Published in final edited form as:

Exp Eye Res. 2014 June ; 123: 131–140. doi:10.1016/j.exer.2013.07.012.

Regulation of Müller Glial Dependent Neuronal Regeneration in the Damaged Adult Zebrafish Retina

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Abstract

This article examines our current knowledge underlying the mechanisms involved in neuronal regeneration in the adult zebrafish retina. Zebrafish, which has the capacity to regenerate a wide variety of tissues and organs (including the fins, kidney, heart, brain, and spinal cord), has become the premier model system to study retinal regeneration due to the robustness and speed of the response and the variety of genetic tools that can be applied to study this question. It is now well documented that retinal damage induces the resident Müller glia to dedifferentiate and reenter the cell cycle to produce neuronal progenitor cells that continue to proliferate, migrate to the damaged retinal layer and differentiate into the missing neuronal cell types. Increasing our understanding of how these cellular events are regulated and occur in response to neuronal damage may provide critical information that can be applied to stimulating a regeneration response in the mammalian retina. In this review, we will focus on the genes/proteins that regulate zebrafish retinal regeneration and will attempt to critically evaluate how these factors may interact to correctly orchestrate the definitive cellular events that occur during regeneration.

Keywords

Müller glia; neuronal progenitor cells; TNF α ; Ascl1a; dedifferentiation

Introduction

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

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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 is 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 innermost 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 coexpress 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). 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 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 expresses GFP from a Müller glial-specific promoter, to successfully characterize changes in gene expression during Müller glial 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 this review, we 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.

Molecular Consequences of Cell Death

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 glial 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 glial proliferation (Ramachandran et al., 2011). However, expression of *dkk1b* decreased throughout the retina following the puncture lesion, rather than specifically in the 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 TNF α 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 TNF α , as ouabain-induced retinal damage resulted in increased TNF α expression in the dying INL and ganglion cell neurons (Nelson et al., 2013). It is unclear if the Müller glia responding to the TNF α produced from dying photoreceptors (primary proliferating Müller glia, PPMG) are in direct contact or if the diffusion of TNF α allows for nearby Müller glia to respond to this initial production of TNF α (Figure 1). Regardless, TNF α appears to be a general signal produced by all dying neurons in the zebrafish retina.

To determine if TNF α 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 TNF α expression significantly reduced Müller glial proliferation relative to the controls. Preliminary results also show that intravitreal injection of TNF α is sufficient to induce Müller glial proliferation in the undamaged retina (Hobgood and Hyde, unpublished data). These data provide compelling evidence for TNF α being the first identified factor produced by dying retinal neurons that directly signal Müller glia to dedifferentiate and proliferate in the regeneration program.

This general mechanism of dying photoreceptors signaling a positive response in Müller glia is not limited to zebrafish. It was previously shown that dying photoreceptors in the murine retina secrete endothelin2 to signal the Müller glia (Rattner and Nathans, 2005). Additionally, the light damage resulted in increased expression of the endothelin receptor in the Müller glia. Subsequently, endothelin2 was shown to have a neuroprotective effect on photoreceptors (Braunger et al., 2013). Thus, dying photoreceptors in both the zebrafish and

mouse retinas possess the ability to signal to Müller glia and induce a response that either repairs or limits the damage.

Because TNF α is most commonly associated with inflammation during the mammalian immune response, the zebrafish regeneration program may involve a similar inflammatory response. Recently, acute inflammation was shown to be necessary and sufficient to initiate the regeneration program in the zebrafish telencephalon (Kyritsis et al., 2012). Expression of *tnfa* (as well as additional proinflammatory cytokines) increases significantly following brain lesion, and coincides with local activation of resident microglia and infiltration of leukocytes. These microglia and/or leukocytes can then secrete cytokines that directly stimulate proliferation of radial glia and drive regeneration of new neurons. Interestingly, activation and invasion of microglia also occurs rapidly following retinal injury (Bailey et al., 2010), suggesting a similar inflammatory response may play a role in zebrafish retinal regeneration. The impact of microglia in zebrafish retinal regeneration has not been extensively studied, and it appears TNF α is expressed exclusively by dying photoreceptors and a subset of Müller glia (Nelson et al., 2013), but it is possible that TNF α functions to activate and recruit microglia, which, in turn, may directly signal to Müller glia. Further studies aimed at microglial behavior in the regenerating retina will reveal whether the inflammation-dependent program described in the regenerating telencephalon is a conserved phenomenon in the teleost CNS.

