \textit{dkk1b} leads to increased canonical Wnt signaling, potentially via Wnt4a and Fzd2, and translocation of \(\beta\)-catenin to the nucleus. This model is also consistent with recent findings that genetic and pharmacological disruption of Wnt signaling depletes the CMZ stem cell compartment, and impairs regeneration of light-damaged larval zebrafish retinas (Meyers et al., 2012). Evidence from retinal development in \textit{Xenopus} has shown that \(\beta\)-catenin regulates Sox2, which is responsible for maintaining progenitor identity (Agathocleous et al., 2009). Similarly, pharmacological inhibition of Wnt signaling inhibits Sox2 expression in the CMZ of the larval zebrafish (Meyers et al., 2012). Expression of the \textit{sox2} transcript increases in the puncture-damaged zebrafish retina (Ramachandran et al., 2010) and the Sox2 protein expression increases in PCNA-positive Müller glia and NPCs following light lesion in the adult zebrafish retina (Gorsuch and Hyde, unpublished data). These results suggest that the regeneration of the zebrafish retina could be mediated by \(\beta\)-catenin, which is necessary for Müller glia proliferation and induction of Sox2 to maintain dedifferentiated Müller glia and/or NPC identity. While this is an attractive model, the interaction between \(\beta\)-catenin and Sox2 has not yet been addressed in the regenerating zebrafish retina.

Because Ascl1a functions primarily as a transcriptional activator, it is curious that \textit{ascl1a} expression was required to inhibit \textit{dkk1b} expression. The most obvious explanation is that Ascl1a activates one or more transcriptional repressors during Müller glia dedifferentiation. Microarray experiments of FACS-sorted Müller glia and Müller glia-derived NPCs revealed \(\sim 1,500\) known transcriptional repressors with significant changes in gene expression following injury (Ramachandran et al., 2012). One of these
genes, *insm1a*, was confirmed via quantitative PCR to increase immediately following puncture injury in a pan-retinal fashion, and then becomes restricted to Müller glia by 2 days post-injury (Ramachandran et al., 2012). Importantly, morpholino-mediated knockdown of Ascl1a protein prevented expression of *insm1a*, making *insm1a* a prime candidate for *ascl1a*-dependent *dkk1b* repression (Ramachandran et al., 2012). Indeed, loss of *insm1a* prevents the necessary reduction in *dkk1b* expression and Müller glia proliferation (Ramachandran et al., 2012). These experiments not only identified *insm1a* as the likely molecular effector of the *ascl1a*-dependent repression of *dkk1b*, but also highlighted the importance of transcriptional repression in retinal regeneration.

Another class of genes that likely plays a significant role in Müller glia dedifferentiation is the stem cell-associated pluripotency factors (Takahashi et al., 2007). Ramachandran et al. (2010) reported increased expression of the stem cell-associated factor mRNAs *nanog*, *sox2*, *lin28*, *klf4*, *myca*, *mycb* and *oct4*. To date, the spatial expression of only *lin28* mRNA and Sox2 protein was localized to dedifferentiated Müller glia by 2 days post injury (Ramachandran et al., 2010; Gorsuch and Hyde, unpublished results, respectively).

Functional studies revealed that expression of *lin28* is necessary for Müller glia dedifferentiation and proliferation (Ramachandran et al., 2010). The function of Lin28 during Müller glia dedifferentiation appears to be, at least in part, to inhibit function of the miRNA *let7*, which is expressed globally in the undamaged retina and repressed following retinal injury. Additionally, coexpression of *let7* and various zebrafish pluripotency factors, each harboring potential *let7* binding sites, in HEK293 cells
revealed a dose-dependent inhibition of pluripotency protein expression (Ramachandran et al., 2010). The authors proposed a model in which basal amounts of hspd1, myca and oct4 are expressed in Müller glia of the undamaged retina (although neither myca nor oct4 RNA expression have been investigated spatially), but protein expression is inhibited by let7. In response to injury, Lin28 inhibits let7, which stabilizes expression of the pluripotency factors. However, it remains unclear if inhibition of let7 alone can induce Müller glial dedifferentiation in the undamaged retina.

The relationship between lin28 and ascl1a expression is not entirely clear. Ramachandran et al. (2010) demonstrated that lin28 expression is dependent on ascl1a expression, due to the observation that morpholino-dependent knockdown of ascl1a decreased lin28 transcript expression 4 days after puncture damage. However, this is several days after the initial Müller glia dedifferentiation. In contrast, Nelson et al. (2012) demonstrated that Lin28 may actually function upstream of Ascl1a protein expression in dedifferentiated Müller glia. This study differed from the earlier report in two key ways: 1) analysis was limited to the time at which Müller glia are dedifferentiating and just beginning to proliferate, and 2) Ascl1a protein expression was assayed. One potential explanation for this discrepancy could be that Lin28 stimulates translation of Ascl1a. It has become clear that Lin28 can function to enhance translation of some mRNAs (reviewed in Huang, 2012), perhaps by shuttling transcripts to polysomes. It is also possible Lin28 is necessary for increased Ascl1a protein levels, without drastically affecting the levels of ascl1a mRNA. Lin28 functioning upstream of Ascl1a is also consistent with the let7-dependent inhibition of zebrafish Ascl1a
expression in HEK293 cells (Ramchandran et al., 2010). If let7 represses Ascl1a expression, it is counterintuitive for Ascl1a expression to increase prior to the let7-repressing factor Lin28. Finally, it is possible that Ascl1a and Lin28 function in a regulatory loop, where a small increase in Ascl1a promotes Lin28, which increases Ascl1a translation further. While it is well established Lin28 and Ascl1a are both required for Müller glia dedifferentiation and proliferation, understanding the direct regulatory interactions between these proteins will require more attention. Procedures such as cross-linking immunoprecipitation followed by next generation sequencing (CLIP-Seq), a protocol that can detect interactions between RNA and RNA-binding proteins, could be used to directly investigate Lin28 regulation of ascl1a transcript. This also highlights the need to characterize the expression of target proteins at the correct time in the Müller glia dedifferentiation and retinal regeneration process to define the regulatory pathways in Müller glia and NPCs.

While many of these changes in gene expression are likely affected by epigenetic modifications that occur during Müller glia dedifferentiation, only one study has attempted to determine the role of the epigenome during retinal regeneration in zebrafish (Powell et al., 2012). This study observed that expression of the cytidine deaminase genes apobec2a and apobec2b increases in dedifferentiating Müller glia following stab lesion (Powell et al., 2012). Functional analyses revealed that Ascl1a is required for apobec2b expression, and both Apobec proteins are necessary to sustain ascl1a expression, placing these proteins in a positive feedback loop. While loss of Apobec proteins prevented Müller glia proliferation at 4 days post injury (Powell et al.,
2012), their role during Müller glia dedifferentiation and proliferation were not reported. This rather late time point makes it difficult to conclusively state the importance of these proteins in Müller glia dedifferentiation relative to NPC amplification. While DNA methylation and Müller glia dedifferentiation were not directly monitored in this study, it is the first to demonstrate that CpG modifying enzymes are necessary for zebrafish retinal regeneration. Future experiments utilizing bisulfite sequencing of CpG methylation throughout the genome and ChIP to analyze histone modification states will provide a deeper understanding of the role of epigenetic regulation.

1.4 Recruitment of secondary proliferating Müller glia

Recent studies suggest that Müller glia dedifferentiate and proliferate in waves, allowing additional Müller glia to be "recruited" into the regeneration response as needed (Nelson et al., 2012; Nelson et al., 2013). This provides the zebrafish retina with a cellular/molecular "rheostat" that incorporates the necessary number of Müller glia into the regeneration program. In the previous section, we described the events necessary for the initial Müller glia to dedifferentiate and proliferate following stimulation by dying photoreceptors, likely via TNFα. Many of the pathways required for primary proliferating Müller glia (PPMG) to dedifferentiate and reenter the cell cycle are also likely required for secondary proliferating Müller glia (SPMG) dedifferentiation. Here we will focus on how the PPMG directly recruit the SPMG to dedifferentiate and proliferate (Figure 1.3).
Figure 1.3: Recruitment of SPMG and maintenance of QMG. PPMG secrete TNFα (green triangles) in a Stat3- and Ascl1a-dependent manner, and HB-EGF (purple circles), It is not entirely clear what stimulates HB-EGF expression, although Ascl1a may be involved. These secreted factors bind their respective receptors in neighboring Müller glia, which activates a secondary dedifferentiation program (SPMG). A concentration gradient of TNFα and HB-EGF likely activates neighboring Müller glia at a certain threshold, below which Müller glia do not dedifferentiate and remain quiescent (QMG). Inhibition of Notch signaling results in dedifferentiation of QMG (Wan et al., 2012), suggesting Notch expression may be negatively correlated to the TNFα/HB-EGF gradient. Because Ascl1a can canonically activate Delta ligands, Delta expression in PPMG/SPMG may reinforce Notch expression in neighboring Müller glia outside of the TNFα/HB-EGF gradient. Black lines indicate active signaling, while grey lines represent repressed signaling. Dashed objects represent inactive components. While Notch expression/activity is likely repressed in the SPMG, the identity and location, either the photoreceptors and/or INL cell type, of the corresponding ligand remains unknown. Question marks indicate uncertainty between the specified signaling interactions. Ascl1a, Achaete-scute homolog 1a; EGFR, epidermal growth factor receptor; ERK1/2, Extracellular signal-regulated kinase 1 and 2; HB-EGF, Heparin-binding EGF-like growth factor; Insm1a, Insulinoma associated 1a; PCNA, Proliferating cell nuclear antigen; PPMG, primary proliferating Müller glia; QMG, Quiescent Müller glia; SPMG, Secondary proliferating Müller glia; Stat3, Signal activator and transducer of transcription 3; TNFα, Tumor necrosis factor-alpha; TNFSR, TNF superfamily receptor.
Müller glia in the injured retina express the Jak/Stat signaling protein, Stat3, in a Lin28/Ascl1a-dependent manner (Nelson et al., 2012). While Lin28 and Ascl1a are necessary for all proliferating Müller glia, only ~40%-50% of the proliferating Müller glia (SPMG) require increased Stat3 expression. We hypothesized that Stat3 activates expression of an intercellular signal to stimulate the SPMG to dedifferentiate and reenter the cell cycle. Interestingly, following injury, TNFα is first expressed in dying neurons and subsequently in a subset of Müller glia that are PCNA-positive (Nelson et al., 2013). Müller glia expression of TNFα required Lin28/Ascl1a/Stat3 expression, suggesting that TNFα is the Stat3-dependent signal to the SPMG. This was supported by the observation that Müller glia expression of TNFα, like Stat3, was necessary for a subset of, but not all, Müller glia to proliferate (SPMG). Additionally, Müller glia-derived TNFα is required for Ascl1a and Stat3 expression in the SPGM, which is consistent with Stat3 functioning through a cytokine receptor/Jak/Stat pathway; however, the link between TNFα activation of Ascl1a in SPMG is less clear.

While a model of TNFα/Stat3 signaling in the Müller glia to induce the SPMG to proliferate is attractive and supported by functional data, it is unclear why Stat3 expression rapidly increases in all Müller glia following injury and the PPMG appear to proliferate in a Stat3-independent fashion (Kassen et al., 2007; Nelson et al., 2012). Thus, the link between TNFα activation of Lin28/Ascl1a in the PPMG, and activation of Stat3 in all Müller glia remains unclear. These observations could be explained by a model in which basal amounts of TNFα and/or another, unidentified damage signal stimulates increased Stat3 expression in all Müller glia. In this model, pan-Müller glia
Stat3 expression could function as a molecular "transistor," providing little output on its own, but rapidly amplifying an input signal. While the mechanism that induces Stat3 expression in all Müller glia and the role of Stat3 in PPMG dedifferentiation/proliferation remains unknown, it is clear that Ascl1a expression is necessary for Stat3 expression in PPMG and Stat3 function is required to yield the maximal number of Ascl1a-expressing Müller glia.

One potential Stat3 transcriptional target is *hspd1*, which encodes a mitochondrial chaperone protein. Hspd1 functions primarily with its co-chaperone, Hspe1/Hsp10, in the mitochondrial matrix, where it aids in folding mitochondrial proteins to promote mitochondrial biogenesis and/or cell survival following stress (reviewed by Stetler et al., 2010). Additionally, mammalian Hspd1/Hspe1 expression is tightly regulated by a bidirectional promoter harboring a functional Stat3 binding site that is activated in response to ischemic stress (Kim and Lee, 2010). In the zebrafish retina, *hspd1* expression increases in dedifferentiated Müller glia 48 hours following acute intense light lesion (Qin et al., 2009). The temperature-sensitive *hspd1* mutant line, *no blastema (nbl)*, revealed a ~50% reduction in the number of proliferating Müller glia at the restrictive temperature (Qin et al., 2009), which is similar to what was observed following Stat3 knockdown (Nelson et al., 2012). Taken together, these findings suggest a model in which Hspd1 (likely in association with Hspe1) is required to maintain Müller glia mitochondria in response to retinal damage, and expression of these mitochondrial chaperones may be regulated directly by Stat3.
A signaling cascade involving the heparin binding EGF-like growth factor, HB-EGF, EGF receptor (EGFR) and mitogen activated protein kinase (MAPK) pathway may also function to stimulate proliferation of SPMG. While *hb-egf* expression has not been detected in damaged or dying neurons, it has been localized in INL cells with Müller glia morphology in the light damage model after 36 hours of constant light treatment (Nelson et al., 2013) and in clusters of NPCs in the puncture-damaged retina (Wan et al., 2012). While loss of HB-EGF expression resulted in significantly fewer proliferating NPCs at 4 days following stab lesion (Wan et al., 2012), there are conflicting reports if HB-EGF is sufficient to induce Müller glia proliferation in the absence of retinal damage (Wan et al., 2012; Nelson et al., 2013). Combined with the absence of *hb-egf* expression in the damaged/dying retina neurons, it is unlikely that HB-EGF plays a role in initiating PPMG proliferation in the damaged retina.

As a member of the EGF family, HB-EGF can signal through EGFR and MAPK to regulate multiple cellular processes. In the injured retina, *egfr* is expressed by differentiated Müller glia and dedifferentiated Müller glia/NPCs (Wan et al., 2012). Pharmacological inhibition of EGFR or Erk1/2 MAP kinases using PD1530305 or PD98059 and SL327, respectively, reduced the number of proliferating NPCs at 4 days post injury (Wan et al., 2012). Inhibition of EGFR/MAPK, or HB-EGF knockdown, also reduced expression of genes associated with Müller glia dedifferentiation, such as *lin28* and *ascl1a* (Wan et al., 2012). Taken together, these data suggest that Müller glia expression of HB-EGF likely functions as a paracrine signal that initiates the dedifferentiation response in nearby Müller glia (SPMG), similar to how the Müller glia
expression of TNFα recruits additional Müller glia to reenter the cell cycle (Nelson et al., 2013).

1.5 Maintaining quiescent Müller glia

The regenerating zebrafish retina is capable of activating and recruiting large numbers of Müller glia to dedifferentiate and proliferate (PPMG and SPMG), as well as maintaining a subset of fully differentiated, quiescent Müller glia (Thummel et al., 2008b). These three Müller glial populations could represent a mechanism to regulate Müller glia proliferation so that: 1) healthy tissue is not unnecessarily remodeled, 2) excessive numbers of NPCs are not generated resulting in either excessive neurogenesis and/or cell death, and 3) retinal homeostasis is maintained for the remaining healthy retinal neurons by a subset of the Müller glia remaining quiescent. Several mechanisms have been identified to restrict the zone of regeneration and limit excessive Müller glia dedifferentiation.

During vertebrate retinal development, Ascl1a plays a central role in regulating a Notch/Delta program responsible for progenitor cell maintenance and proper neurogenesis (Nelson et al., 2009). Many studies have documented increased expression of notch and delta genes during zebrafish retinal regeneration (Raymond et al., 2006; Yurco and Cameron, 2007; Wan et al., 2012). These observations certainly suggest a prominent role for Notch signaling during retinal regeneration; however, the spatial and temporal expression of various pathway components has yet to be studied sufficiently. While these detailed experiments will be necessary, some functional data
have revealed that Notch is required to prevent unnecessary Müller glia proliferation. Wan et al. (2012) demonstrated that application of the \( \gamma \)-secretase inhibitor, DAPT, expanded the zone of Müller glia proliferation following puncture lesion. Additionally, overexpression of the cleaved Notch effector, NICD, inhibited proliferation, though this effect seemed to be most prominent at 4 days post puncture injury (Wan et al., 2012), suggesting a role in recruiting SPMG or NPC amplification. While Notch signaling remains elusive, it is clear that one of its functions is maintaining Müller glia quiescence.

More recently, Conner et al. (2014) showed that inhibition of Notch signaling via intraperitoneal injection of the \( \gamma \)-secretase inhibitor RO4029097 could also expand Müller glia proliferation in the light damaged retina (Conner et al., 2014). More importantly, intraperitoneal injection of RO4929097 could also stimulate Müller glia proliferation in the undamaged retina (Conner et al., 2014). Additionally, heat shock-induced expression of NICD was able to block the RO4929097-induced Müller glia proliferation (Conner et al., 2014). Together, these studies demonstrated that Notch signaling also maintains Müller glia quiescence in the undamaged retina.