Primary Müller glial dedifferentiation and proliferation

Müller glia respond to TNF α and potentially other signals from dying photoreceptors by initiating gene programs that are characteristic of both dedifferentiation and proliferation (Figure 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 secreted growth factor *midkine-a* (Calinescu et al., 2009); 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 has also been confirmed in the PCNA-positive Müller glia (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 glial dedifferentiation and promote the initial mitotic event that gives rise to the transient population of NPCs (Figure 2).

The first gene identified to be essential for zebrafish Müller glial dedifferentiation and proliferation was *ascl1a*. Expression of the *ascl1a* transcript increased within 6 hours of the puncture damage in the proliferating Müller glia near the wound (Fausett 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 glial 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 glial proliferation (Ramachandran et al., 2011), Wnt signaling may be necessary for Müller glial 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 clusters of NPCs at 4 days post-injury. Pharmacological stabilization of the β -catenin destruction complex also inhibited Müller glial proliferation. Perhaps the most interesting finding from these experiments was that pharmacological inhibition of GSK3 β was sufficient to activate the expression of several genes associated with the normal Müller glial dedifferentiation program (*ascl1a*, *lin28*, *myc*, *wnt4a* and *fzd2*) and stimulate Müller glial proliferation in the absence of damage (Ramachandran et al., 2011). The increase in *ascl1a* expression following GSK3 β inhibition is surprising, given that *ascl1a* is required for *dkk1b* suppression and activation of *wnt4a*, which would logically occur prior to β -catenin stabilization (Ramachandran et al., 2011). Because the GSK3 β inhibitor-injected eyes were not analyzed until 4 days post-injection, it is possible that exogenous Wnt activation is sufficient to stimulate Müller glial 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 glial dedifferentiation.

As a whole, these experiments provided strong evidence for a Müller glial dedifferentiation program in which *Ascl1a* inhibits *dkk1b* expression (Figure 2). This reduction in *dkk1b* leads to increased canonical Wnt signaling, potentially via *Wnt4a* and *Fzd2*, and translocation of β -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 *Xenopus* has shown that β -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 *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 β -catenin, which is necessary for Müller glial proliferation and induction of Sox2 to maintain dedifferentiated Müller glia and/or NPC identity. While this is an attractive model, the interaction between β -catenin and Sox2 has not yet been addressed in the regenerating zebrafish retina.

Because *Ascl1a* functions primarily as a transcriptional activator, it is interesting that *ascl1a* expression was required to inhibit *dkk1b* expression. The most obvious explanation is that *Ascl1a* activates one or more transcriptional repressors during Müller glial dedifferentiation. Microarray experiments of FACS-sorted Müller glia and Müller glia-derived NPCs revealed ~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 glial 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 glial dedifferentiation is the stem cell-associated pluripotency factors (Takahashi et al., 2006). 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 glial dedifferentiation and proliferation (Ramachandran et al., 2010). The function of Lin28 during Müller glial 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 glial 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 (Ramachandran 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 glial 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 glial 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 glial 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 glial proliferation at 4 days post injury (Powell et al., 2012), their role during Müller glial dedifferentiation and proliferation were not reported. This rather late time point makes it difficult to conclusively state the importance of these proteins in Müller glial dedifferentiation relative to amplification of NPCs. While DNA methylation and Müller glial 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.

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 TNF α .

Characterization of the relationship between Stat3 expression and Müller glia proliferation suggested that Müller glia may undergo one of three different responses to retinal damage (Nelson et al. 2012). The PPMG represent Müller glia that proliferate in a Stat3-independent manner. In contrast, the secondary proliferating Müller glia (SPMG) require Stat3 expression to proliferate. Finally, the quiescent Müller glia (QMG) express Stat3, but do not proliferate in the damaged retina. Subsequent experiments suggest that the PPMG are stimulated to proliferate by the dying photoreceptors (through TNF α expression), while the SPMG require Müller glial expression of TNF α to proliferate (Nelson et al., 2013). Many of the pathways required for PPMG to dedifferentiate and reenter the cell cycle are also likely required for dedifferentiation of SPMG. Here we will focus on how the PPMG directly recruit the SPMG to dedifferentiate and proliferate (Figure 3).

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, TNF α is first expressed in dying neurons and subsequently in a subset of Müller glia that are PCNA-positive (Nelson et al., 2013). Müller glial expression of TNF α required Lin28/Ascl1a/Stat3 expression, suggesting that TNF α is the Stat3-dependent signal to the SPMG. This was supported by the observation that Müller glial expression of TNF α , like Stat3, was necessary for a subset of, but not all, Müller glia to proliferate (SPMG). Additionally, Müller glial-derived TNF α is required for Ascl1a and Stat3 expression in the SPMG, which is consistent with Stat3 functioning through a cytokine receptor/Jak/Stat pathway; however, the link between TNF α activation of Ascl1a in SPMG is less clear.

While a model of TNF α /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 TNF α 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 TNF α and/or another, unidentified damage signal stimulates increased Stat3 expression in all Müller glia. In this model, pan-Müller glial 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 glial 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 glial 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 glial 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 glial dedifferentiation, such as *lin28* and *ascl1a* (Wan et al., 2012). Taken together, these data suggest that Müller glial 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 glial expression of TNF α recruits additional Müller glia to reenter the cell cycle (Nelson et al., 2013).

Restricting the zone of dedifferentiation/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 responses could represent a mechanism to regulate Müller glial 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 that function to restrict the zone of regeneration and limit excessive Müller glial 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., 2011). 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 glial proliferation. Wan et al. (2012) demonstrated that application of the γ -secretase inhibitor, DAPT, expanded the zone of Müller glial 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 amplification of NPCs. While Notch signaling remains elusive, it is clear that one of its functions is to maintain Müller glial quiescence at the site of injury.

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 *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 glial 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 *insm1a* expression (Ramachandran et al., 2012). Thus, Notch signaling likely promotes *insm1a* expression, which represses *hb-egf* expression to limit the zone of Müller glial 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

notch and *insmla* expression in quiescent Müller glia will be necessary to confirm this model. Wan et al. (2012) also showed that ectopic HB-EGF-induced Müller glial 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 *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).

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 glial processes to the site(s) of damage and differentiating into the appropriate cell types (Vihtelic and Hyde, 2000; Fausett and Goldman, 2006; Bernardos et al., 2007).

During amplification of NPCs, the Pax6 paralogs, Pax6a and Pax6b, play non-redundant roles. Pax6 protein expression is first observed following Müller glial 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 division of NPCs, while having no effect on the initial Müller glial division (Thummel et al., 2010). Interestingly, preventing proliferation of NPCs 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 division of NPCs, but prevented subsequent proliferation of NPCs (Thummel et al., 2008). While the identity of the Pax6a and Pax6b transcriptional targets that promote proliferation of NPCs are unknown, several studies have identified factors that can regulate *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 *pax6b* expression following retinal damage. Additionally, the temperature-sensitive *hspd1* allele, *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 glial proliferation (thus, genesis of NPCs) and not direct regulation of Pax6 within NPCs.

The mitotic checkpoint kinase, *monopolar spindle 1* (*mps1*), also appears to regulate proliferation of NPCs (Qin et al., 2009). Expression of *mps1* mRNA levels increased following 36 hours of light lesion, after Müller glial dedifferentiate and begin dividing (Qin et al., 2009). Use of a temperature-sensitive *mps1* allele, *nightcap* (*nep*), revealed that MPS1 does not function in the initial Müller glial dedifferentiation/proliferation, but impairs proliferation of NPCs 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 migration of NPCs. While these observations provide insight into the likely mechanisms controlling migration of NPCs, functional studies are still lacking.

Differentiation of new neurons and glia

The final step in the zebrafish retinal regeneration program is differentiation of NPCs 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 glial 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 glial renewal (Conner and Hyde, unpublished data). This recapitulates the role of Notch signaling during vertebrate retinal development, where Notch signaling is known to drive progenitors to a Müller glial cell fate (Furukawa et al., 2000; Bernardos et al., 2005). These data are also consistent with Müller glial dedifferentiation requiring loss of Notch signaling, as reestablishment of Notch signaling can drive redifferentiation of the activated Müller glia.