The transcriptional repressor, Insm1a, is initially expressed in all Müller glia across the retina within 6 hours of the puncture damage (Ramachandran et al., 2012). By 2 days post injury, however, insm1a expression became restricted to the dedifferentiated Müller glia (Ramachandran et al., 2012). Furthermore, loss of Insm1a expression resulted in increased hb-egf expression and ChIP analysis revealed that Insm1a can directly bind the hb-egf promoter (Ramachandran et al., 2012). These observations suggest that Müller glia neighboring dedifferentiated Müller glia are
maintained in a quiescent state via Insm1a-mediated transcriptional repression of \textit{hb-egf}.

Morpholino-mediated knockdown of Insm1a expression also expanded the zone of proliferating Müller glia at 4 days post puncture injury (Ramachandran et al., 2012), indicating that Insm1a restricts Müller glia proliferation at the injury site. This is reminiscent of the expansion of the proliferation zone following DAPT-mediated Notch inhibition (Wan et al., 2012). Additionally, DAPT treatment decreased \textit{insm1a} expression (Ramachandran et al., 2012). Thus, Notch signaling likely promotes \textit{insm1a} expression, which represses \textit{hb-egf} expression to limit the zone of Müller glia dedifferentiation at the damage site. While the Müller glia likely express the Notch receptor, it remains to be determined what cells express the Notch ligand. Future studies that colocalize increased \textit{notch} and \textit{insm1a} expression in quiescent Müller glia will be necessary to confirm this model.

Wan et al. (2012) also showed that ectopic HB-EGF-induced Müller glia dedifferentiation was dependent on MMP-mediated cleavage of the HB-EGF ectodomain. If unregulated, this signal could propagate a widespread proliferative gradient. Thus, HB-EGF activity could be restricted to the injury site by MMP expression. This model is supported by microarray experiments that revealed increased expression of several \textit{mmp} genes very early following constant light treatment (Kassen et al., 2007; Qin et al., 2009). Additionally, expression of MMP2 protein increased significantly in dedifferentiated Müller glia and NPCs following light damage (Gorsuch and Hyde, unpublished data).
1.6 Neuronal progenitor cell amplification and migration

Following dedifferentiation, Müller glia divide asymmetrically to produce a transiently amplifying neuronal progenitor cell lineage, as evidenced by one daughter cell maintaining some of its glial identity (Bernardos et al., 2007), and the other expressing the homeobox transcription factor Pax6 (Thummel et al., 2008b; Thummel et al., 2010). The NPC then undergoes several rounds of cell division before migrating along the dedifferentiated Müller glia processes to the site(s) of damage and differentiating (Vihtelic and Hyde, 2000; Fausett and Goldman, 2006; Bernardos et al., 2007).

During NPC amplification, the Pax6 paralogs, Pax6a and Pax6b, play non-redundant roles. Pax6 protein expression is first observed following Müller glia division, with \( pax6b \) mRNA levels increasing prior to \( pax6a \), suggesting that Pax6b is expressed in the initial NPC (Thummel et al., 2010). This is supported by the observation that morpholino-mediated knockdown of Pax6b, but not Pax6a, prevented the first NPC division, while having no effect on the initial Müller glia division (Thummel et al., 2010). Interestingly, preventing NPC proliferation via Pax6b knockdown in the light-damaged retina prevented cone cell regeneration at one month post injury, but extensive proliferation of resident rod precursor cells in the ONL were sufficient to regenerate rod photoreceptors (Thummel et al., 2010). In contrast, loss of Pax6a expression had no effect on the first NPC division, but prevented subsequent NPC proliferation (Thummel et al., 2008). While the identity of the Pax6a and Pax6b transcriptional targets that promote NPC proliferation are unknown, several studies have identified factors that can
regulate \textit{pax6} expression. For example, either knockdown of Ascl1a expression (Fausett et al., 2008) or inhibition of the HB-EGF/EGFR/MAPK pathway (Wan et al., 2012) prevents \textit{pax6b} expression following retinal damage. Additionally, the temperature-sensitive \textit{hspd1} allele, \textit{nbl}, reduces Pax6 expression relative to controls (Qin et al., 2009). It should be noted, though, that while Pax6 levels were affected by perturbation of these pathways, this may be due to reduced Müller glia proliferation (thus, NPC genesis) and not direct regulation of Pax6 within NPCs.

The mitotic checkpoint kinase, \textit{monopolar spindle 1 (mps1)}, also appears to regulate NPC proliferation (Qin et al., 2009). Expression of \textit{mps1} mRNA levels increased following 36 hours of light lesion, after Müller glia dedifferentiate and begin dividing (Qin et al., 2009). Use of a temperature-sensitive \textit{mps1} allele, \textit{nightcap (ncp)}, revealed that MPS1 does not function in the initial Müller glia dedifferentiation/proliferation, but impairs NPC proliferation and/or migration to the ONL (Qin et al., 2009). Interestingly, MPS1 plays a similar role during zebrafish fin regeneration, as it is not required for the initial blastema formation, but rather for rapid progenitor cell proliferation during outgrowth (Poss et al., 2002). This suggests that a conserved mechanism exists for controlling epimorphic regeneration in multiple zebrafish tissues (Qin et al., 2009).

Proliferating NPCs eventually migrate in close association with the radial processes of the dedifferentiated Müller glia to the appropriate sites/layers of damage (Vihtelic and Hyde, 2000; Fausett and Goldman, 2006; Bernardos et al., 2007). This behavior is reminiscent of neuronal tracking along radial glia in the cortex (Noctor et al. 2001), suggesting NPCs may use the radial fibers as mechanical guides to migrate along
to the different retinal layers. Spatial expression of factors associated with changes in cell adhesion and matrix composition support this hypothesis. For example, N-cadherin expression increases in dedifferentiated Müller glia and NPCs (Raymond et al., 2006) in a pattern similar to β-catenin expression (Ramachandran et al., 2011), suggesting the extracellular environment of neurogenic clusters may be coupled directly to intracellular actin dynamics. Additionally, we have observed a similar pattern of MMP2 expression in neurogenic clusters following light damage (Gorsuch and Hyde, unpublished data), suggesting active turnover of the extracellular matrix is necessary for proper NPC migration. While these observations provide insight into the likely mechanisms controlling NPC migration, functional studies are still lacking.

1.7 Differentiation of new neurons and glia

The final step in the zebrafish retinal regeneration program is NPC differentiation and integration into the existing retinal circuitry, as well as renewal of the dedifferentiated Müller glia. Expression studies revealed that some migrating NPCs increase transcription from the olig2 promoter after only 68 hours of light damage (Thummel et al., 2008b) and from the atoh7 promoter at 7 days following intravitreal ouabain injection (Fimbel et al., 2007), suggesting they become committed to a retinal neuron lineage as they reach their terminal location. In contrast, the proneural transcription factor, Neurogenin1 (Ngn1), is expressed by dedifferentiated Müller glia as early as 51 hours of light damage, and maintained in redifferentiated Müller glia until 17
days post light damage, suggesting Ngn1 plays a role specifically in Müller glia renewal (Thummel et al., 2008b).

Similar to Ngn1, activation of the Tg(EPV.Tp1-Mmu.Hbb:EGFP) Notch reporter line shows Notch activity in redifferentiated Müller glia, and inhibition of Notch activity via γ-secretase inhibitors prevents Müller glia renewal (Conner et al., 2014). This recapitulates the role of Notch signaling during vertebrate retinal development, where Notch signaling is known to drive progenitors to a Müller glia cell fate (Furukawa et al., 2000; Bernardos et al., 2005). These data are also consistent with Müller glia dedifferentiation requiring loss of Notch signaling, as reestablishment of Notch signaling can drive redifferentiation of the activated Müller glia.

In the undamaged retina, Notch inhibition via RO4929097 injection stimulates Müller glia proliferation, but these proliferating cells fail to differentiate into functional neurons and die (Conner et al., 2014). Co-injection of RO4929097 and recombinant zebrafish TNFα was sufficient to stimulate a robust regeneration response similar to the light-damaged retina. Remarkably, these cells are able to differentiate into multiple neuronal cell types (Conner et al., 2014), suggesting that both Notch inhibition and TNFα signaling are necessary for initiating a complete regeneration response that culminates in neuronal differentiation.

How NPCs are specified to regenerate neurons as diverse as photoreceptors and ganglion cells remains elusive. During vertebrate retinal patterning, apical-basal gradients of various signaling environments can control cell-type specification (Del Bene et al., 2008; reviewed by Willardsen and Link, 2011). Similar mechanisms may play a
role in zebrafish retinal regeneration. For example, components of the FGF signaling pathway are differentially expressed along the apical-basal axis of the adult zebrafish retina (Qin et al., 2011; Hochmann et al., 2012), with \textit{fgfr1a} mRNA (Qin et al., 2012) and Fgfr1a protein (Hochmann et al., 2012) expression detected in the ONL, basal INL and GCL, while \textit{fgfr2} and \textit{fgfr3} mRNAs were detected only in the basal INL and apical INL, respectively (Hochmann et al., 2012). Genes encoding FGF ligands were also differentially expressed in the adult zebrafish retina, with \textit{fgf8a} and \textit{fgf24} being expressed in the INL and GCL, and \textit{fgf20a} being expressed in all three nuclear layers (Hochmann et al., 2012). The differential expression of these FGF components may represent a mechanism in which cell identity is reinforced by each unique signaling environment. There is some evidence to support this, as heterozygous \textit{Tg(hsp70:dn-fgfr1)}/+ zebrafish, which express a dominant-negative Fgfr1, fail to regenerate rods following light damage, but cone regeneration and Müller glia proliferation are unaffected (Qin et al., 2011). In contrast, homozygous \textit{Tg(hsp70:dn-fgfr1)} zebrafish displayed impaired Müller glia proliferation and disruption of both rod and cone regeneration following light damage (Hochmann et al., 2012). These data reveal that FGF signaling plays a role in maintaining and regenerating rod photoreceptors following light damage, however, detailed experiments will be necessary to tease apart these two roles.

In addition to the intracellular and extracellular signals that dictate proper differentiation of NPCs during retinal regeneration, cell cycle exit is necessary to produce the requisite number of progenitor cells to regenerate the correct number of...
retinal neurons. However, the details surrounding the underlying mechanisms and timing of NPC cell cycle exit remain poorly understood. PCNA-positive NPCs almost completely migrate out of the INL and into the ONL by 3 days of recovery following 4 days of constant light treatment (Thummel et al., 2008b; Thummel et al., 2010), which may indicate a time at which most of the NPCs have gone through a terminal cell division. While this information is useful for studying photoreceptor regeneration following constant light treatment, other damage paradigms likely do not share this timeline (Fimbel et al., 2007; Sherpa et al., 2008), making markers for cell cycle exit imperative.

The transcriptional repressor Insm1a, which mediates earlier events in the regeneration program (see above) is also necessary for proper NPC cell cycle exit (Ramachandran et al., 2012). Between 4 days post puncture lesion, when NPCs are amplifying in number, and 6 days post lesion, when NPCs are likely exiting the cell cycle, the proportion of BrdU-positive cells that also express *insm1a* increased from 40% to 80%, respectively (Ramachandran et al., 2012). In addition, at 6 days post injury, cell cycle regulators, such as *ccna2*, *ccnb1*, *ccnd1*, *cdk1* and *cdk2* were reduced in expression. Loss of Insm1a expression resulted in increased levels of these cell cycle genes, suggesting Insm1a functions in late-stage NPCs to repress cell cycle regulators (Ramchandran et al., 2012). In contrast, expression of the cyclin-dependent kinase inhibitor, *p57kip2* (*cdkn1c*) was maintained at high levels in an Insm1a-dependent manner at later time points. As a transcriptional repressor, Insm1a maintains *p57kip2* expression by inhibiting another transcriptional repressor, *bcl11a* (Ramachandran et al., 2012).
2012). While this study is the first to closely analyze NPC cell cycle exit, further experiments will be necessary to understand the biological relevance of the dynamic gene expression levels for many of these factors. Importantly, this study indicates that p57kip2 may be a suitable marker for terminal division and cell cycle exit of NPCs during retinal regeneration.

1.8 Sox2 background

Sox2 is a member of the highly conserved B1 family of high mobility group (HMG) domain transcription factors. In mammals and birds, this family is comprised of SOX1, SOX2 and SOX3. Because of the teleost whole genome duplication, this family in zebrafish includes sox1a, sox1b, sox2, sox3, as well as two additional members sox19a and sox19b (Okuda et al., 2006). The latter two are likely orthologous to the mammalian Sox15 (SoxG group). Though Sox2 functions primarily as a transcriptional activator, by interacting with a multitude of other factors, such as Oct4 (Reményi et al., 2003), Otx2 (Danno et al., 2008), Pax6 (Kamachi et al., 2001; Danno et al., 2008) and chromatin remodeling proteins (Cimadamore et al., 2013), recent evidence suggests Sox2 may also recruit transcriptional repressors (Liu et al., 2014). The ability of Sox2 to interact with such a vast array of partners makes it a highly versatile regulator of gene expression, but the complexity also makes studying Sox2 difficult.

Sox2 is required to maintain pluripotency of ES cells and is one of the original Yamanaka factors required for generation of iPSCs (Takahashi et al., 2007). Building off of this work, researchers have begun to develop transdifferentiation strategies, forcing
functional conversion of one cell state directly to another via different reprogramming cocktails. For example, human pericyte cells from the adult cerebral cortex can be reprogrammed directly into functional neurons by overexpressing SOX2 and MASH1 (Karow et al., 2012). More recently, Sox2 alone was sufficient to convert NG2 glia into functional neurons in vivo following stab lesion to the adult mouse cerebral cortex (Heinrich et al., 2014).

In the developing vertebrate retina, Sox2 is necessary to maintain both neurogenic and gliogenic potential of retinal progenitor cells. In frogs and fish, sox2 expression in retinal progenitor cells (RPCs) is maintained by canonical Wnt/β-catenin signaling (Agathocleous et al., 2009; Meyers et al., 2012). Using sophisticated genetic manipulations available in the mouse, Taranova et al. (2006) showed that Sox2 functions in a dose-dependent manner to regulate retinal development. Together these studies revealed that Sox2 is required to both maintain RPCs in a proliferative progenitor state and to promote differentiation of neuronal and glial lineages. It would seem that the processes of maintaining a progenitor state and promoting differentiation are strongly juxtaposed, but given the wide array of interacting partners for Sox2, small changes in the signaling environment could lead to significant changes in gene regulation and the resulting cellular responses.

In the mature vertebrate retina, Sox2 expression is maintained in the Müller glia and a subset of amacrine cells (Lin et al., 2009; Whitney et al., 2014). In the postnatal mouse retina, Sox2 is required to prevent terminal mitotic division of Müller glia (Surzenko et al., 2013). Conditional ablation of Sox2 in Müller glia causes loss of radial
morphology, loss of quiescence and global disruption of retinal morphology. The maintenance of these progenitor qualities of Müller glia is the fundamental basis of retinal regeneration in zebrafish, suggesting a significant role for Sox2 in this process.

1.9 Summary

In response to retinal damage, zebrafish Müller glia reprogram their genome to partially dedifferentiate and divide, giving rise to a population of neuronal progenitor cells (NPCs). The NPCs will amplify and migrate to the site of damage, where they will differentiate to regenerate the lost or damage photoreceptors. Several key molecular pathways that regulate this regeneration response are being elucidated. Ascl1a is one of the most well-characterized factors required for Müller glia proliferation in response to retinal damage (Fausett et al., 2008; Ramachandran et al., 2010; Nelson et al., 2012). Ascl1a also regulates Müller glia proliferation induced following Notch-inhibition and TNFα exposure in the undamaged retina (Conner et al., 2014). In addition, Stat3 has also been shown to regulate Müller glia proliferation in multiple damage models (Nelson et al., 2012; Wan et al., 2014) and damage-free Müller glia proliferation models (Conner et al., 2014). Müller glial expression of Ascl1a and Stat3 are induced following retinal damage, likely via TNFα produced by dying photoreceptors (Nelson et al., 2012; Nelson et al., 2013). The increased Ascl1a and Stat3 expression in the Müller glia that respond directly to the dying photoreceptor-secreted TNFα also stimulated increased TNFα production from the Müller glia (Nelson et al., 2013). The increased Müller glial secretion of TNFα was necessary to recruit additional Müller glia.
to reenter the cell cycle in a similar Ascl1a and Stat3-dependent manner. These studies illustrated the dynamic and complex nature of Müller glia proliferation and highlight two of the key regulators of this process. As we continue to identify new molecular regulators of Müller glia proliferation, we will utilize Ascl1a and Stat3 as "core" regulators for comparison.