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., 2012; Hochmann et al., 2012), with *fgfr1a* mRNA (Qin et al., 2012) and Fgfr1a protein (Hochmann et al., 2012) expression detected in the ONL, basal INL and GCL, while *fgfr2* and *fgfr3* mRNAs 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 *fgf8a* and *fgf24* being expressed in the INL and GCL, and *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 *Tg(hsp70:dn-fgfr1)/+* zebrafish, which express a dominant-negative Fgfr1, fail to regenerate rods following light damage, but cone regeneration and Müller glial proliferation are unaffected (Qin et al., 2012). In contrast, homozygous *Tg(hsp70:dn-fgfr1)* zebrafish displayed impaired Müller glial 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.

Galectin 1-like 2 (*drgal1-l2*) is a member of a family of secreted glycoprotein-binding proteins that can function as an extracellular signaling molecule in neuronal regeneration (reviewed in Camby et al., 2006). In the regenerating zebrafish retina, *drgal1-l2* expression increases in dedifferentiated Müller glia following light-induced photoreceptor cell death and the Drgal1-L2 protein is secreted from Müller glial-derived NPCs (Craig et al., 2010). Despite this expression profile, which is indicative of a role in Müller glial dedifferentiation and/or amplification of NPCs, Drgal1-L2 appears to only function in regeneration of rod photoreceptors in the light-damaged retina (Craig et al., 2010). This suggests there may be spatially restricted glycoproteins that bind Drgal1-L2, which may dictate the function of Drgal1-L2 at different locations/cell types throughout the regenerating retina. Additionally, because *drgal1-l2* was identified by microarray analyses of laser captured ONL tissue following light-damage, there may be other, unidentified Galectin family members that function redundantly in the INL during regeneration. Regardless, these data clearly demonstrate the importance of the extracellular matrix during retinal regeneration.

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 cell cycle exit of NPCs 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 cell cycle exit of NPCs (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 (Ramachandran 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). While this study is the first to closely analyze cell cycle exit of NPCs, 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.

Conclusion

Retinal regeneration in zebrafish is an incredibly complex process that requires a number of events: 1) dedifferentiation and proliferation of the primary proliferating Müller glia, 2) recruitment of the secondary proliferating Müller glia (if the extent of damage is severe), 3) amplification of the number of NPCs, 4) migration of the NPCs to the proper retinal layer, and 5) differentiation into the missing retinal neurons. While many of the molecular details of these different steps are being extensively studied and have already been identified, many key questions remain to be resolved. How many different signals are necessary to initiate the regeneration process? What signals determine the number of Müller glia (PPMG and SPMG) that will dedifferentiate in response to the level of retinal damage? How do the different signaling pathways integrate to provide a unified message to the Müller glia and NPCs to properly regenerate the retina? What specifies the NPCs to commit to the neuronal lineages that are lost in the damaged retina? What causes the NPCs to exit the cell cycle when a sufficient number of neurons is produced to accurately replace those that are lost in the damaged retina?

The development of new techniques, such as morpholino-mediated knockdown of specific proteins, transgenics to transiently express candidate proteins (wild-type and mutant) in specific cell types, and targeted mutagenesis of genes using TALENS, will be critical to elucidate the details underlying Müller glial-dependent retinal regeneration in zebrafish. A deeper understanding of the factors and mechanisms regulating Müller glia to accurately regenerate the lost zebrafish retinal neurons could provide important information to test if the Müller glia in the damaged mammalian retina can be induced to regenerate lost neurons.

Acknowledgments

Support Information: This study was supported by a grant from the National Eye Institute of NIH to DRH (R01-RY018417) and the Center for Zebrafish Research, University of Notre Dame, Notre Dame, IN.

References

- Agathocleous M, Iordanova I, Willardsen MI, Xue XY, Vetter ML, Harris WA, Moore KB. A directional Wnt/beta-catenin-Sox2-proneural pathway regulates the transition from proliferation to differentiation in the Xenopus retina. *Development*. 2009; 136:3289–3299. [PubMed: 19736324]
- Bernardos RL, Barthel LK, Meyers JR, Raymond PA. Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. *J Neurosci*. 2007; 27:7028–7040. [PubMed: 17596452]
- Bernardos RL, Lentz SI, Wolfe MS, Raymond PA. Notch-Delta signaling is required for spatial patterning and Muller glia differentiation in the zebrafish retina. *Dev Biol*. 2005; 278:381–395. [PubMed: 15680358]
- Braunger BM, Ohlmann A, Koch M, Tanimoto N, Volz C, Yang Y, Bosl MR, Cvekl A, Jagle H, Seeliger MW, Tamm ER. Constitutive overexpression of Norrin activates Wnt/beta-catenin and endothelin-2 signaling to protect photoreceptors from light damage. *Neurobiol Dis*. 2013; 50:1–12. [PubMed: 23009755]
- Calinescu AA, Vihtelic TS, Hyde DR, Hitchcock PF. Cellular expression of midkine-a and midkine-b during retinal development and photoreceptor regeneration in zebrafish. *J Comp Neurol*. 2009; 514:1–10. [PubMed: 19263476]
- Camby I, Le Mercier M, Lefranc F, Kiss R. Galectin-1: a small protein with major functions. *Glycobiology*. 2006; 16:137R–157R.
- Cameron DA. Cellular proliferation and neurogenesis in the injured retina of adult zebrafish. *Vis Neurosci*. 2000; 17:789–797. [PubMed: 11153658]
- Cameron DA, Gentile KL, Middleton FA, Yurco P. Gene expression profiles of intact and regenerating zebrafish retina. *Mol Vis*. 2005; 11:775–791. [PubMed: 16205622]
- Craig SE, Calinescu AA, Hitchcock PF. Identification of the molecular signatures integral to regenerating photoreceptors in the retina of the zebra fish. *J Ocul Biol Dis Infor*. 2008; 1:73–84. [PubMed: 20072637]
- Craig SE, Thummel R, Ahmed H, Vasta GR, Hyde DR, Hitchcock PF. The zebrafish galectin Drgal1-l2 is expressed by proliferating Muller glia and photoreceptor progenitors and regulates the regeneration of rod photoreceptors. *Invest Ophthalmol Vis Sci*. 2010; 51:3244–3252. [PubMed: 20071673]
- Del Bene F, Wehman AM, Link BA, Baier H. Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal notch gradient. *Cell*. 2008; 134:1055–1065. [PubMed: 18805097]
- Fausett BV, Goldman D. A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. *J Neurosci*. 2006; 26:6303–6313. [PubMed: 16763038]
- Fausett BV, Gumerson JD, Goldman D. The proneural basic helix-loop-helix gene *ascl1a* is required for retina regeneration. *J Neurosci*. 2008; 28:1109–1117. [PubMed: 18234889]
- Fimbel SM, Montgomery JE, Burket CT, Hyde DR. Regeneration of inner retinal neurons after intravitreal injection of ouabain in zebrafish. *J Neurosci*. 2007; 27:1712–1724. [PubMed: 17301179]
- Furukawa T, Mukherjee S, Bao ZZ, Morrow EM, Cepko CL. *rax*, *Hes1*, and *notch1* promote the formation of Müller glia by postnatal retinal progenitor cells. *Neuron*. 2000; 26:383–394. [PubMed: 10839357]
- Hochmann S, Kaslin J, Hans S, Weber A, Machate A, Geffarth M, Funk RH, Brand M. Fgf signaling is required for photoreceptor maintenance in the adult zebrafish retina. *PLoS One*. 2012; 7:e30365. [PubMed: 22291943]
- Huang Y. A mirror of two faces: Lin28 as a master regulator of both miRNA and mRNA. *Wiley Interdiscip Rev RNA*. 2012; 3:483–494. [PubMed: 22467269]
- Kassen SC, Ramanan V, Montgomery JE, T Burket C, Liu CG, Vihtelic TS, Hyde DR. Time course analysis of gene expression during light-induced photoreceptor cell death and regeneration in albino zebrafish. *Dev Neurobiol*. 2007; 67:1009–1031. [PubMed: 17565703]