Because reprogramming the Müller glia genome is likely the rate-limiting step for retinal regeneration, I hypothesized that canonical reprogramming factors (Takahashi et al., 2007) may regulate this process. Ramachandran et al. (2010) showed that expression of many of these reprogramming factors increased following retinal stab-lesion, however no spatial expression or functional data was characterized. In particular, we were interested in sox2, which is a well established neural stem cell marker in the neurogenic regions of the adult vertebrate brain (Favaro et al. 2009). Additionally, Sox2 expression is maintained in the Müller glia of the mature mammalian retina and is required to maintain Müller glial identity (Surzenko et al., 2013). The goal of this work is to begin characterizing the role Sox2 plays during Müller glia reprogramming in the regenerating zebrafish retina, and how Sox2 interacts with known regulators of Müller glia reprogramming.

I will show a key role for Sox2 in Müller glia reprogramming during zebrafish retinal regeneration. I confirmed that basal Sox2 expression is maintained in amacrine cells and Müller glia of the mature zebrafish retina, recapitulating the expression pattern observed in other vertebrates. Following light-induced photoreceptor cell death, Sox2 expression significantly increased in reprogrammed Müller glia as they
began proliferating. I found that Sox2 expression was necessary, and overexpression of Sox2 was sufficient, for Müller glia proliferation. While investigating the relationship between Wnt/β-catenin signaling and Sox2 in Müller glia reprogramming, we determined that β-catenin 2, but not β-catenin 1 was required for Müller glia proliferation, but neither regulated sox2 expression. I concluded that Sox2 expression was required for ascl1a expression, most likely through induction of lin28a-dependent repression of let7 miRNA biogenesis. In addition the role Sox2 plays during Müller glia reprogramming, I also found that Sox2 expression is required for amplification of NPCs, which likely downregulate Sox2 as they become committed neurons and differentiate. This work is the first to describe a functional role for Sox2 during zebrafish retinal regeneration.
CHAPTER 2:

MATERIALS AND METHODS

2.1 Zebrafish maintenance and light-lesion protocol

Wild-type AB, albinob⁴, albinob⁴; Tg(gfap:EGFP)⁷¹⁻¹ (Kassen et al., 2007), and Tg(hsp70l:sox2)x²¹ (Millimaki et al., 2010) zebrafish lines were maintained in the Center for Zebrafish Research at the University of Notre Dame Freimann Life Science Center. Adult zebrafish used for these studies were between 6-12 months old (4-5 cm) and maintained under a standard 14 hour light-10 hour dark cycle at 28.5°C (Westerfield, 1993). Rod and cone cell death was carried out according to established protocols (Vihtelic and Hyde, 2000; Vihtelic et al., 2006). Briefly, adult fish were dark adapted for 14 days, then transferred to clear polycarbonate tanks placed between 4 fluorescent bulbs (15,000-20,000 lux) for various times, up to 4 days. After light treatment, fish were euthanized by anesthetic overdose of 0.2% 2-phenoxyethanol and eyes were enucleated for further processing. All experimental protocols were approved by the animal use committee at the University of Notre Dame and are in compliance with the ARVO statement for the use of animals in vision research.
2.2 Heat shock and injection protocol

Adult *Tg(hsp70l:sox2)*\textsuperscript{21} transgenic zebrafish and wild type siblings were genotyped using the following primers: sox2 R (5'- CTTCAGCTCGGTTCATCATG-3') and hsp70l F (5'- CTCCTCTCAATGACAGCTG-3'). Fish were heat shocked daily at 38°C for two or four days. Fish were transferred to 3-inch diameter polycarbonate tubes (3-4 fish per tube) with mesh screen bottoms in a circulating water bath. Water temperature was set to 28°C and gradually ramped up to 38°C over the course of 30 minutes. Fish were maintained at 38°C for one hour before being transferred back to plastic tanks filled with 38°C water. Water temperature was allowed to cool slowly to room temperature before being placed back on the system.

Injections of RO4929097 and recombinant zebrafish TNF\textalpha were performed as previously described (Conner et al., 2014). Briefly, adult AB zebrafish were injected intraperitoneally with \textasciitilde 25 \textmu L of 1 mM RO4929097 using a 30 gauge beveled needle. Recombinant TNF\textalpha (0.5-1 \textmu L at \textasciitilde 1 mg/mL concentration; Conner et al., 2014) was intravitreally injected into left eyes with Hamilton syringe (World Precision Instruments) and a 30 gauge blunt end needle after using a sapphire blade (World Precision Instruments) to cut a small hole in the cornea. Control fish were intraperitoneally injected with the 10% DMSO and left eyes injected with Ni-NTA elution buffer. Injections were carried out every 12 hours for three days. For each fish, the right eye was used as RO4929097 or DMSO control, and the left eyes was used as RO4929097/TNF\textalpha or DMSO/elution buffer control.
2.3 Injection and electroporation of morpholinos into adult zebrafish retinas

Morpholino-mediated knockdown of protein expression in adult zebrafish retinas was performed as previously described (Thummel et al., 2008; Thummel et al., 2011). Briefly, a 1 mM solution of lissamine-tagged morpholinos was intravitreally injected into the left eye of dark-adapted albinob4 zebrafish prior to the initiation of light treatment. The morpholinos (MOs) used in this study are listed in Table 2.1. Platinum plate electrode tweezers (CUY650-P3, Protech International Inc.) were used to deliver two 50 ms pulses (75 V with a 1 second pause between pulses) to the left eye using a CUY21 Square Wave Electroporator (Protech International Inc.). Fish were allowed to recover and returned to the dark or placed in constant light for various periods of time.

2.4 Immunofluorescence

Fish were euthanized in 0.2% 2-phenoxyethanol. Eyes were enucleated and fixed in either 4% paraformaldehyde/1x PBS or in 9:1 ethanolic formaldehyde. After fixation in 4% PFA, eyes were briefly rinsed with 1x PBS, then washed 3 x 5 minutes in 5% sucrose/1x PBS. Eyes fixed in 9:1 were rehydrated through an ethanol series (90%, 80%, 70%, 50% v/v ethanol in water) and then washed with 5% sucrose as described above. Eyes were incubated in 30% sucrose/1x PBS at 4°C overnight. The next day, eyes were transferred to a 2:1 mixture of tissue freezing media (TFM, Triangle Biomedical Sciences) and 30% sucrose/1x PBS and incubated at 4°C overnight. Eyes were then
TABLE 2.1
MORPHOLINOS USED IN THESE STUDIES

<table>
<thead>
<tr>
<th>Morpholino</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std Control</td>
<td>5'-CCTCTTACCTCAGTACCTTTATA-3'</td>
<td>Gene Tools</td>
</tr>
<tr>
<td>ctnnb1</td>
<td>5'-ATCAAGTCAGACTGGGTAGCCATGA-3'</td>
<td></td>
</tr>
<tr>
<td>ctnnb2</td>
<td>5'-AGCCATCGTTGCATCTATTTTAG-3'</td>
<td></td>
</tr>
<tr>
<td>sox2</td>
<td>5'-GAAATGTCTACCCACCAGCCTTAA-3'</td>
<td>Kamachi et al., 2008</td>
</tr>
<tr>
<td>ascl1a</td>
<td>5'-ATCTTTGGGATGTCCATTTTCG-3'</td>
<td>Fausett et al., 2008</td>
</tr>
<tr>
<td>lin28</td>
<td>5'-TGAGATGCGGATTTGCGGGGGG-3'</td>
<td>Ramachandran et al., 2010</td>
</tr>
</tbody>
</table>
embedded in 100% TFM and frozen at -80°C before 12-14 µm sections were obtained and stored at -80°C.

Frozen retinal sections were dried at 55°C for 20 minutes before rehydrating in 1x PBS for 20 minutes. Sections were blocked for one hour in blocking buffer (1x PBS, 4% normal goat serum, 0.4% Triton X-100, 1% DMSO). Primary antibodies were diluted in blocking buffer, applied to sections and incubated overnight at 4°C. The primary antibodies used in these studies and their dilutions were as follows: mouse monoclonal anti-PCNA antibody (1:1000; P8825, clone PC10, Sigma-Aldrich); rabbit anti-Sox2 polyclonal antiserum (1:100; GTX124477, GeneTex); goat anti-Sox2 polyclonal antiserum (1:100; AF2018, R&D Systems); rabbit anti-β-catenin polyclonal antiserum (1:100; ab6302, Abcam); rabbit anti-GFP polyclonal antiserum (1:500; ab6556, Abcam); rabbit anti-Ascl1a polyclonal antiserum (1:50; HPA029217, Sigma-Aldrich); mouse anti-HuC/D monoclonal antibody (1:250; A21271, clone 16A11, Life Technologies); mouse anti-Glutamine synthetase monoclonal antitbody (1:500; MAB302, clone GS-6, Millipore). Sections were washed 3 x 10 minutes in PBST (1x PBS with 0.5% Tween-20). Fluorescent-labeled secondary antibodies were diluted 1:500 in PBST and applied to sections for 1 hour at room temperature. Secondary antibodies used in these studies were as follows: goat anti-mouse lgG or goat anti-rabbit lgG conjugated to Alexa Fluor 488, 568, 594, or 647; chicken anti-goat lgG conjugated to Alexa Fluor 488 (Life Technologies).

In experiments utilizing goat anti-Sox2 polyclonal antiserum, the blocking buffer was prepared with an equivalent amount of normal chicken serum rather than normal...
goat serum. If the goat anti-Sox2 polyclonal antiserum was used for colabeling with
either rabbit or mouse antibodies, the secondary antibody staining was carried out in
two steps. First, chicken anti-goat Alexa Fluor 488 was incubated for 1 hour and washed
as described above for secondary antibody staining. This process was then repeated
with a goat anti-rabbit and/or goat anti-mouse Alexa Fluor antibodies required to detect
the additional primary antibodies.

To detect Sox2, Ascl1a, HuC/D and PCNA (in retinas fixed in 4% PFA) antigen
retrieval was required (Raymond et al., 2006; Nelson et al., 2012; Nelson et al. 2013).
Coplin jars were filled with citrate buffer (10 mM citrate pH 6.0 with 0.1% Tween-20)
and placed in boiling water baths. Warmed slides were placed directly into the hot
citrate solution and incubated for 20 minutes, covered. Coplin jars were then removed
from water baths and allowed to cool gradually at room temperature (~45 minutes).
Slides were then briefly rinsed 3 x 2 minutes in 1x PBS, processed, and transferred to
block as described above.

2.5 TUNEL

Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end
labeling (TUNEL) was performed using the ApoAlert DNA Fragmentation Assay kit
(CloneTech) as previously described (Bailey et al., 2010; Nelson et al., 2013).
Biotinylated dNTPs were detected by using Alexa Fluor 488-conjugated streptavidin (Life
Technologies) diluted 1:200 in PBS for 20 minutes at room temperature. Slides were
mounted with glass coverslips and VECTASHIELD (Vector Labs).
2.6 Immunoblot analysis

Total protein lysates were obtained by pooling 5-10 adult dorsal retinas per treatment and homogenizing in protein extraction buffer (1x PBS, 1% Triton-X-100, protease inhibitors, 10 µL per retina; Roche Applied Science) using a polypropylene micro pestle. Lysates were incubated for one hour on ice then centrifuged briefly to remove Triton-X-100-insoluble debris and the supernatant was collected and stored at -80°C. Protein concentration was determined using Bradford assay (Pierce) and 10 µg of lysate was combined with Novex 5x tris-glycine-SDS sample buffer and 10x sample reducing agent (Life Technologies). Samples were heated at 95°C for 5 minutes and then electrophoresed through Novex 4%-12% tris-glycine gels (Life Technologies). Proteins were transferred to Hybond-P PVDF membrane (GE Healthcare) and then blocked for 1 hour at room temperature blocking buffer composed of PBST (1x PBS with 0.1% Tween-20) with 5% (w/v) nonfat dry milk. Membranes were probed with the following antibodies diluted in blocking buffer overnight at 4°C: rabbit anti-β-catenin polyclonal antiserum (1:5000; ab6302, Abcam); rabbit anti-Sox2 polyclonal antiserum (1:2000; GTX124477, GeneTex); mouse anti-Glutamine synthetase monoclonal antibody (1:1000; MAB302, clone GS-6, Millipore); rabbit anti-GFAP polyclonal antiserum (1:5000; Z0334, Dako); mouse anti-Actin monoclonal antibody (1:5000, clone AC-40, Sigma-Aldrich). Membranes were washed three times for 5 minutes, then once for 15 minutes in PBST before incubating with ECL HRP-conjugated secondary antibodies (1:10,000, GE Healthcare) diluted in blocking buffer. Membranes were washed as before and then developed using the ECL Prime kit (GE Healthcare). Exposed film was
imaged using a Carestream Gel Logic 2200 Pro imaging station (Carestream Health). Actin was used as a loading control for all blots.

2.7 Quantitative real-time PCR

RNA was prepared from light treated retinas as previously described (Nelson et al., 2012; Nelson et al., 2013). Briefly, 8-10 dorsal retinas per time point or treatment group were dissected and pooled. RNA was extracted using TRIzol reagent (Life Technologies) and purified RNA was DNase treated using the Turbo DNA-free Kit (Life Technologies). Total cDNA was generated from 1 µg of RNA using qScript cDNA SuperMix (Quanta Biosciences).

Reactions were assembled using PerfeCta SYBR Green SuperMix (low ROX; Quanta Biosciences). For most targets, 25 ng of cDNA was sufficient for amplification within an optimal Ct range. For 18S controls, 0.25 ng of cDNA were used. The gene specific primers for these studies (Table 1) were used at a final concentration of 300 nM. Reactions were carried out and data acquired using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with data collection occurring after extension for each cycle. Dissociation curve analysis verified that single products were produced with each set of the primer pairs used in these experiments. For each gene examined by qRT-PCR cDNA from each time point or treatment was run in triplicate and the median Ct value was normalized against the 18S rRNA Ct. The comparative ∆∆Ct method was used for data analysis, with 0 hours of light
TABLE 2.2
PRIMERS USED FOR QUANTITATIVE REAL-TIME PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primers for qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>sox2</td>
<td>F: 5'-GCTCCAGTACAACCTCCATGAC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTGCGAATAGGACATGCTGTAG-3'</td>
</tr>
<tr>
<td>ctnnb1</td>
<td>F: 5'-CAAGAGCAAGTAGCAGACATCG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTGCGAATAGGACATGCTGTAG-3'</td>
</tr>
<tr>
<td>ctnnb2</td>
<td>F: 5'-AACTACCAGGACGTAGCAGAG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTGCGAATAGGACATGCTGTAG-3'</td>
</tr>
<tr>
<td>stat3</td>
<td>F: 5'-GAGGAGGCGTTGGGCAAA-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TGTGTCAGGGAACTCAGTCTG-3'</td>
</tr>
<tr>
<td>ascl1a</td>
<td>F: 5'-GCCAGACGGAACGAGAGA-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGGTTGCAAAGCCTGTTG-3'</td>
</tr>
<tr>
<td>lin28</td>
<td>F: 5'-TAACGTCGGGATGGGCTCGAT-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ATTGGGTCCACAGTTGAAAGCAGT-3'</td>
</tr>
<tr>
<td>18s</td>
<td>F: 5'-TCGGCTACCATCCAGGAAGGCAGC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TTGCTGGAATCCAGGCCGCTG-3'</td>
</tr>
</tbody>
</table>
serving as the reference time point and the 18S rRNA as the reference gene to generate a log2-fold difference in gene expression levels (Johnson et al., 2000; Vong et al., 2003). For knockdown experiments measuring gene expression among different treatments at the same time point, data are presented in linear fold-change.

2.8 Confocal imaging and statistical analysis

Confocal imaging was performed with a Nikon A1R laser scanning confocal microscope. Low-intensity signals were enhanced and background signals were reduced in representative images using the levels function in Adobe Photoshop (Adobe Systems). Levels were adjusted identically to all layers within a panel and to all panels in a figure. Red-green color scheme images were converted to magenta-green using Adobe Photoshop as described previously (Montgomery et al., 2010).

To maintain consistency between eyes and experimental groups, only retinal sections encompassing or immediately adjacent to, the optic nerve were utilized. Quantification of all markers was performed on 5 µm thick confocal z-stacks from the middle of the 14 µm sections. All images were acquired across a 350 µm region of the central/dorsal retina that consistently received the same degree of damage and reliably resulted in comparable subsequent high levels of Müller glia proliferation (Thomas et al., 2012). Retinas from ten different individual fish were examined for every control and experimental group at each timepoint in this study. Statistical significance was determined between control and experimental groups of all experiments using a two-
tailed Student’s t-test, in which p-values less than 0.05, 0.01, and 0.005 were considered significant, highly significant, and very highly significant, respectively.