- Kassen SC, Thummel R, Burket CT, Campochiaro LA, Harding MJ, Hyde DR. The Tg(ccnb1:EGFP) transgenic zebrafish line labels proliferating cells during retinal development and regeneration. *Mol Vis.* 2008; 14:951–963. [PubMed: 18509551]
- Kyritsis N, Kizil C, Zocher S, Kroehne V, Kaslin J, Freudenreich D, Iltzsche A, Brand M. Acute inflammation initiates the regenerative response in the adult zebrafish brain. *Science.* 2012; 338:1353–1356. [PubMed: 23138980]
- Lombardo F. La rigenerazione della retina negli adulti di un Teleosteo. [Regeneration of the retina in an adult Teleost]. *Accad Lincei-Rendiconti Scienze Fis Mat Nat Ser.* 1968; 845:631–635.
- Lombardo F. Andamento e localizzazione della mitosi durante la rigenerazione della retina di un Teleosteo adulto. [Time course and localization of mitoses during regeneration of the retina an adult teleost.]. *Accad Lincei-Rendiconti Scienze Fis Mat Nat Ser.* 1972; 853:323–327.
- Maier W, Wolburg H. Regeneration of the goldfish retina after exposure to different doses of ouabain. *Cell Tissue Res.* 1979; 202:99–118. [PubMed: 509506]
- Meyers JR, Hu L, Moses A, Kaboli K, Papandrea A, Raymond PA. beta-catenin/Wnt signaling controls progenitor fate in the developing and regenerating zebrafish retina. *Neural Dev.* 2012; 7:30–8104. 7–30. [PubMed: 22920725]
- Mitashov VI. Mechanisms of retina regeneration in urodeles. *Int J Dev Biol.* 1996; 40:833–844. [PubMed: 8877458]
- Montgomery JE, Parsons MJ, Hyde DR. A novel model of retinal ablation demonstrates that the extent of rod cell death regulates the origin of the regenerated zebrafish rod photoreceptors. *J Comp Neurol.* 2010; 518:800–814. [PubMed: 20058308]
- Morris AC, Forbes-Osborne MA, Pillai LS, Fadool JM. Microarray analysis of XOPS-mCFP zebrafish retina identifies genes associated with rod photoreceptor degeneration and regeneration. *Invest Ophthalmol Vis Sci.* 2011; 52:2255–2266. [PubMed: 21217106]
- Nelson BR, Hartman BH, Ray CA, Hayashi T, Bermingham-McDonogh O, Reh TA. Acheate-scute like 1 (Ascl1) is required for normal delta-like (Dll) gene expression and notch signaling during retinal development. *Dev Dyn.* 2009; 238:2163–2178. [PubMed: 19191219]
- Nelson CM, Ackerman KM, O'Hayer P, Bailey TJ, Gorsuch RA, Hyde DR. Tumor Necrosis Factor-Alpha Is Produced by Dying Retinal Neurons and Is Required for Muller Glia Proliferation during Zebrafish Retinal Regeneration. *J Neurosci.* 2013; 33:6524–6539. [PubMed: 23575850]
- Nelson CM, Gorsuch RA, Bailey TJ, Ackerman KM, Kassen SC, Hyde DR. Stat3 defines three populations of Muller glia and is required for initiating maximal Muller glia proliferation in the regenerating zebrafish retina. *J Comp Neurol.* 2012; 520:4294–4311. [PubMed: 22886421]
- Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR. Neurons derived from radial glial cells establish radial units in neocortex. *Nature.* 2001; 409:714–720. [PubMed: 11217860]
- Otteson DC, Hitchcock PF. Stem cells in the teleost retina: persistent neurogenesis and injury-induced regeneration. *Vision Res.* 2003; 43:927–936. [PubMed: 12668062]
- Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. *Science.* 2002; 298:2188–2190. [PubMed: 12481136]
- Powell C, Elsaedi F, Goldman D. Injury-dependent Muller glia and ganglion cell reprogramming during tissue regeneration requires Apobec2a and Apobec2b. *J Neurosci.* 2012; 32:1096–1109. [PubMed: 22262907]
- Qin Z, Barthel LK, Raymond PA. Genetic evidence for shared mechanisms of epimorphic regeneration in zebrafish. *Proc Natl Acad Sci U S A.* 2009; 106:9310–9315. [PubMed: 19474300]
- Qin Z, Kidd AR 3rd, Thomas JL, Poss KD, Hyde DR, Raymond PA, Thummel R. FGF signaling regulates rod photoreceptor cell maintenance and regeneration in zebrafish. *Exp Eye Res.* 2011; 93:726–734. [PubMed: 21945172]
- Ramachandran R, Fausett BV, Goldman D. Ascl1a regulates Muller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signaling pathway. *Nat Cell Biol.* 2010; 12:1101–1107. [PubMed: 20935637]
- Ramachandran R, Zhao XF, Goldman D. Ascl1a/Dkk/beta-catenin signaling pathway is necessary and glycogen synthase kinase-3beta inhibition is sufficient for zebrafish retina regeneration. *Proc Natl Acad Sci U S A.* 2011; 108:15858–15863. [PubMed: 21911394]