2.9 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed on adult retinal tissue using the MAGnify ChIP system (Life Technologies) with several modifications. Dark-adapted adult albino zebrafish were subjected to very intense ultraviolet light (~100,000 lux) for 30 minutes before initiating the constant light treatment protocol described above (Thomas et al., 2012). After 48 hours of constant light, 20 whole retinas (~30 mg tissue) were dissected into ice-cold Hanks’ balanced salt solution (HBSS). The same number of undamaged retinas were collected as controls. Retinal tissue was dissociated on ice by 10 passages through an 18 G needle followed by 20 passages through a 21 G needle. Dissociated retinal cells were washed twice with ice-cold PBS (400 x g for 5 minutes at 4°C) and resuspended in 300 µL of room temp PBS with 1% formaldehyde (prepared from ampules of methanol free, 16% formaldehyde solution; Pierce) and incubated for 12 minutes at room temperature on a nutating rocker. The crosslinking reaction was quenched by adding 1.25 M glycine to a final concentration of 0.125 M and incubated for 5 minutes at room temperature on a nutating rocker. Crosslinked cells was washed twice with ice-cold PBS supplemented with 1x protease inhibitor cocktail (MAGnify ChIP system, Life Technologies). Pelleted cells were resuspended in 90 mL of MAGnify ChIP Lysis Buffer supplemented with ice cold 1x protease inhibitor cocktail. Chromatin was sheared at 4°C with a Covaris S220 sonicator.
using the following settings: 600 seconds; 5% duty cycle; 70 Watts peak incident power; 200 cycles per burst. Sheared chromatin was centrifuged at 20,000 x g for 5 minutes at 4°C and the supernatant was aliquoted, snap frozen on dry ice and stored at -80°C until ready to use for ChIP.

Chromatin was diluted 1:10 in Dilution Buffer (MAGnify ChIP system; Life Technologies) and precipitated with 5 µg of rabbit anti-RNA polymerase II antibody (Abcam, ab5131) or 5 µg rabbit IgG control antibody (MAGnify ChIP system, Life Technologies). Binding, washing and DNA elution were carried out according to the manufacturer's protocol (MAGnify ChIP system, Life Technologies).

Quantitative real-time PCR was carried out using 5 µL of eluted DNA and SYBR Green Supermix (Quanta Biosciences). The following primers were used to amplify the promoter region of the indicated genes: rho-F, 5'-GCCAGATATGCAGTGCAATGATG-3'; rho-R, 5'-GCGGTAATCTCTAGATGTCTGG-3'; gapdh-F, 5'-GTACGACCTTGACATTCTAAGGG-3'; gapdh-R, 5'-CGTTTGTGAGAAGCCTCTGTTC-3'; stat3-F, 5'-GAGGAGGCGTTTGGCAAA-3'; stat3-R, 5'-TGTGTCAGGGAAACTCAGTTCTAGTGCTG-3'. Data was calculated as a percent of input chromatin according to the manufacturer's protocol (MAGnify ChIP system, Life Technologies).

2.10 Genome editing

CRISPR target sites were identified using the ZiFiT Targeter website (http://zifit.partners.org/ZiFiT/). A gRNA targeting the 5'-UTR of sox2 was generated by annealing the ZiFiT Targeter-designed oligos (Oligo 1, 5'-TAGGGTAGACTTTTCGAGAAAAT-
3'; Oligo 2, 5'-AAACATTTTCTCGAAAGTCTAC-3') and ligating the annealed fragment into
*BsaI*-digested pDR274 (Addgene). PCR was used to amplify a fragment containing the T7
promoter and gRNA sequence from the recombinant pDR742 using the following
primers: pDR274 F, 5'-CTAGCTAATACGACTCACTATAGG-3'; pDR274 R, 5'-
AAAAGCACCAGACTCGGTGCC-3'. *In vitro* transcription of gRNA was carried out using the
MEGAshortscript kit (Life Technologies). *Cas9* mRNA was transcribed from pMLM3613
(Addgene) using the mMessage mMACHINE T7 Ultra kit (Life Technologies). Transcribed
RNAs were cleaned up with phenol/chloroform/isoamyl alcohol extraction and
resuspended in nuclease-free water at a concentration of 250 ng/µL (gRNA) or 500
ng/µL (*Cas9* mRNA).

RNAs were co-injected into one-to-four cell stage zebrafish embryos at
concentrations of 25 ng/µL (gRNA) and 250 ng/µL (*Cas9* mRNA). Control embryos were
injected with *Cas9* mRNA alone. At 24 hours post fertilization (hpf), embryos were
collected for genomic DNA extraction. An ~600 base pair (bp) fragment surrounding the
CRISPR target site in the 5'-UTR of *sox2* was amplified with AccuPrime Pfx SuperMix (Life
Technologies) using the following primers: *sox2* T7EI F, 5'-TTATGCAAACCGAGGGAGCAG-
3'; *sox2* T7EI R, 5'-TCGATGAATGGTCGCTTCTC-3'. PCR products were purified using
PureLink PCR Purification columns (Life Technologies). T7 endonuclease I (T7EI; New
England Biolabs) assays were performed according to the manufacturer's protocol.
Briefly, 200 ng of purified PCR product were hybridized in 1x T7EI reaction buffer using
the following thermocycler settings: 95°C for five minutes; ramp to 85°C at -2°C per
second; ramp to 25°C at -0.1°C per second; hold at 4°C. Following hybridization, 1 µL of
T7E1 enzyme was added to each reaction and incubated at 37°C for 15 min. T7E1-mediated cleavage was analyzed via agarose gel electrophoresis and ethidium bromide staining.
CHAPTER 3:

RESULTS

3.1 Sox2 is necessary and overexpression of Sox2 is sufficient to induce Müller glia proliferation

3.1.1 Sox2 expression is conserved in the adult vertebrate retina

Expression of Sox2 in the developing vertebrate retina has been widely studied in rodent, chick, amphibians and fish (Van Raay et al., 2005; Taranova et al., 2006; Lin et al., 2009; Agathocleous et al., 2009; Meyers et al., 2012). In addition, Sox2 expression persists into adulthood in a subpopulation of amacrine cells localized in the inner nuclear layer (INL) and ganglion cell layer (GCL) and all Müller glia in most vertebrates (Taranova et al., 2006; Lin et al., 2009; Surzenko et al., 2013). This Sox2 expression pattern is also present in juvenile zebrafish (Meyer et al., 2012); however, it was not conclusively demonstrated in the mature adult zebrafish retina.

To examine Sox2 expression in the adult zebrafish retina, immunofluorescence on cryosections of undamaged zebrafish retinas were colabeled with the amacrine cell marker HuC/D (Fig. 3.1). We observed a subset of Sox2-positive cells colabeling with HuC/D-positive amacrine cells in the INL and displaced amacrine cells in the GCL (Fig. 3.1 A-C, arrows). Additionally, Sox2 expression was detected in a population of HuC/D-
Figure 3.1: Sox2 expression is maintained in the Müller glia and a subset of amacrine cells in the adult zebrafish retina. Cryosections of undamaged adult zebrafish retinas were prepared and labeled with Sox2 antibodies (A-F, green) and either HuC/D (A-C, magenta) or glutamine synthetase (GS; D-F, magenta). Sox2 labeled cells with round nuclei in the INL and GCL (A and D, arrows) and fusiform nuclei in the INL (A and D, arrowheads). Co-labeling with the amacrine cell marker HuC/D revealed that the round Sox2-positive nuclei were also HuC/D-positive. Fusiform Sox2-positive nuclei also expressed the Müller glial marker glutamine synthetase. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel A is 25 µm and is the same for panels B-F.
negative INL cells with fusiform-shaped nuclei, indicative of Müller glia (Fig. 3.1 A-C, arrowheads). Costaining undamaged retinal sections for Sox2 and the Müller glial marker glutamine synthetase (GS) confirmed that a subset of Sox2-positive INL cells were Müller glia (Fig. 3.1 C-D). Thus, Sox2 expression in the adult zebrafish retina recapitulates that of other vertebrates. It is worth noting that, despite Sox2 being expressed in the ciliary marginal zone (CMZ) of embryonic and juvenile zebrafish retinas (Meyer et al., 2012), we did not detect Sox2 expression in the CMZ of the adult retina (data not shown).

3.1.2 Sox2 expression increases in proliferating Müller glia following light damage

Because Sox2 expression persists in the Müller glia in adult zebrafish retinas and Sox2 is widely considered an adult neural stem cell marker, we hypothesized that Sox2 may be required for Müller glia reprogramming and proliferation in the damaged retina. Using qRT-PCR, we found a significant increase in sox2 expression following 31 hours of light damage (Fig. 3.2 A). This time point corresponds to the initiation of Müller glia proliferation (Kassen et al., 2007). The sox2 expression profile through the 96 hour light-treatment time course is similar to what we observed in a microarray study of light-damaged retinas (Fig. 3.2 A; Kassen et al., 2007).

To investigate the spatial localization of the increased Sox2 protein expression in the light-damaged retina, we utilized immunofluorescence on retinal cryosections. We observed increased Sox2 expression in a population of fusiform-shaped INL nuclei following 31 hours of light, relative to the weak basal INL expression observed in the
Figure 3.2: Sox2 expression increases in proliferating Müller glia after 31 hours of light. RNA was isolated from adult albino zebrafish retinas either prior to, or immediately following 16, 31, 51, 68 and 96 hours of constant intense light. Total cDNA was prepared and sox2 expression was analyzed via qRT-PCR (A). After 31 hours of constant light, sox2 expression increased >2 log2-fold and remained elevated through the remainder of the time course. We observed a similar, but less pronounced, expression profile (A) in the microarray data generated in our lab (Kassen et al., 2007). Retinal cryosections were prepared from either undamaged (B-D) or 31 hour light-damaged eyes (E-K). As we previously observed, Sox2 expression was expressed at a low level in Müller glia of the undamaged retina (B and D, arrows), but was expressed much more strongly in PCNA-positive cells in the INL following 31 hours of light (E-G, arrows). Note that the acquisition settings for these images were adjusted for an optimal dynamic range for Sox2 expression at the 31 hour time point (E and G), accounting for the much weaker expression observed at 0 hours (B and D) with the same settings. Co-labeling of Sox2, PCNA and GFP in Tg(gfap:EGFP) transgenic zebrafish retinas (H-K), which expresses GFP in the Müller glia (Kassen et al., 2007), revealed more robust Sox2 expression in the PCNA-positive Müller glia compared to PCNA-negative Müller glia after 31 hours of light. The insets in panels H-K show a PCNA-negative Müller glial cell with low levels of Sox2 expression (arrow) and a PCNA-positive Müller glial cell with robust Sox2 expression (arrowhead). PCNA, proliferating cell nuclear antigen; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panels B and H are 25 µm and apply to panels C-G and I-K, respectively. Scale bars in panels B and H are 25 µm and are the same for panels C-G and I-K, respectively.
A. *sox2* expression during light damage

![Graph showing *sox2* expression during light damage](image)

- **qRT-PCR**
- **Microarray**

### Images

**B. 0 hr**
- **Sox2**
- **PCNA**
- **Overlay**

**C. 31 hr**
- **Sox2**
- **gfap:EGFP**
- **PCNA**
- **Overlay**
undamaged retina (Fig. 3.2 E and B, respectively). At this time point, the cells expressing increased levels of Sox2 also colabeled with PCNA and GFP in Tg(gfap:EGFP) transgenic zebrafish retinas (Fig. 3.2 E-K), which express EGFP in all Müller glia (Kassen et al., 2007). Importantly, non-proliferating (PCNA-negative) Müller glia displayed basal levels of Sox2 expression (Fig. 3.2 H-K, insert, arrowhead). Together, these data suggest that Sox2 expression, which is maintained at a low level in amacrine cells and Müller glia in the undamaged retina, increases in reprogrammed Müller glia when they begin proliferating in response to light-induced photoreceptor cell death.

3.1.3 Sox2 expression increases during Notch inhibitor/TNFα-induced Müller glia proliferation in the undamaged retina

Conner et al. (2014) recently showed that inhibition of Notch signaling via intraperitoneal injection of the γ-secretase inhibitor RO4929097 could induce Müller glia proliferation in the uninjured retina. Intravitreal injection of recombinant zebrafish TNFα was also sufficient to induce Müller glia proliferation (Conner et al., 2014). Interestingly, combination of these treatments had a synergistic effect on the amount of Müller glia proliferation (Conner et al., 2014). To determine if sox2 expression increases in this injury-free model of Müller glia proliferation, we utilized qRT-PCR. We observed 2.44 ± 0.01-fold increase in sox2 expression after 3 days of RO4929097 injections (RO) and the combination of RO492 and TNFα (RO/TNF) resulted in a 2.08 ± 0.02-fold increase in sox2 expression compared to control retinas (Fig. 3.3 A). To see how the increased sox2 expression correlated with other genes known to regulate Müller glia
Figure 3.3: Expression of sox2 increases following Notch inhibition and TNFα-induced Müller glia proliferation in the undamaged retina. Adult AB zebrafish were intraperitoneally (ip) injected with RO4929097 (RO) or DMSO. To the same fish, the left eyes were intravitreally injected with recombinant zebrafish TNFα (TNF) or protein elution buffer (EB). Injections were carried out every 12 hours for 3 days. Because ip injection results in each eye receiving an equivalent dose of RO or DMSO, the right eyes represent RO-only or DMSO-only, and the left eyes received RO and TNFα (RO/TNF) or DMSO and EB (DMSO/EB). RNA was prepared from retinas from each group and total cDNA was prepared. Analysis of sox2 (A), ascl1a (B), lin28a (C), or stat3 (D) gene expression was carried out using qRT-PCR. Expression of sox2, ascl1a and stat3 increased to a similar degree in both RO and RO/TNF retinas relative to their respective controls. We observed a higher increase of lin28a expression in RO/TNF co-injected retinas compared to RO-only retinas.
reprogramming and proliferation, we also assayed for \textit{ascl1a}, \textit{lin28} and \textit{stat3}. We saw a similar increases in \textit{ascl1a} and \textit{stat3} expression from RO4929097 and RO/TNF\(\alpha\)-injected retinas (Fig. 3.3 B,D). Interestingly, \textit{lin28a} expression increased to a greater extent in the RO/TNF injected retinas relative to the RO retinas (Fig. 3.3 C). It is important to note that gene expression for each treatment (RO or RO/TNF) was normalized to its relative control (DMSO Ctrl or DMSO/TNF Ctrl, respectively). This is significant, as normalizing RO/TNF data to the DMSO control revealed further increased gene expression relative to RO alone, but was also accompanied by increased gene expression in DMSO/TNF control retinas relative to the DMSO controls (not shown). This suggests that corneal incision and/or intravitreal injection of fluid causes some level of stress that is sufficient to induce moderate increases in regeneration-associated gene expression. Together, these data demonstrate that the RO4929097/TNF\(\alpha\)-induced Müller glia proliferation in the undamaged retina display a gene expression profile similar to what we observe following light-damage. More importantly, this gene expression program includes increased \textit{sox2} expression, suggesting Notch inhibition and/or TNF\(\alpha\) may function upstream of Sox2 during regeneration.

\textbf{3.1.4 Sox2 knockdown prevents Müller glia proliferation}

Based on the spatial and temporal expression of Sox2 following retinal damage, and because \textit{sox2} expression increased following RO4929097 and TNF\(\alpha\)-induced Müller glia proliferation in the undamaged retina, we hypothesized that Sox2 may be necessary for Müller glia proliferation. To test this, we electroporated morpholinos to knockdown
Sox2 expression in adult retinas immediately prior to initiating light damage (Thummel et al., 2008a). For these experiments, we utilized an anti-sox2 morpholino empirically determined to provide >80% knockdown of Sox2 in developing zebrafish embryos (Kamachi et al., 2008). We performed immunoblots for Sox2 on zebrafish embryos to verify the efficacy of the anti-sox2 morpholino. At 72 hpf, we observed global reduction in Sox2 expression in sox2 morphant (sox2 MO) embryo lysates relative to uninjected and standard control morphant lysates (Fig. 3.4 A; SC MO). Following injection and electroporation of morpholinos into the adult zebrafish retinas immediately prior to light-treatment, Sox2 expression was virtually undetectable by immunofluorescence in the central-dorsal region of sox2 morphant retinas relative to SC morphant and uninjected control retinas (Fig. 3.4 B-D), confirming successful Sox2 knockdown. The minimal Sox2 expression observed in these retinas was generally relegated to a few circular nuclei in the INL or GCL, which are likely amacrine cells (Fig. 3.4 D, arrows), but not in the fusiform Müller glia nuclei.

To assess the effect of Sox2 knockdown on Müller glia proliferation, we labeled sox2 morphant, SC morphant and uninjected retinal sections with PCNA. Following 31 hours of light, uninjected and SC morphant retinas contained an average of 38.5±2.0 and 36.0±3.9 PCNA-positive cells per 350 µm of retinal section, respectively (Fig. 3.4 E,F,H). In contrast, sox2 morphant retinas displayed significantly fewer PCNA-positive cells per section (Fig. 3.4 G,H; 12.1±2.3; p < 0.001). Thus, Sox2 expression is required for Müller glia proliferation following light-induced retinal damage.
Figure 3.4: Sox2 is required for Müller glia proliferation in the light-damaged zebrafish retina. Protein lysates were prepared from 72 hpf embryos that were either uninjected (UI), or injected with standard control morpholino (SC MO) or anti-sox2 morpholino (sox2 MO) at the 1-4 cell stage. Western blot analysis of these lysates revealed a dramatic reduction of Sox2 protein expression levels in the sox2 morphant embryos compared to controls (A). Actin was used as a loading control (A). Adult eyes were either uninjected, or injected and electroporated with SC morphant or sox2 morphant immediately prior to light treatment. Retinal sections were prepared and labeled with Sox2 antibodies (B-D). Compared to control retinas (B and C), Sox2 expression was undetectable in virtually all Müller glia and amacrine cells in sox2 morphant retinas (D). We observed a few round Sox2-positive nuclei in sox2 morphant retinas (D, arrows), but very rarely observed Sox2 expression in Müller glia of the central-dorsal retina. These retinal sections were also labeled with PCNA antibodies (E-G). Fewer PCNA-positive cells were observed in sox2 morphant retinas (G) compared to controls (E and F). The number of PCNA-positive cells in each treatment was quantified (H) and showed a significant reduction in the number of proliferating cells in the INL of sox2 morphant compared to controls (N ≥ 9; *p < 0.001). PCNA, proliferating cell nuclear antigen; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars in panels B and E are 25 µm and are the same for panels C-D and F-G, respectively.
3.1.5 Sox2 knockdown does not affect photoreceptor cell death

One possible explanation for reduced Müller glia proliferation following Sox2 knockdown is a reduction in photoreceptor cell death. To determine if the reduced Sox2 expression was neuroprotective, we performed TUNEL analysis of SC morphant and sox2 morphant retinal sections following 16 hr of light (the time of peak photoreceptor cell death; Nelson et al., 2013). We observed equivalent numbers of TUNEL-positive photoreceptors in both SC morphant and sox2 morphant retinas (36.4 ± 3.5 and 37.6 ± 2.9, respectively; Fig. 3.5; n = 5, p =0.8). These data demonstrate that the reduced number of proliferating Müller glia in light-damaged sox2 morphant retinas is not due reduced photoreceptor cell death, but likely due to a Sox2-dependent proliferation process in Müller glia.

3.1.6 Sox2 is not required to maintain Müller glia identity

A recent study by Surzenko et al. (2013), utilized conditional ablation of Sox2 in P0 mouse retinas to show that Sox2 is required for maintenance of the limited quiescent progenitor state of mammalian Müller glia. Specifically, the authors observed that conditional knockout of Sox2 in cultured P0 retinas caused Müller glia to lose their radial morphology, reenter the cell cycle and terminally divide. These changes were also accompanied by global disruptions in retinal architecture, mostly likely due to the loss of Müller glia.

To determine if Sox2 is required to maintain Müller glia identity, we knocked down Sox2 expression in non-dark adapted Tg(gfap:EGFP) zebrafish retinas and allowed
Adult albino zebrafish eyes were either uninjected (UI) or injected and electroporated with either standard control morpholino (SC MO) or anti-sox2 morpholino (sox2 MO), prior to exposure to constant intense light. After 16 hours of light treatment, retinal cryosections were prepared and TUNEL analysis was performed. We observed robust TUNEL signal in the ONL of uninjected, SC morphant and sox2 morphant retinas (A-C). Quantification of TUNEL-positive cells revealed no difference in the number between SC morphant (36.4 ± 3.5) and sox2 morphant (37.6 ± 6.6) retinas (D; n = 5, p = 0.8). TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel A is 25 µm and is the same for panels B and C.
them to recover for either 2 or 5 days under standard light conditions. At 2 days post electroporation (dpe), Müller glial morphology in sox2 morphant retinas was indistinguishable from Müller glia in SC morphant or uninjected retinas (Fig. 3.6 A-C, F-H). Quantification of GFP-positive cells at 2 dpe revealed no difference in the number of Müller glia present in uninjected or either morphant retinas (Fig. 3.6 K, blue bars). At 5 dpe, Müller glia in sox2 morphant retinas were still indistinguishable from uninjected retinas by morphology (Fig. 3.6 E,J) and quantity (Fig. 3.6 K), but observed fewer GFP-positive Müller glia in SC morphant retinas (Fig. 3.6 K). The reduced number of GFP-positive Müller glia at 5 dpe correlated with increased numbers of PCNA-positive cells in the INL of SC morphant retinas (Fig. 3.6 D,I, arrows). This indicates either intravitreal injection and/or electroporation of morpholinos into the retina causes some inherent level of damage. Additionally, the reduction of GFP signal from the Tg(gfap:EGFP) transgenic zebrafish during later stages of regeneration is well documented (Thummel et al., 2008b), but may represent an artifact of the transgene, rather than suggest a true decrease of GFAP (Bernardos et al., 2007). Indeed, immunoblot analysis of the Müller glia-specific proteins glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP) revealed no significant difference in expression levels between uninjected, SC morphant or sox2 morphant retinal lysates 5 dpe (Fig 3.6 L). Finally, TUNEL analysis revealed Müller glia cell death was not caused by Sox2 knockdown (Fig. 3.5). Together, these data suggest that Sox2 is not required to maintain Müller glia identity in the damaged retina.
Figure 3.6: Sox2 is not required to maintain Müller glia identity in the undamaged zebrafish retina. Eyes of adult Tg(gfap:EGFP);albino zebrafish were either uninjected or injected and electroporated with either standard control morpholino (SC MO) or anti-sox2 morpholino (sox2 MO) and placed back in a standard light-dark cycle to recover. Retinal sections were prepared after either 2 or 5 days post electroporation (dpe) and labeled with GFP antibodies (A-J). At 2 dpe, no differences in the number or morphology of Müller glia were observed between treatments (A-C). At 5 dpe, however, we observed fewer Müller glia in SC morphant retinas (D) compared to uninjected and sox2 morphant retinas (A and E, respectively). The numbers of GFP-positive Müller glia were quantified (K). Retinal sections from uninjected, 2 dpe and 5 dpe retinas were labeled with PCNA antibodies (F-J). Few PCNA-positive cells were observed in the INL of uninjected (F) or sox2 morphant (H and J) retinas at either time point. In contrast, multiple PCNA-positive cells were observed in the INL of SC morphant retinas at both time points (G and I). Protein lysates were prepared from uninjected (UI), standard control morphant (SC MO) and sox2 morphant (sox2 MO) retinas after 5 dpe. Immunoblot analysis revealed no change in total levels of Müller glia specific proteins glutamine synthetase (GS) or glial fibrillary acidic protein (GFAP) (L). Actin was used as a loading control. PCNA, proliferating cell nuclear antigen; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel A is 25 μm and is the same for panels B-J.
3.1.7 Overexpression of Sox2 induces Müller glia proliferation

Because Sox2 expression is required for Müller glia proliferation, we hypothesized that elevating Sox2 expression above the basal levels observed in the undamaged retina could induce Müller glia proliferation, even in the absence of a damage stimulus. To test this hypothesis, we utilized the Tg(hsp70l:sox2)x21 transgenic zebrafish line (Millimaki et al., 2010), which ubiquitously overexpresses sox2 upon heat shock. Adult Tg(hsp70l:sox2) fish were subjected to heat shock at 38°C for one hour per day for either two or four days. Eyes were collected for analysis at 2 and 4 days post initial heat shock (dphs; Fig. 3.7 A). Sox2 labeling on cryosections revealed ubiquitously increased Sox2 expression throughout the retina of Tg(hsp70l:sox2) fish, but not wild-type siblings at 2 dphs (Fig. 3.7 B,C). Additionally, Western blot analysis revealed substantially elevated levels of Sox2 in Tg(hsp70l:sox2) retinal lysates relative to controls (Fig. 3.7 D).

To determine if the ubiquitous overexpression of Sox2 was sufficient to induce Müller glia proliferation in the undamaged retina, we stained Tg(hsp70l:sox2) retinal sections for PCNA. At 2 dphs, individual PCNA-positive cells were observed in the INL of Tg(hsp70l:sox2) retinas (Fig. 3.7 E,H), but rarely, if ever, in controls (data not shown). At 4 dphs, large clusters of PCNA-positive cells were present in the INL and appeared to be actively migrating to the ONL in Tg(hsp70l:sox2) retinas (Fig. 3.7 F,I). Large clusters of proliferating cells were never observed in wild-type control retinas at 4 dphs (Fig. 3.7 G,J). Interestingly, we observed this induced proliferation predominantly in the ventral retina. Though we did detect some disruption in outer segment morphology in
Figure 3.7: Overexpression of Sox2 can induce Müller glia proliferation in the undamaged zebrafish retina. Tg(hsp70l:sox2) transgenic zebrafish (hs:sox2) or wild type (WT) sibling control zebrafish were exposed to one hour of heat shock daily at 38°C for either two or four days (A). Retinal sections were prepared after 2 days post-initial heat shock (2 dphs) and labeled with Sox2 antibodies (B and C). Sox2 expression in WT siblings (B) was consistent with normal Sox2 expression in the undamaged retina (Fig. 1), but strong ubiquitous expression of Sox2 was observed in hs:sox2 retinas (C). Protein lysates were prepared from WT sibling (WT) and hs:sox2 retinas and Sox2 protein expression analyzed by Western blot (D), confirming increased Sox2 levels specifically in hs:sox2 retinas. PCNA-staining of hs:sox2 retinas at 2 dphs (E and H) and 4 dphs (F and I) revealed increased INL proliferation compared to WT sibling retinas at 4 dphs (G and J). Quantification of PCNA-positive cells confirmed increased proliferation in both the INL Land ONL following Sox2 overexpression relative to WT siblings (k). Scale bars in panels B and E are 25 µm and is the same for panels C and E-J, respectively.
Tg(hsp70l:sox2) retinas, particularly at 4 dphs, when large clusters of proliferating cells were observed migrating into the ONL, we did not detect elevated TUNEL-labeling at either 2 dphs or 4 dphs (Fig. 3.8 A-J). Together, these data suggest Sox2 overexpression is sufficient to induce proliferation in a subset of Müller glia in the undamaged retina.

One possible explanation for the variable results we observed following Sox2 overexpression involves the nature of zebrafish retinal growth (Otteson and Hitchcock, 2003). The zebrafish retina undergoes persistent neurogenesis throughout the life of the fish, with slowly cycling Müller glia throughout the central retina producing new rod photoreceptors in the growing retina (Otteson and Hitchcock, 2003). It is possible that overexpressing Sox2 might cause hyperproliferation of the Müller glia involved in persistent neurogenesis to generate more progenitors. To test this, we injected EdU four hours prior to the first heat shock to label any Müller glia currently in the process of persistent rod neurogenesis (Fig. 3.8 K). If Sox2 overexpression expands these rare proliferating Müller glia, we would expect to see many EdU-positive/PCNA-positive cells following Sox2 overexpression. At 2 dphs, we observed few, if any, EdU-positive cells in the INL in both Tg(hsp70l:sox2) (Fig. 3.8 M) and control retinas (not shown), consistent with thymidine-analog tracing studies in this short timeframe (Otteson et al., 2001).

More importantly, we never observed EdU-positive cells among the Sox2-induced proliferating INL cells at 2 dphs (Fig. 3.8 L-N). These data demonstrate that induced proliferation following Sox2 overexpression is not the result of expanded persistent neurogenesis mechanisms.
Figure 3.8: Sox2-induced ectopic Müller glia proliferation does not require cell death or expansion of non-quiescent Müller glia. Tg(hsp70l:sox2) transgenic zebrafish (hs:sox2) or wild type (WT) sibling control zebrafish were exposed to one hour of heat shock daily at 38°C for either two or four days (Fig. 3.7 A). Retinal sections were prepared and TUNEL assays were performed (A–J). DNase I-treated AB control retinal sections displayed ubiquitous TUNEL-positive cells throughout cell nuclei in the retina (E and J). We did not observe TUNEL-positive cells in either genetic background, at either time point analyzed (A–D, F–I). Tg(hsp70l:sox2) transgenic zebrafish injected with EdU 4 hours prior to beginning the 2 day heat shock regiment (K). Co-labeling of PCNA and EdU revealed no PCNA-positive cells that were also EdU-positive (L–N). We occasionally observed EdU-positive cells in the ONL (L–N, arrowhead), consistent with more frequently cycling rod precursor cells. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; PCNA, proliferating cell nuclear antigen; EdU, 5-ethynyl-2’-deoxyuridine; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars in panels A and L are 25 µm and is the same for panels B–J and M–N, respectively.
3.2 β-catenin 2 is required for Müller glia proliferation, but not sox2 expression during Müller glia reprogramming

3.2.1 β-catenin is required for Müller glia proliferation

Several studies recently showed that Wnt/β-catenin can directly regulate sox2 expression in the developing vertebrate retina (Agathocleous et al. 2009, Meyers et al. 2012). Additionally, canonical Wnt signaling is a key regulator of Müller glia proliferation in the regenerating adult zebrafish retina following stab lesion (Ramachandran et al. 2011). While this study clearly showed increased β-catenin expression at the site of injury following retinal damage, the functional data was obtained using pharmacological agents that act on molecules upstream of β-catenin. Furthermore, the targeted efficacy of these drugs on the specific pathways that regulate β-catenin have not been extensively characterized in zebrafish. Thus, we sought to determine if β-catenin itself is required for Müller glia proliferation, and if β-catenin regulates Müller glia expression of sox2 in the light-damaged retina.

Zebrafish possess two beta-catenin (ctnnb) paralogs, ctnnb1 and ctnnb2. To test the requirement of total β-catenin in the light-damaged retina, we used a mixture of two morpholinos (ctnnb1/2 MO) to simultaneously knockdown the expression of both β-catenin paralogs prior to light treatment. After 31 hours of light damage, the ctnnb1/2 morphant retinas exhibited a large reduction in β-catenin expression in the Müller glia relative to the uninjected or SC morphant retinas (Fig. 3.9 A-C). Furthermore, the
Figure 3.9: β-catenin is required for Müller glia proliferation following light-induced retinal damage. Adult albino zebrafish eyes were either uninjected, or injected and electroporated with either standard control morpholino (SC MO) or a cocktail of anti-ctnnb1 and anti-ctnnb2 morpholinos (ctnnb1/2 MO). Retinal sections were prepared following 31 hours of light and labeled with β-catenin (A-C) or PCNA (D-F) antibodies. Strong Sox2 expression was observed in Müller glia-shaped cells (arrows) in uninjected (A) and SC morphant (B) retinas, but was drastically reduced in cttnb1/2 morphant (C) retinas. PCNA labeling revealed fewer proliferating cells in cttnb1/2 morphant (F) retinas compared to uninjected (D) and SC morphant (E) control retinas. Quantification of PCNA-positive cells in the INL confirmed significantly fewer proliferating INL cells in the cttnb1/2 morphant retinas (14.8 ± 5.6) compared to either the uninjected (56.4 ± 7.3) or SC MO (42.7 ±5.0) controls (N = 8; *p < 0.005; **p < 0.0005). PCNA, proliferating cell nuclear antigen; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars in panels A and D are 25 µm and is the same for panels B-C and E-F, respectively.
ctnnb1/2 morphant retinas possessed significantly fewer PCNA-positive Müller glia in the INL relative to the uninjected and SC morphant retinas (Fig. 9D-G). These data demonstrate that knockdown of β-catenin expression impairs Müller glia proliferation following retinal damage.

### 3.2.2 Differential expression of ctnnb paralogs in the regenerating zebrafish retina

Interestingly, Ramachandran et al. (2011) observed little-to-no change in expression of either ctnnb transcript following the retinal stab injury. However, microarray data previously obtained in our lab (Kassen et al., 2007) revealed differential regulation of these genes following light damage. We performed qRT-PCR to determine how the two ctnnb paralogs were temporally regulated during the initial regeneration response to light damage. Consistent with the microarray data, we observed increased ctnnb2 gene expression very early in the regeneration response, prior to the increased ctnnb1 expression (Fig. 3.10 A). These increases in ctnnb transcripts correlate to large increases in total β-catenin protein, as shown via Western blots using pan-β-catenin antibodies (Fig. 3.10 B).

### 3.2.3 β-catenin 2, but not β-catenin 1, is required for Müller glia proliferation

We examined the spatial pattern of increased β-catenin expression on retinal sections from undamaged and 31 hour light-treated fish relative to the PCNA-positive Müller glia. In the undamaged eye, β-catenin is predominantly expressed in the outer plexiform layer (Fig. 10 C,E, arrows), outer limiting membrane (Fig. 10 C,E, double arrowheads), and the sites of cell-cell contact between photoreceptors (Fig. 10, C,E,
Figure 3.10: Differential expression of ctnnb paralogs leads to increased β-catenin expression in proliferating Müller glia after 31 hours of light. RNA was prepared from zebrafish retinas either immediately prior to (0 hr), or following 16, 31, 51, 68 or 96 hours of constant light treatment. Quantification of ctnnb1 and ctnnb2 expression during this time course was determined via qRT-PCR (A). We observed ctnnb2 expression increase following 16 and 31 hours of constant light, while ctnnb1 expression did not increase until 51 hours (A). Protein lysates were prepared from retinas that were light treated for the same time course. Western blot analysis revealed levels of total β-catenin protein expression gradually increased over the first 31 hours of light treatment and remained elevated until 96 hours (B). Retinal sections were prepared from undamaged and 31 hour light-treated zebrafish eyes. Labeling with β-catenin and PCNA antibodies in undamaged retinas (C-E) revealed β-catenin expression was predominantly in the outer plexiform layer (OPL, arrows), outer limiting membrane (OLM, double arrowheads) and focal sites of cell-cell contact between photoreceptors (arrowheads). After 31 hours of light (F-H), β-catenin expression was observed in the processes and soma of PCNA-positive Müller glia (arrowheads). PCNA, proliferating cell nuclear antigen; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel C is 25 µm and is the same for panels D-H.
arrowheads). After 31 hours of constant light, robust β-catenin expression was observed in PCNA-positive Müller glia (Fig. 3.10 F-H, arrows). Based on these temporal and spatial expression data, we hypothesized that β-catenin 2, but not β-catenin 1, may play a role in Müller glia reprogramming and proliferation. To determine the individual contribution of both β-catenin proteins to Müller glia proliferation, we knocked down both proteins independently prior to constant light treatment. We quantified the number of PCNA-positive INL cells and found significantly fewer proliferating Müller glia in cttnb2 morphant retinas (7.11 ± 2.3) relative to both SC morphant and cttnb1 morphant retinas (27.63 ± 1.9 and 27.67 ± 1.5, respectively) after 31 hours of light (Fig 3.11). These data confirm that β-catenin 2, but not β-catenin 1, is required for Müller glia proliferation in the light-damaged zebrafish retina.