- Ramachandran R, Zhao XF, Goldman D. Insm1a-mediated gene repression is essential for the formation and differentiation of Muller glia-derived progenitors in the injured retina. *Nat Cell Biol.* 2012; 14:1013–1023. [PubMed: 23000964]
- Rattner A, Nathans J. The genomic response to retinal disease and injury: evidence for endothelin signaling from photoreceptors to glia. *J Neurosci.* 2005; 25:4540–4549. [PubMed: 15872101]
- Raymond PA, Barthel LK, Bernardos RL, Perkowski JJ. Molecular characterization of retinal stem cells and their niches in adult zebrafish. *BMC Dev Biol.* 2006; 6:36. [PubMed: 16872490]
- Raymond PA, Reifler MJ, Rivlin PK. Regeneration of goldfish retina: rod precursors are a likely source of regenerated cells. *J Neurobiol.* 1988; 19:431–463. [PubMed: 3392530]
- Sherpa T, Fimbel SM, Mallory DE, Maaswinkel H, Spritzer SD, Sand JA, Li L, Hyde DR, Stenkamp DL. Ganglion cell regeneration following whole-retina destruction in zebrafish. *Dev Neurobiol.* 2008; 68:166–181. [PubMed: 18000816]
- Sperry RW. Patterning of central synapses in regeneration of the optic nerve in teleosts. *Physiol Zool.* 1948; 21:351–361. [PubMed: 18891154]
- Stetler RA, Gan Y, Zhang W, Liou AK, Gao Y, Cao G, Chen J. Heat shock proteins: cellular and molecular mechanisms in the central nervous system. *Prog Neurobiol.* 2010; 92:184–211. [PubMed: 20685377]
- Thummel R, Enright JM, Kassen SC, Montgomery JE, Bailey TJ, Hyde DR. Pax6a and Pax6b are required at different points in neuronal progenitor cell proliferation during zebrafish photoreceptor regeneration. *Exp Eye Res.* 2010; 90:572–582. [PubMed: 20152834]
- Thummel R, Kassen SC, Enright JM, Nelson CM, Montgomery JE, Hyde DR. Characterization of Muller glia and neuronal progenitors during adult zebrafish retinal regeneration. *Exp Eye Res.* 2008; 87:433–444. [PubMed: 18718467]
- Thummel R, Kassen SC, Montgomery JE, Enright JM, Hyde DR. Inhibition of Muller glial cell division blocks regeneration of the light-damaged zebrafish retina. *Dev Neurobiol.* 2008; 68:392–408. [PubMed: 18161852]
- Vihtelic TS, Hyde DR. Light-induced rod and cone cell death and regeneration in the adult albino zebrafish (*Danio rerio*) retina. *J Neurobiol.* 2000; 44:289–307. [PubMed: 10942883]
- Wan J, Ramachandran R, Goldman D. HB-EGF is necessary and sufficient for Muller glia dedifferentiation and retina regeneration. *Dev Cell.* 2012; 22:334–347. [PubMed: 22340497]
- Willardsen MI, Link BA. Cell biological regulation of division fate in vertebrate neuroepithelial cells. *Dev Dyn.* 2011; 240:1865–1879. [PubMed: 21761474]

Highlights

1. Müller glia are the source of regenerated neurons in damaged zebrafish retinas.
2. TNF α is produced by dying retinal neurons to signal Müller glia to proliferate.
3. Müller glial dedifferentiation and proliferation requires Ascl1a and Stat3.
4. Müller glia undergo either Stat3-dependent or independent cell cycle reentry.
5. Müller glia dedifferentiation involves both positive and negative regulation.

Figure 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.

Figure 2.

Signaling events necessary for Müller glial dedifferentiation and proliferation. Dying retinal neurons (e.g. rod and cone photoreceptors) secrete TNF α (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; TNF α , Tumor necrosis factor-alpha; TNFSR, TNF superfamily receptor.

Figure 3.

Recruitment of SPMG and maintenance of QMG. PPMG secrete TNF α (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 TNF α 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 TNF α /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 TNF α /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; TNF α , Tumor necrosis factor-alpha; TNFSR, TNF superfamily receptor.