3.2.4 β-catenin 2 does not regulate sox2 expression in Müller glia reprogramming

We examined whether sox2 expression in proliferating Müller glia is regulated by β-catenin 2, as it is in neuronal progenitors during retinal development (Agathocleous et al., 2009). We analyzed sox2 expression via qRT-PCR after 31 hours of light in cttnb1 morphant and cttnb2 morphant retinas and observed no difference in sox2 levels in either cttnb morphant compared to controls (Fig 3.12 G). Interestingly, Sox2 staining on 31 hour light-damaged retinal cryosections was qualitatively different in cttnb1/2 morphant and cttnb2 morphant retinas compared to SC morphant and cttnb1 morphant retinas. The spatial arrangement of Sox2-positive Müller glia in cttnb1/2
Figure 3.11: β-catenin 2, but not β-catenin 1, is required for Müller glia proliferation following light damage. Adult albino zebrafish retinas were injected and electroporated with either standard control morpholino (SC MO), anti-ctn nb1 morpholino (ctn nb1 MO), or anti-ctn nb2 morpholino (ctn nb2 MO) immediately prior to light treatment. Retinal sections were prepared after 31 hours of constant light, labeled with PCNA antibodies (A-F) and counterstained with DAPI (D-F). Multiple PCNA-positive cells were observed in the INL of SC morphant (A and D) and ctn nb1 morphant (B and E) retinas after 31 hours of light. In contrast, significantly fewer PCNA-positive cells were observed in the INL of ctn nb2 morphant retinas (C and F). Quantification of PCNA-positive cells in the INL of the three morphant retina backgrounds (G) confirmed a significant reduction of proliferating cells in the ctn nb2 morphant (7.11 ± 2.3) retinas compared to both SC morphant (27.6 ± 1.8) and ctn nb1 morphant (27.7 ± 1.5) retinas (N ≥ 8; *p < 5 x 10^{-6}). PCNA, proliferating cell nuclear antigen; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel A is 25 μm and is the same for panels B-F.
morphant and cttnb2 morphant retinas was much more varied along the apical-basal axis of the retina than either the SC morphant or cttnb1 morphant retinas (Fig. 3.12 A-F arrows vs. arrowheads). Additionally, the morphology of these Sox2-positive Müller glia was more diffuse in cttnb1/2 morphant and cttnb2 morphant retinas compared to control and cttnb1 morphant retinas. Thus, β-catenin does not regulate sox2 expression in proliferating Müller glia following retinal damage, though we cannot rule out the possibility that either β-catenin paralog regulates sox2 expression in the Müller glia-derived NPCs at later time points.

We also assessed expression of a ccnd1 (the gene encoding Cyclin D1), a well characterized transcriptional target of β-catenin (Tetsu and McCormick, 1999), by qRT-PCR. We observed significant decreases in ccnd1 gene expression in both sox2 morphant and cttnb2 morphant retinas relative to controls (Fig. 3.12 H), suggesting that Sox2 and/or β-catenin 2 may regulate Müller glia proliferation by modulating expression of key cell cycle genes.

3.3 Sox2 regulates Müller glia reprogramming via expression of ascl1a and lin28a, but not stat3

Ascl1a and Stat3 are two extremely well characterized transcription factors required for Müller glial reprogramming/proliferation in the regenerating zebrafish retina (Kassen et al., 2007; Fausett et al., 2008; Ramachandran et al., 2010; Nelson et al., 2012; Nelson et al., 2013; Ramachandran et al., 2014). Transcription of both proteins increases rapidly following retinal damage, and knockdown of either protein results in
Figure 3.12: β-catenin does not regulate sox2 expression in the regenerating zebrafish retina. Adult albino zebrafish eyes were either uninjected, or injected an electroporated with standard control morpholino (SC MO), anti-sox2 morpholino (sox2 MO), anti-ctnnb1 morpholino (ctnnb1 MO), anti-ctnnb2 morpholino (ctnnb2 MO) or a cocktail of anti-ctnnb1 and anti-ctnnb2 morpholinos (ctnnb1/2 MO) immediately prior to light treatment. Retinal sections were prepared after 31 hours of constant light and labeled with Sox2 antibodies (A-F). In uninjected (A), SC morphant (B), and ctnnb1 morphant (E) retinas, intense Sox2 expression was observed in Müller glia nuclei located predominantly in the basal INL (arrows). In ctnnb1/2 morphant (D) and ctnnb2 morphant (F) retinas, we observed variable intensity of Sox2 in Müller glia scattered along the apical-basal axis of the INL (arrows). Some of these Sox2-positive cells displayed unusual spindle morphology (arrowheads). We observed few if any Sox2-positive cells in sox2 morphant retinas (C). Quantification of sox2 gene expression was analyzed via qRT-PCR after 31 hours of light (G). While we observed a large increase in sox2 expression in sox2 morphant retinas, we observed no change in sox2 expression following knockdown of β-catenin 2, relative to the SC morphant control (NS p = 0.07). qRT-PCR analysis of ccnd1 expression was significantly reduced in both sox2 morphant and ctnnb2 morphant retinas relative to SC morphant after 31 hours of light (*p < 1 x 10^{-6}). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel A is 25 µm and is the same for panels B-F.
reduced expression of the other (Nelson et al., 2012). To determine if Sox2 regulates transcriptional induction of either transcription factor, we performed qRT-PCR to analyze \textit{ascl1a} and \textit{stat3} expression in sox2 morphant retinas. We observed a significant reduction in \textit{ascl1a} expression after 31 hours of light treatment (Fig. 3.13 A), which correlated to reduced Ascl1a expression on retinal cryosections in sox2 morphant retinas compared to controls (Fig. 3.13 D-F,J-L). Interestingly, \textit{stat3} expression was unaffected by Sox2 knockdown (Fig 3.13 B). This represents the first instance we observed whereby a loss of \textit{ascl1a} occurs without affecting \textit{stat3} levels.

In cultured human neuronal progenitor cells, SOX2 directly induces \textit{LIN28} expression, which inhibits \textit{let7} miRNAs and, in turn, destabilizes \textit{MASH1} mRNA (a mammalian ortholog of zebrafish \textit{ascl1a}) and reduces translation of MASH1 protein. In zebrafish, Lin28a is also required for Müller glia reprogramming and proliferation in the injured retina (Ramachandran et al., 2010; Nelson et al., 2012). To investigate whether Sox2 regulates \textit{lin28} expression, we analyzed \textit{lin28a} expression in sox2 morphant retinas via qRT-PCR. After 31 hours of light, we observed a drastic reduction of \textit{lin28a} expression in sox2 morphant retinas compared to controls (Fig. 3.13 C). While we cannot confirm that this reduction in \textit{lin28a} expression is directly responsible for the reduced \textit{ascl1a} expression via unsuppressed \textit{let7} miRNAs, Ramachandran et al. (2010) demonstrated that two \textit{let7} family members (\textit{let7a} and \textit{let7f}) remain elevated following knockdown of \textit{lin28a} morphant in retinas following stab lesion.
Figure 3.13: Sox2 regulates *ascl1a* and *lin28a*, but not *stat3*, expression in the regenerating zebrafish retina. Adult albino zebrafish eyes were either uninjected, or injected and electroporated with either standard control morpholino (SC MO) or anti-sox2 morpholino (sox2 MO) immediately prior to the onset of light treatment. Total RNA was isolated from retinas after 31 hours of constant light. Expression of *ascl1a*, *stat3*, and *lin28a* gene expression was assayed via qRT-PCR. Knockdown of Sox2 expression had no affect on *stat3* expression (B). In contrast, both *ascl1a* and *lin28a* expression decreased in sox2 morphant retinas compared to SC morphant retinas (A, n = 3, *p = 0.03; C, n = 3, *p = 0.02, respectively). Retinal sections were prepared from eyes following 31 hours and labeled with Ascl1a and PCNA antibodies (D-L). PCNA-positive cells in the INL of uninjected (D, G, J, arrows) and SC morphant (E, H, K, arrows) retinas also expressed Ascl1a; however, sox2 morphant retinas displayed a reduced number of cells expressing both Ascl1a and PCNA (F, I, L). PCNA, proliferating cell nuclear antigen; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel D is 25 mm and is the same for panels E-L.
3.4 Roles for Sox2 in NPC amplification and neuronal differentiation

3.4.1 Sox2 expression in early and late-stage NPCs

Following Müller glia reprogramming during the regeneration response (~8-31 hours post injury), the Müller glia-derived neuronal progenitor cells (NPCs) begin to amplify, migrate and differentiate (51-96+ hours). Expression of sox2 remained elevated after 51 and 68 hours of light-treatment, but began to decrease after 96 hours (Fig. 3.2). This temporal gene expression data suggested Sox2 may be active in proliferating NPCs, but expression may be reduced in differentiating late-stage NPCs.

We labeled retinal sections with antibodies to Sox2 and PCNA following 51, 68 and 96 hours of light (Fig. 3.14). After 51 hours, many PCNA-positive NPCs also expressed Sox2 (Fig. 3.14 A,D,G, arrows). Quantification of the total numbers of Sox2-positive cells, PCNA-positive cells, and cells positive for both markers in the INL revealed a similar number of PCNA-positive cells and cells co-expressing Sox2 and PCNA (Fig. 3.14 J, 27.83 ± 1.2 and 26.83 ± 1.2, respectively). Thus, 96 ± 0.17% of PCNA-positive cells also express Sox2 after 51 hours of light (Fig. 3.14 K). At both 68 and 96 hours, some PCNA-positive cells still express Sox2 (Fig. 3.14 B,E,H and C,F,I, arrows, respectively). At these time points, there are also more PCNA-positive cells that do not express Sox2 (Fig. 3.14 B,E,H and C,F,I, arrowheads, respectively). We quantified the number INL cells expressing PCNA and Sox2 after 68 and 96 hours of light (Fig. 3.14 J) and observed that the percentage of PCNA-positive cells co-expressing Sox2 was reduced to 40 ± 2% at 68 hours and further to 31 ± 2% at 96 hours (Fig. 3.14 K). Together, these data suggest that
Figure 3.14: Sox2 expression dynamics in early and late-stage neuronal progenitor cells. Adult albino zebrafish were exposed to constant intense light for either 51, 68 or 96 hours. Retinal cryosections were prepared and labeled with antibodies to Sox2 (A-F) and PCNA (G-I). After 51 hours of constant light, many small clusters of INL cells were observed expressing both PCNA and Sox2 (A,D,G, arrows). After 68 hours, some PCNA-positive cells in the INL still expressed Sox2 (B,E,H, arrows), but some cells within these clusters did not express Sox2 (B,E,H, arrowheads). At 96 hours, even fewer cells expressed both markers (C,F,I, arrows), as the majority of PCNA-positive cells had stopped expressing Sox2 (C,F,I, arrowheads). We quantified the number of INL cells expressing only Sox2, only PCNA, or those expressing both markers (J). We used these cell counts to determine the percentages of all of the PCNA-positive INL cells also expressing Sox2 at each time point (K). We observed 96 ± 0.17% of PCNA-positive cells also expressing Sox2 after 51 hours of light treatment, compared to 40 ± 2% and 31 ± 2% of cells at 68 and 96 hours, respectively (n = 6, *p < 3 x 10^-10). PCNA, proliferating cell nuclear antigen; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel A is 25 µm and is the same for panels B-I.
proliferating NPCs express Sox2 early in the regeneration response, but stop expressing Sox2 as they become committed to a neuronal lineage.

### 3.4.2 Sox2 knockdown prevents amplification of neuronal progenitor cells

To determine if Sox2 is required for NPC amplification following Müller glia reprogramming, we exposed fish to constant intense light for 31 hours, at which time we knocked down Sox2 expression. After 68 hours of total light treatment (37 hours after Sox2 knockdown), we labeled retinal sections with antibodies to Sox2 and PCNA (Fig. 3.15). We observed large clusters of PCNA-positive NPCs in the INL of uninjected and SC morphant retinas, some of which also expressed Sox2 (Fig. 3.15 A,D,H and B,E,I, arrows, respectively). Following Sox2 knockdown, we observed individual PCNA-positive cells in the INL rather than cell clusters (Fig. 3.15 F,J, arrowheads). Importantly, the PCNA-positive cells in the INL of sox2 morphant retinas did not express Sox2 (Fig. 3.15 C,J, arrowheads). While these data could suggest a Sox2-independent pathway for Müller glia proliferation, we hypothesize that the individual PCNA-positive INL cells observed in sox2 morphant retinas represent the Müller glia that were already reprogramming at the time of Sox2 knockdown (31 hours of light treatment) and Sox2 is required for NPC amplification following Müller glia proliferation in the light-damaged zebrafish retina.
Figure 3.15: Sox2 expression is required for NPC proliferation following Müller glia reprogramming. Adult *albino* zebrafish were exposed to constant intense light for 31 hours. At this time, we injected and electroporated either standard control morpholino (SC MO) or anti-sox2 morpholino (sox2 MO) and placed the fish back in light for 37 hours (total of 68 hours from the onset of light-treatment). Retinal cryosections were prepared and labeled with antibodies to Sox2 (A-C, H-J) and PCNA (D-J). In both uninjected (UI) and SC morphant retinas, large clusters of PCNA-positive cells were observed in the INL, some of which also expressed Sox2 (A,D,H and B,E,I, arrows, respectively). In sox2 morphant retinas, we observed primarily individual PCNA-positive cells that did not express Sox2 in the INL (C,F,J, arrowheads). PCNA, proliferating cell nuclear antigen; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel A is 25 µm and is the same for panels B-J.
ZHbrafish Müller glia respond to retinal damage by reprogramming their genome and proliferating to generate a pool of neuronal progenitor cells that will eventually replenish the lost retinal neurons. Recent studies have begun to elucidate the molecular basis for Müller glia reprogramming and proliferation, but, to date, none have directly studied the functional requirement of known cell reprogramming factors, such as Sox2.

Sox2 is a well established neuronal stem cell associated transcription factor required for: 1) maintaining pluripotency in ESCs; 2) reprogramming somatic cells to generate iPSCs (Takahashi et al., 2007) or direct conversion of non-neuronal cells to functional neurons (Karow et al., 2012; Heinrich et al., 2014); and 3) regulation of neural development (Okuda et al., 2006) and adult neurogenesis in vertebrates (Favaro et al., 2009). In the developing vertebrate retina, Sox2 is required to maintain both the progenitor state and neuronal competence of retinal progenitor cells (Taranova et al., 2006; Agathocleous et al., 2009). In fish and amphibians, Sox2 expression in retinal progenitors is maintained by canonical Wnt/β-catenin signaling (Agathocleous et al., 2009; Meyers et al., 2012). Sox2 expression is downregulated as retinal progenitors
differentiate (Agathocleous et al., 2009), and in the adult retina, only the Müller glia and a subset of amacrine cells maintain Sox2 expression (Taranova et al., 2006; Lin et al. 2009). Conditional loss of SOX2 in Müller glia of the postnatal mouse retina causes loss of glial identity, loss of Müller glia quiescence and global defects in retinal morphology.

The goal of this work was to begin defining the role Sox2 plays during Müller glia reprogramming in the light-damaged zebrafish retina. I found that Sox2 expression is maintained in the Müller glia and a subset of amacrine cells in the undamaged adult zebrafish retina, recapitulating that of other well-studied vertebrates. I then investigated the function of Sox2 during Müller glia reprogramming in the light-damaged zebrafish retina. Expression of sox2 transcript increased significantly in retinas following 31 hours of light treatment, correlating with increased Sox2 expression in proliferating Müller glia. I also observed increased sox2 expression in undamaged retinas following γ-secretase inhibition and TNFα-induced Müller glia proliferation. Knockdown of Sox2 expression decreased the number of proliferating Müller glia, demonstrating that Sox2 is required for Müller glia reprogramming and proliferation. In contrast, forced overexpression of Sox2 induced proliferation of Müller glia in the absence of retinal damage. I investigated known regulators of Sox2 and determined that, unlike developing retinal progenitors, Müller glial expression of sox2 is independent of β-catenin expression. Investigating the relationship between Sox2 and several well-characterized genes that regulated Müller glia reprogramming, I determined that Sox2 regulates expression of ascl1a and lin28a, but not stat3 expression following retinal damage. Finally, preliminary characterization of Sox2
expression during later stages of regeneration revealed that Sox2 expression is required for the amplification of neuronal progenitor cells (NPCs) and this expression is reduced in late-stage NPCs as they commit to a neuronal lineage. This work will serve as a foundation for future studies aimed at understanding how the Müller glia genome is reprogrammed to efficiently regenerate the vertebrate retina.

4.1 Sox2 is necessary and overexpression is sufficient for Müller glia proliferation

To date, the only study investigating sox2 expression in the adult zebrafish retina observed an increase in sox2 transcript at 2 days post stab-lesion, but concluded sox2 was not expressed in the undamaged eye (Ramachandran et al., 2010). This was a novel observation, as Sox2 expression has been shown to be maintained in the mature retina of mammalian and avian models. In contrast to that study, I show Sox2 expression in the zebrafish retina recapitulates that of the other vertebrate systems (Fig. 3.1). The importance of this observation is that it reflects another molecular similarity between zebrafish and mammalian Müller glia, though the latter do not maintain the regenerative capacity as the former. Obvious questions raised by the conserved expression pattern is whether the inability of the mammalian Müller glia to efficiently initiate a regeneration response, as it pertains to Sox2, occurs because of: 1) differential intracellular regulation of Sox2 targets in Müller glia of the undamaged retina, 2) differential regulation of Sox2 expression or transcriptional targets following damage, or 3) differential signaling environment/niche that mitigates the intracellular similarities between mammalian and zebrafish Müller glia. While our studies have laid the
groundwork to answering this question in zebrafish, future studies will be required to determine the similarities and differences between Sox2 targets in both the fish and mammalian Müller glia. Furthermore, simply determining whether Sox2 expression is upregulated in mammalian Müller glia following retinal damage will be invaluable in determining where the mammalian regeneration response diverges from fish.

Similar to Ramachandran et al. (2010), I observed increased sox2 transcript expression after 31 hours of light (Fig. 3.2), when reprogrammed Müller glia begin proliferating (Kassen et al., 2007). This correlated to increased Sox2 protein expression in the PCNA-positive Müller glia (Fig. 3.2) and knockdown of Sox2 prior to light-treatment reduced the number of proliferating Müller glia (Fig. 3.4). Importantly, the reduced Müller glia proliferation we observed was not caused by reduced photoreceptor cell death following Sox2 knockdown (Fig. 3.5).

A possible explanation for the reduced Müller glia proliferation observed following Sox2 knockdown is a loss of Müller glia identity. In the postnatal mouse retina, conditional loss of SOX2, specifically in the Müller glia, resulted in loss of glial identity (Surzenko et al., 2013). In zebrafish, however, knockdown of Sox2 in the absence of light-damage revealed no differences in Müller glial markers GFAP or GS after two or five days of recovery (Fig. 3.6). While these data suggest that Sox2 is not required to maintain Müller glial identity in the undamaged zebrafish retina, there are two mitigating factors when comparing our experimental protocol to the mouse studies. First of all, Surzenko et al. (2013) utilized conditional ablation of Sox2, specifically in Müller glia, whereas electroporation of morpholinos provides a variable and transient
reduction of protein levels in all retinal cell types. Because the level of knockdown will almost certainly never reach what is observed in a true null or knockout allele, more elaborate tools will be required to unequivocally confirm that Sox2 does not maintain Müller identity in the adult zebrafish retina. Second, based on the amount of proliferation induced in the standard control morphant retinas (Fig. 3.6), the electroporation protocol itself is inherently damaging to the retina. Thus, while these retinas were not subjected to light-damage, to say anything conclusive about Sox2 function in the undamaged retina would be inaccurate. Despite these two caveats, this work shows that the level of Sox2 knockdown we achieve by electroporating morpholinos does not lead to a loss of Müller glial identity within five days. It is important to note that even we do not see a loss of Müller glial identity based on traditional Müller glial markers, we cannot discount the possibility that knockdown of Sox2 expression alters an intracellular signaling program required for Müller glia to respond to damage signals, but not to maintain their glial differentiation status.

Perhaps one of the more intriguing observations in this study was that ectopic overexpression of Sox2 could result in proliferation of Müller glia in the undamaged retina (Fig. 3.7). Curiously, ectopic proliferation was only observed in a subset of the retinas, and primarily in the ventral retina. Because of the dose-dependent nature of Sox2, it is not entirely surprising to see a variable response to a variable heat shock-inducible overexpression. In human ES cells, for example, too much or too little SOX2 induces differentiation (Kopp et al., 2008). Similarly, hypomorphic Sox2 mutations cause distinct phenotypes compared to true null mutations in developing mouse retinas.
(Taranova et al., 2008). We were more intrigued by the ventral preference for ectopic proliferation. While it is clear from previous studies that Müller glia along the entire length of the retina maintain the capacity to proliferate and regenerate retinal neurons (Fimbel et al., 2007; Montgomery et al., 2010; Conner et al., 2014), this data suggests an inherent molecular difference exists along the dorsoventral axis of the retina. While such asymmetries exist during retinal development to pattern the dorsoventral axis, no factors have been identified to distinguish these retinal regions at the molecular level. During our characterization of β-catenin expression in the undamaged retina, we observed an obvious difference in Müller glial expression of β-catenin along the dorsoventral axis of the retina (data not shown). Compared to weak expression in the central dorsal retina, β-catenin expression was detected strongly in Müller glia of the ventral retina and CMZ. While we could not directly test whether this expression influenced the response to ectopic Sox2, it is the first factor, to our knowledge, that indicates an intrinsic difference exists within Müller glia along the dorsoventral axis of the retina.

Taken together, these data demonstrate that Sox2 expression is required for, and overexpression is sufficient to induce, Müller glia reprogramming and proliferation. An important question remains as to whether it is the basal Sox2 expression in Müller glia of the undamaged retina or the damage-induced increase in Sox2 expression that is required for Müller glia proliferation. While we saw no changes in Müller glial markers after knocking down Sox2 in the absence of light-damage, it remains possible that intracellular Sox2 signaling is disrupted such that Müller glia can no longer properly
respond to damage signals, but can maintain their function as differentiated glial cells. In contrast to this model, overexpressing Sox2 in the undamaged retina can induce Müller glia proliferation, but the basal levels normally observed in the undamaged eye maintain Müller glia quiescence, suggesting that it is the increased Sox2 expression required for Müller glia reprogramming. In reality, Sox2 likely plays a dynamic role in reprogramming the Müller glia genome in response to damage. To better understand this dynamic process, future studies must gain a more in-depth understanding of Sox2 transcriptional targets in Müller glia of the undamaged retina and how those targets change in response to damage.

4.2 β-catenin 2 is required for Müller glia proliferation, but does not regulate sox2 expression in the regenerating zebrafish retina

Sox2 is required to maintain the progenitor state and neuronal competence of retinal progenitor cells (RPCs) during vertebrate retinal development (Taranova et al., 2006; Agathocleous et al., 2009). In fish and frogs, Sox2 expression in RPCs is regulated by Wnt/β-catenin signaling (Agathocleous et al., 2009; Meyers et al., 2012). When evaluating the role of a particular pathway in retinal regeneration, it is often helpful to first determine whether it recapitulates its function in the developing retina. Several studies have recently established a critical role for Wnt signaling during zebrafish retinal regeneration (Ramachandran et al., 2011; Wan et al., 2014). While these studies did show increased β-catenin protein expression in proliferating NPCs, they concluded ctnnb1 and ctnnb2 transcript expression did not change following stab-lesion.
Furthermore, these studies utilized pharmacological manipulation of canonical Wnt signaling, but these drugs are not well characterized in the zebrafish and there were no controls to show they specifically modulated β-catenin as the terminal effector molecule. I determined not only that ctnnb2 expression increases very early following retinal damage, while ctnnb1 expression increases during the NPC amplification phase (Fig. 3.10), but also that β-catenin 2 was the only β-catenin paralog required for Müller glia proliferation (Fig. 3.11). These data support the previous studies by showing that β-catenin itself is required for Müller glia proliferation, but also refine the pathway by identifying non-redundant roles for both β-catenin paralogs.

While directly modulating β-catenin expression was necessary to support the conclusions made by previous studies utilizing pharmacological manipulation of the Wnt pathway, β-catenin has cellular functions beyond canonical Wnt signaling. One prominent role for β-catenin outside of Wnt signaling is linking cell adhesion molecules, such as cadherins, to the actin cytoskeleton (Stepniak et al., 2009). Consistent with this role, we observed β-catenin protein expression predominantly at the outer limiting membrane (OLM) and focal adhesion sites between photoreceptors. The OLM serves as the apical membrane of the neural retina, and defines the apical domain of Müller glia (Paffenholtz et al., 1999; Wei et al., 2006). Importantly, the OLM was previously shown to be maintained by N-cadherin, β-catenin, α-catenin and p120 catenin (Paffenholtz et al., 1999). N-cadherin expression increases in the apical domain of reprogrammed Müller glia and the Müller glia-derived NPCs following retinal damage (Raymond et al., 2006). Recently, Nagashima et al. (2013) showed that suppression of N-cadherin
impaired NPC cell cycle progression. Interestingly, β-catenin expression in Müller glia largely overlaps with N-cadherin expression (Ramachandran et al., 2011; personal observations, data not shown). Thus, it is possible that loss β-catenin 2 knockdown could antagonize N-cadherin-mediated processes during retinal regeneration. While this remains a possibility, the defects observed in N-cadherin mutants were observed during NPC amplification, but not during Müller glia reprogramming or the initial cell division (Nagashima et al., 2013).

When I assayed sox2 expression following β-catenin knockdown, I found that the characteristic increase in sox2 levels after 31 hours of light was not dependent upon β-catenin 2 (Fig. 3.12), but expression of the canonical β-catenin target gene ccnd1 (gene encoding Cyclin D1) was (Fig. 3.12). Interestingly, when I looked at Sox2 expression on retinal sections, I observed a subjective reduction of staining in ctnnb2 morphant retinas compared to control or ctnnb1 morphant retinas. However, this was accompanied by distinct morphological changes of the Müller glia in ctnnb2 morphant retinas, in which they adopted a more wispy, spindle morphology compared to the rounder and denser nuclei of the control and ctnnb1 morphant Müller glia (Fig. 3.12). Thus, I hypothesize the perceived decrease in Sox2 staining in ctnnb2 morphant Müller glia may be attributable to more diffuse expression influenced by the noted morphological changes. Additionally, the Müller glia nuclei of ctnnb2 morphant retinas were located more sporadically along the apical-basal axis of the retina, compared to the uniform basal-INL localization in control and ctnnb1 morphant retinas. Because Müller glia undergo interkinetic nuclear migration when they proliferate (Nagashima et al., 2013; M. Lahne,
personal communication), and because β-catenin can directly regulate multiple cell cycle proteins, it's possible that a variable reduction of β-catenin 2 from cell-to-cell could account for cell cycle arrest at various points along the INKM axis.

The contradictory relationship between β-catenin and Sox2 in the developing and regenerating zebrafish retina suggest that the process of Müller glia reprogramming is not equivalent to the maintenance of cycling RPCs. However, it is possible that one or both of the β-catenin paralogs may regulate sox2 expression in proliferating NPCs at later time points. Consistent with the notion of a context-dependent relationship between Sox2 and β-catenin, a recent study determined that, in contrast to the observations of retinal development in fish and frogs (Agathocleous et al., 2009; Meyers et al., 2012), SOX2 maintains RPCs in the central mouse retina by directly repressing β-catenin (Heavner et al., 2014). They also observed that forced expression of activated Ctnnb1 early in the optic cup could antagonize Sox2 expression and promote a ciliary epithelial cell fate. This study, coupled with those in the lower vertebrates, demonstrate the complexity of the relationship between Sox2 and β-catenin, both between species and likely, as the authors note, between developmental time points.

4.3 Sox2 regulates Müller glia reprogramming via lin28a and ascl1a

We investigated whether increased ascl1a or stat3 expression are regulated by Sox2 following retinal damage. These represent two very well-characterized factors required for Müller glia proliferation in zebrafish (Fausett et al., 2008; Ramachandran et al., 2009; Nelson et al., 2012; Nelson et al., 2013; Wan et al., 2014). To our surprise, we
observed that while *ascl1a* expression was dependent on Sox2 expression, *stat3* expression was not (Fig. 3.13). Our previous work indicated that loss of Ascl1a resulted in reduced Stat3 expression by immunofluorescence (Nelson et al., 2012). The discrepancy between these two studies may be explained by a less severe decrease in Ascl1a expression following Sox2 knockdown, compared to directly knocking down Ascl1a expression. Alternatively, it is possible that the loss of Sox2 still allows the pathway upstream of Stat3 to function, up to the point that it converges with Ascl1a.

Because Sox2 knockdown resulted in just a 40% reduction in *ascl1a* gene expression, we hypothesized that Sox2 may not directly regulate *ascl1a* transcription, but function through another transcription factor or post-transcriptional regulatory pathway. We also observed an 88% reduction in *lin28a* expression following Sox2 knockdown (Fig. 3.13). In cultured human NSCs, SOX2 maintains *LIN28* expression by recruiting chromatin remodeling factors to the *LIN28* locus (Cimadamore et al., 2013). In this system, LIN28 prevents biogenesis of mature *let7* miRNA family members that directly destabilize *MASH1* mRNA (the mammalian ortholog of *ascl1a*) and prevent translation of MASH1 protein. Thus, in cultured NSCs, SOX2 indirectly regulates *MASH1* expression via direct regulation of *LIN28*.

*Lin28a* is also required for zebrafish Müller glia reprogramming (Ramachandran et al., 2010). Conflicting reports have suggested that Ascl1a directly regulates *lin28* expression and vice versa (Ramachandran et al., 2010; Nelson et al., 2012). While some level of feedback is likely involved throughout the pathways regulating retinal regeneration, the earlier study that concluded Lin28a does not regulate *ascl1a*
expression suffers from several flaws, many of which have been highlighted by the more recent publication. Two particularly interesting observations from Ramachandran et al. (2010) are that: 1) Lin28a is required to antagonize let7 miRNA biogenesis, just as it is in cultured human NSCs (Cimadamore et al., 2013) and 2) let7 suppressed zebrafish Ascl1a translation in HEK293 cells in a dose-dependent manner. These observations, along with the results of the current study, suggest Müller glia reprogramming may utilize a conserved Sox2/Lin28/let7/Ascl1a pathway to generate NSCs in the regenerating zebrafish retina.

Further studies will be required to determine if the Sox2-dependent loss of lin28a expression directly impact let7 family members. Furthermore, in order to more accurately understand the role Sox2 plays in Müller glia reprogramming, more elaborate experiments will be required to identify additional transcriptional targets of Sox2. For example, Sox2 ChIP experiments, coupled with Next-Generation sequencing of gene expression following both Sox2 knockdown and overexpression, will yield a wealth of Sox2 target candidates. The work presented here will serve as a solid starting point for such studies, providing genes whose expression can be used to evaluate the validity of large data sets.

4.4 Sox2 regulates NPC dynamics following Müller glia reprogramming

These studies focused primarily on understanding the role Sox2 plays in Müller glia reprogramming in the light-damaged zebrafish retina. However, Sox2 likely plays a role in other cellular events during the regeneration response. Expression of sox2
remained elevated after 51 hours and 68 hours of light-treatment, but began to decline by 96 hours (Fig. 3.2). This correlated with Sox2 protein expression remaining high in early NPCs, but decreasing in late-stage NPCs (Fig. 3.14). We hypothesize that the late-stage NPCs with weak or no Sox2 expression have committed to a neuronal lineage and started to differentiate into new neurons. Further studies will be required to characterize Sox2 expression with known markers for neuronal commitment, such as the transgenic zebrafish lines Tg(olig2:EGFP) (Thummel et al., 2008b) or Tg(atoh7:EGFP) (Conner et al., 2014), which express GFP under control of the olig2 or atoh7 promoters, respectively.

Because Sox2 expression remained elevated in early NPCs, we knocked down Sox2 expression just prior Müller glia division and observed the effect on NPC amplification after 68 hours of light. Knocking down Sox2 expression at this time point prevented amplification of NPCs after 68 hours of light-treatment (Fig. 3.15), demonstrating that Sox2 also regulates NPC dynamics to some degree following Müller glia reprogramming. In order to better investigate the function of Sox2 during specific stages of the regeneration time course, more elaborate genetic tools will necessary. For example, our current system of in vivo electroporation of morpholinos is variable both within and between morpholino/gene target combinations (Kamachi et al., 2008). Knockdown is also ubiquitous in this system, posing a problem when investigating protein function in a heterogeneous population of cells. For example, it would be nearly impossible to distinguish the function of Sox2 during proliferation and differentiation in a population of cells at later stages of regeneration, in which some Sox2-positive cells
are proliferating (PCNA expression), some are expressing markers for neuronal
differentiation, and some are expressing markers for both. With the advances in
designer nucleases, such as TALE nucleases and the CRISPR/Cas9 system, we will soon
be able to engineer the zebrafish genome with targeted, sophisticated tools that will
allow us to address these more complex problems. For example, a conditional sox2
allele, in which the endogenous sox2 locus is flanked by loxP sites, could be combined
with various CreER drivers for spatial and temporal control of true Sox2 knockout
(Appendix, Fig. A 10). Not only would this system provide more consistent reductions in
protein expression from cell-to-cell (knockout versus transient knockdown), but it will
also allow us to target specific cell populations for Sox2 ablation (i.e. proliferation-
dependent tuba1 promoter for proliferating cells versus ath5 promoter for
differentiating cells). These tools will be invaluable assets to increase the scope and
rigor of our studies in the future.

4.5 Conclusion

In response to light-induced photoreceptor cell death, zebrafish Müller glia
reprogram their genome to partially dedifferentiate and divide, giving rise to a
population of neuronal progenitor cells (NPCs) that will amplify and migrate to the outer
nuclear layer, where they will differentiate to regenerate the lost or damage
photoreceptors. Because Müller glia reprogramming and proliferation is likely the rate-
limiting step in this regeneration response, we investigated the role of the
reprogramming factor Sox2 during light-induced retinal regeneration in zebrafish. In
this work, I have shown that Sox2 is necessary, and overexpression of Sox2 is sufficient, to stimulate Müller glia reprogramming. We also expanded upon previous research to show that β-catenin 2, but not β-catenin 1, is required for Müller glia proliferation. Unlike the retinal progenitor cells in the developing fish and frog retinas, β-catenin does not regulate sox2 expression during Müller glia reprogramming. Expression of ascl1a and lin28a were dependent on Sox2 expression following light-damage. It remains unclear whether Sox2 directly regulates transcription of these genes, or if they are regulated through a conserved Sox2/Lin28/let7/Ascl1a pathway. Overall, I propose a model for Müller glia reprogramming in which retinal damage stimulates Sox2 to promote expression of lin28a and ascl1a (Fig. 4.1). This occurs independently of β-catenin 2, which is also required to drive Müller glia proliferation through a separate pathway (Fig. 4.1).

Future work utilizing high throughput transcriptomic and targetomic datasets will be necessary to determine what additional genes are regulated by Sox2 during Müller glia reprogramming. Additionally, the role of Sox2 in other aspects of retinal regeneration will require novel genetic tools. Finally, Müller glia of the undamaged mammalian and zebrafish retina maintain Sox2 expression into adulthood, but the former cannot provide the robust regenerative response observed in the fish. Comparative studies between Sox2 targets and Sox2 expression dynamics in both animal models will be invaluable to increase our understanding where the mammalian regeneration response fails and provide novel therapeutic targets to stimulate Müller glia to regenerate the human retina.
Figure 4.1: Pathways that drive Müller glia reprogramming and proliferation in the regenerating zebrafish retina. In response to retinal damage, dying neurons signal Müller glia, via TNF\textsubscript{α} or other unidentified factors, to reprogram their genome and proliferate. My work has shown that Müller glia reprogramming is dependent upon damage-induced modulation of Sox2-dependent gene expression. I have determined that Sox2 is required for expression of \textit{lin28a} and \textit{ascl1a}, however, it remains unclear whether this relationship is direct or indirect. A favorable model involves direct regulation of \textit{lin28a} transcription by Sox2. Lin28a then antagonizes let7 miRNA biogenesis, which relieves let7-dependent repression of Ascl1a translation (Ramachandran et al., 2010; Cimadamore et al., 2013). Interestingly, \textit{stat3} expression was not dependent upon Sox2 expression. This was an unexpected observation, as previous work showed Ascl1a is required for increased Stat3 expression in Müller glia following retinal damage (Nelson et al., 2012; Nelson et al., 2013). It is not yet clear how the relationship between Ascl1a and Stat3 changes after knockdown of Sox2 expression. Finally, I determined that β-catenin 2, but not β-catenin 1, is required for Müller glia proliferation in response to retinal damage, but unlike retinal progenitors in the developing vertebrate retina, β-catenin 2 does not regulate \textit{sox2} expression.
APPENDIX A:

SUPPLEMENTAL FIGURES
Figure A. 1: Levels of *mmp2* transcript and MMP2 protein expression increased during regeneration of the light-damaged retina. Dark-adapted albino zebrafish were treated in constant intense light for various lengths of time and retinal lysates were prepared at each time point to extract either total RNA for qRT-PCR (A) or total protein for immunoblot (B, C). Total cDNA was synthesized from RNA using random hexamers, while gene-specific primers against *mmp2* were used for qRT-PCR. Results of qRT-PCR (A, red) were used to validate previously obtained microarray data (A, blue; Kassen et al., 2007). Expression levels of *mmp2* mRNA in both data sets decreased initially by 16 hours, then began to rise steadily throughout the remainder of the light treatment time course (A). To monitor MMP2 protein expression, total protein lysate from light-damaged retinas was separated via SDS-PAGE and transferred to PVDF for immunodetection using antibodies specific to zebrafish MMP2 (B). Similar to mRNA levels, MMP2 protein expression increased gradually throughout the time-course, reaching a maximum at 96 hours (B, C). Notably, the pro-form of MMP never increased in expression, while the active cleaved form increased dramatically. Actin was used as a loading control for the immunoblot (B). MMP2 levels were quantified using ImageJ (C). qRT-PCR, quantitative real-time polymerase chain reaction; MMP2, matrix metalloproteinase 2.
A: \textit{mmp2} mRNA expression during constant light-treatment

\begin{center}
\begin{tikzpicture}
\begin{axis}[
width=\textwidth,
height=0.6\textwidth,
axis x line=bottom,
axis y line=left,
axis line style=-,\]
\addplot[mark=none,red,mark options={solid},ERROR bars=misaligned coords=both]
coordinates {
(0, -4)
(16, -2)
(31, 0)
(51, 2)
(68, 4)
(96, 4)
(0, 4)
(16, 2)
(31, 0)
(51, -2)
(68, -4)
(96, -4)
};
\addlegendentry{qRT-PCR}
\addplot[mark=none,blue,mark options={solid}]
coordinates {
(0, 4)
(16, 2)
(31, 0)
(51, -2)
(68, -4)
(96, -4)
(0, -4)
(16, -2)
(31, 0)
(51, 2)
(68, 4)
(96, 4)
};
\addlegendentry{microarray}
\end{axis}
\end{tikzpicture}
\end{center}

B: MMP2 protein expression during constant light treatment

\begin{center}
\begin{tabular}{c|c|c|c|c|c|c}
0 hr & 16 hr & 31 hr & 51 hr & 68 hr & 96 hr \\
\hline
Pro & & & & & \\
Active & & & & & \\
Actin & & & & & \\
\end{tabular}
\end{center}

C: Quantification of MMP2 protein levels during constant intense light-treatment

\begin{center}
\begin{tikzpicture}
\begin{axis}[
width=\textwidth,
height=0.6\textwidth,
axis x line=bottom,
axis y line=left,
axis line style=-,\]
\addplot[mark=none,red,mark options={solid}]
coordinates {
(0, 1)
(16, 1)
(31, 1)
(51, 2)
(68, 3)
(96, 4)
(0, 4)
(16, 3)
(31, 2)
(51, 1)
(68, 0)
(96, -1)
};
\addlegendentry{Active-MMP2}
\addplot[mark=none,blue,mark options={solid}]
coordinates {
(0, 0)
(16, 0)
(31, 0)
(51, 0)
(68, 0)
(96, 0)
(0, 0)
(16, 0)
(31, 0)
(51, 0)
(68, 0)
(96, 0)
};
\addlegendentry{Pro-MMP2}
\end{axis}
\end{tikzpicture}
\end{center}
Figure A. 2: MMP2 localizes to clusters of NPCs during regeneration of the light damaged-retina. Dark-adapted albino zebrafish were exposed to constant intense light and eyes were enucleated and fixed at each time point. 12 micron cryosections were cut and stained with antibodies specific to zebrafish MMP2 and PCNA (green and red, respectively). At 0 and 16 hours of light (A, B), a basal level of MMP2 was expressed throughout the retinal layers. By 31 hours (C), MMP2 expression increased around the soma of proliferating Müller glia (arrowheads). MMP2 expression continued to increase around the surface of clustered NPCs (D-F, arrowheads), as well as along the entire length of the Müller glial processes associated with neurogenic clusters (E, arrows). By 96 hours (F), MMP2 expression persisted on the surface of NPCs in the INL (arrowheads), and was detected at very high levels near the apical domains of migrating NPCs in the ONL (arrows). NPC, neuronal progenitor cell; MMP2, matrix metalloproteinase 2; PCNA, proliferating cell nuclear antigen; ONL, outer nuclear layer; INL, inner nuclear layer. The scale bar in panel A is 25 µm and is the same for panels B-F.
Figure A. 3: Electroporation of morpholinos effectively knocks down MMP2 expression. Dark-adapted albino zebrafish were exposed to intense light for 24 hours, after which their eyes were either uninjected (A, D) or injected and electroporated with a lissamine-tagged anti-\textit{mmp2} morpholino (\textit{mmp2}-MO; C, F) or a lissamine-tagged anti-\textit{mmp2} five-base mismatch control morpholino (5mis-MO; B, E). After electroporation, fish were placed back in constant intense light for an additional 44 hours (total 68 hours constant light), when 12 micron cryosections were cut and stained with anti-MMP2 and DAPI (A-C). MMP2 expression was observed around clusters of NPCs in both the uninjected and 5mis-morphant control eyes (A and B, respectively; arrows), however, no MMP2 expression was detected in the INL of \textit{mmp2}-morphant injected eyes (C). Lissamine signal was imaged to verify successful uptake of either 5mis-morphant or \textit{mmp2}-morphant (D-F). To independently confirm the knockdown of MMP2 protein expression, zebrafish embryos were either uninjected or injected with \textit{mmp2}-morphant at the 1-4 cell stage. Total protein lysate was obtained at 48 hpf and analyzed via immunoblot (G). The large decrease in MMP2 expression in the \textit{mmp2}-morphant injected embryos relative to uninjected controls confirmed that \textit{mmp2}-morphant effectively knocked down MMP2 expression (G). Actin was used as a loading control for immunoblot analysis. MMP2, matrix metalloproteinase 2; hpf, hours post fertilization. Scale bar in panel A is 25 \textmu m and is the same for panels B-F.
Figure A. 4: Knockdown of MMP2 expression decreased NPC proliferation in the regenerating zebrafish retina. Dark-adapted albino zebrafish were exposed to intense light for 24 hours, after which their eyes were either uninjected (A, D, G) or injected and electroporated with lissamine-tagged anti-mmp2 morpholino (mmp2-MO; C, F, I) or lissamine-tagged anti-mmp2 five-base mismatch control morpholino (5mis-MO; B, E, H). After electroporation, fish were placed back in constant intense light for an additional 44 hours (total 68 hours constant light), when 12 micron cryosections were cut and stained with anti-PCNA and DAPI (A-F, green and blue, respectively). Both the uninjected (A, D) and 5mis-morphant control (B, E) retinas contained more PCNA-positive INL cells than the mmp2 morphant (C, F) retinas (J; Student’s t-test *p < 0.05, **p < 5 x 10^-5). To assess whether the decreased INL proliferation in the mmp2 morphant retinas was due to fewer Müller glia reentering the cell cycle or the NPCs not proliferating, the number of Müller glial-derived NPC columns was quantified (K). The NPCs present in a single column are thought to be derived from a single Müller glial cell. There was no statistically significant difference in the number of PCNA-positive columns between any treatment, indicating that the same number of Müller glia reentered the cell cycle in mmp2 morphant retinas as in the controls. Thus, the decreased INL proliferation observed in the mmp2 morphant retinas is most likely due to decreased NPC amplification. MMP2, matrix metalloproteinase 2; INL, inner nuclear layer; NPC, neuronal progenitor cell. Scale bar in panel A is 25 mm and is the same for panels B-I.
Figure A.5: Knockdown of MMP14b prevents Müller glia proliferation after 31 hours of light-treatment. Dark-adapted albino zebrafish were either uninjected (A, D) or injected and electroporated with lissamine-tagged anti-mmp14b morpholino (mmp14b MO; C, F) or lissamine-tagged anti-mmp14b five-base mismatch morpholino (mmp14b 5mis MO; B, E) and exposed to intense light for 68 hours. After 68 hours of light, eyes were collected and 12 micron cryosections were cut and stained with anti-PCNA and DAPI (A-F, green and blue, respectively). Both the uninjected (A, D) and mmp14b 5mis morphant control (B, E) retinas contained more PCNA-positive INL cells than the mmp14b morphant (C, F) retinas (G; Student’s t-test *p < 0.01). We also observed an increase in PCNA-positive cells in the ONL in mmp14b morphant retinas (C, F) compared to uninjected (A, D) and mmp14b 5mis morphant (B, E) control retinas, however, this increase was not statistically significant (G; Student’s t-test p > 0.05). PCNA, proliferating cell nuclear antigen; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar in panel A is 25 μm and is the same for panels B-F.
Figure A. 6: Generating a transgenic sox2 reporter zebrafish line using recombineering of bacterial artificial chromosomes. We PCR amplified a targeting cassette containing GFP and kanamycin with 50 bp homology arms targeting the endogenous sox2 locus. This cassette was recombined with the CH73-242D14-iTol-amp BAC, which harbors the endogenous sox2 locus and ~100 kb of upstream and downstream regulator sequence (A). The recombineering was carried out in the SW105 bacterial strain, which induces recombination following heat shock at 42°C. The recombineered BAC was verified via sequencing before being injected into 1-4 cell-stage zebrafish embryos, along with Tol2 mRNA. One day after injections, multiple embryos displayed mosaic expression of GFP, predominantly along the head, eye and spinal cord (B). These G0 embryos were raised to adulthood, out crossed to wild-type fish and the resulting F1 progeny were screened for the transgene.
Figure A. 7: Acetylation of histone H3 at lysine 9 increases in the regenerating zebrafish retina. Adult albino zebrafish retinas were either untreated or exposed to 36 hours of constant intense light. Retinas were dissected, disrupted with a 21G needle in HBSS and centrifuged to collect a crude nuclear pellet. Histones were extracted overnight at 4°C in 0.1 N HCl. Nuclear extracts were precipitated in acetone overnight at -20°C and resuspended in water. Coomassie staining revealed efficient histone extraction and equal loading of both sample preparations (A). Immunoblots were performed using antibodies to total H3 and H3 acetylated at lysine 9 (H3K9Ac) (B). We observed a 70% increase in intensity of H3K9Ac following 36 hours of light treatment. Densitometry was used to quantify the fold-increase of H3K9Ac at 36 hours relative to 0 hours (C).
Figure A. 8: Histone deacetylase inhibitor treatment causes hyperacytylation of histone H3 at lysine 9 in the adult zebrafish retina. Adult AB zebrafish were exposed either 100 nM or 250 nM doses of the potent HDAC inhibitor, Trichostatin A (TSA). TSA was added to ~500 mL of system water and the fish were bathed in the drug solution for 2 days. Control fish were treated with 1% DMSO. Histones were prepared from dissected retinas by acid extraction and acetone precipitation. Histone pellets were resuspended in water and immunoblots were performed using antibodies to total histone H3 and histone H3 acetylated at lysine 9 (H3K9Ac). We observed increasing band intensity of H3K9Ac as TSA concentration increased (A) and quantified the fold-change of band intensity for H3K9Ac relative to DMSO-treated retinas (B).
Figure A. 9: ChIP can successfully identify changes in gene regulation in the regenerating zebrafish retina. Chromatin was prepared using 100 retinas from adult albino zebrafish that had either been left untreated (0hr) or light-damaged for 48 hours (48hr) using the two-step method described by Thomas et al. (2012). Chromatin was immunoprecipitated using either rabbit anti-RNA Polymerase II C-terminal domain (Pol II) or normal rabbit IgG (IgG). qRT-PCR was performed using primers targeting the TATA-region of rhodopsin, gapdh and stat3 promoters. Following 48 hours of light, the rhodopsin promoter enrichment decreased, consistent with apoptosis of rhodopsin-expressing rods prior to this time point (Vihtelic and Hyde, 2000; Nelson et al., 2013). Conversely, stat3 promoter enrichment is minimal in the undamaged eye, but increases following light damage. This is consistent with stat3 expression increasing significantly in response to light-damage (Kassen et al., 2007). The gapdh promoter did not change between undamaged and light-damaged samples, as expected. All three sequences showed minimal enrichment in IgG control reactions.
Figure A. 10: Overview of genome engineering strategy. CRISPR/Cas9 target sites (red triangles) will generate double strand breaks (DSBs) for homology-directed repair HDR of the endogenous sox2-2a-sfGFP locus (Shin et al., 2014) with a targeting cassette harboring loxP sites and a downstream DsRed element. The engineered locus (B) will undergo Cre-mediated excision of sox2-2A-sfGFP, resulting in loss of sfGFP expression and the generation of DsRed expression under control of the sox2 promoter. T7EI assays (Kim et al., 2009) demonstrate effective DSBs at the 5'-gRNA target site, but not in Cas9-only controls (C).
REFERENCES


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