RESEARCH ARTICLE

Retinal Regeneration in Adult Zebrafish Requires Regulation of TGFβ Signaling

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Müller glia are the resident radial glia in the vertebrate retina. The response of mammalian Müller glia to retinal damage often results in a glial scar and no functional replacement of lost neurons. Adult zebrafish Müller glia, in contrast, are considered tissue-specific stem cells that can self-renew and generate neurogenic progenitors to regenerate all retinal neurons after damage. Here, we demonstrate that regulation of TGFβ signaling by the corepressors Tgif1 and Six3b is critical for the proliferative response to photoreceptor destruction in the adult zebrafish retina. When function of these corepressors is disrupted, Müller glia and their progeny proliferate less, leading to a significant reduction in photoreceptor regeneration. Tgif1 expression and regulation of TGFβ signaling are implicated in the function of several types of stem cells, but this is the first demonstration that this regulatory network is necessary for regeneration of neurons.

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Key words: Müller glia, photoreceptor, tgif1, six3b, stem cell

Introduction

Müller glia are the radial glia of the adult vertebrate retina and provide physical and homeostatic support for proper neuronal function. Mammalian Müller glia respond to retinal injury with reactive gliosis, characterized by increased glial fibrillary acidic protein (GFAP), hypertrophy, and in some cases mitotic activity. These cellular changes are initially neuroprotective, but typically result in a glial scar and no neuron regeneration (Bringmann et al., 2009; Reichenbach and Bringmann, 2013). Despite this, mammalian Müller glia express low levels of two neural progenitor markers, Sox2 and Pax6 (Roesch et al., 2008), and, in response to neuronal damage, increase expression of brain lipid binding protein (BLBP), a marker of young Müller glia in zebrafish retina (Raymond et al., 2006), radial glia, and a subtype of neural precursors in the developing mammalian brain (Chang et al., 2007; Hartfuss et al., 2001). These studies suggest that mammalian Müller glia have a latent ability to regenerate neurons (Karl and Reh, 2010). In fact, cultured mammalian Müller glia proliferate in response to exogenous growth factors (Ikeda and Puro, 1995; Ueki et al., 2012), and rodent Müller glia have a limited capacity to regenerate retinal neurons after damage, but only in the perinatal retina (Karl and Reh, 2010).

In contrast to mammals, adult zebrafish regenerate all retinal neurons through the activation, dedifferentiation, and proliferation of Müller glia. Like vertebrate neural stem cells in the brain, zebrafish Müller glia divide asymmetrically to produce a proliferative neurogenic progenitor, which then forms a neurogenic cluster that ultimately regenerates lost neurons to restore retinal structure and function (Bernardos et al., 2007). Early stages of regeneration in these retinal stem cells are characterized by upregulation of GFAP, BLBP, and proliferating cell nuclear antigen (PCNA), and expression of

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numerous retinal progenitor genes including *rx1, pax6*, and *acsl1a* (Bernardos et al., 2007; Bringmann et al., 2009; Fausett et al., 2008; Hartfuss et al., 2001; Raymond et al., 2006; Thummel et al., 2010).

TGFβ signaling, mediated through Smad2 and Smad3, controls proliferation of mammalian Müller glia and retinal progenitors *in vivo* (Close et al., 2005; Satoh and Watanabe, 2008) and *in vitro* (Ichida et al., 2009; Ikeda and Puro, 1995), and neural stem cells in the brain (Aigner and Bogdahn, 2008). During mammalian retinal development, TGFβ signaling increases when retinal cells differentiate, and Müller glia proliferation continues when TGFβ signaling is inhibited (Close et al., 2005). Mammalian astrocytes respond to TGFβ signaling by becoming gliotic and secreting extracellular matrix proteins, resulting in glial scars (Bringmann et al., 2009; Reichenbach and Bringmann, 2013; Robel et al., 2011), and inhibiting TGFβ prevents scarring (Moon and Fawcett, 2001). Preventing glial scars is likely a critical step in promoting regeneration in the central nervous system (Robel et al., 2011).

Several TGFβ signaling pathway members are differentially regulated in zebrafish Müller glia during retina regeneration: Two corepressors, *tgif1* and *six3b*, are rapidly upregulated prior to the initial mitotic division (Inbal et al., 2007; Kassen et al., 2007; Qin et al., 2009; Wotton et al., 1999) and thus may have overlapping roles to repress TGFβ signaling in Müller glia. Tgif1 has been implicated in maintaining a stem cell identity, as *tgif1* transcripts are enriched in murine organs containing stem cell populations, such as the ovary and testes, and embryonic stem cells (ESC) (Thorrez et al., 2008). Six3b binds to the DNA replication inhibitor Geminin during eye development and promotes proliferation in neural development in fish (Singh and Tsonis, 2010).

In this study, we tested the hypothesis that down-regulation of Smad2/3-mediated TGFβ signaling by the transcriptional corepressors Tgif1 and Six3b is necessary for the injury-induced proliferative response of Müller glia and Müller-glial derived neurogenic progenitors in zebrafish retinas following photoreceptor destruction. We used *tgif1* and *six3b* genetic mutants and Six3a/b translational knock-down to show that these corepressors function after acute photoreceptor damage to promote the proliferative response of Müller glia stem cells that is required for photoreceptor regeneration.

Materials and Methods

Zebrafish Lines and Light Lesions

We maintained fish under standard conditions (Westerfield, 2000). We used transgenic fish lines in which Müller glia are the only retinal cells that express green fluorescent protein (GFP) or enhanced green fluorescent protein (EGFP) (Bernardos et al., 2007): in *Tg(gfap:EGFP)mi2002*, differentiated Müller glia express GFP, and in *Tg(gfap:nGFP)mi2004*, immature Müller glia and Müller glia that are activated in response to retinal damage express nuclear GFP. We identified mutant fish lines with the $tgif1^{fh258}$ allele (Y143→Stop) and the $six3b^{vu87}$ allele (E109→Stop; Inbal et al., 2007) in a cryopreserved TILLING library (Draper et al., 2004). For experiments with $tgif1^{fh258}$ and $six3b^{vu87}$ lines, we destroyed photoreceptors with an acute light exposure (Bernardos et al., 2007). Mutant allele annotations have been abbreviated as $tgif1^{-/-}$ and $six3b^{-/-}$. For Six3 morpholino experiments, we destroyed photoreceptors in albino zebrafish using constant light treatment (Thummel et al., 2010). All procedures were approved by the Committees on Use and Care of Animals at the University of Michigan and Wayne State University.

Morpholinos

We injected 3′ lissamine-tagged morpholinos intravitreously and electroporated immediately prior to light lesion (Thummel et al., 2010). We used the Gene Tools standard control morpholino (5′-CCTCCTACCTCAGTTACAATTTATA-3′), and a morpholino that recognizes both *six3a* and *six3b* in the experimental treatment (5′-GCTCTAAAGGAGACCTGAAAACCAT-3′; Ando et al., 2005).

Immunohistochemistry and Cell Counting

Tissue was collected and processed as previously described (Bernardos et al., 2007). Antibody dilutions and antigen retrieval are provided in Supporting Information Table S1. We sectioned retinas through the dorsoventral axis and counted PCNA-positive nuclei in 100 μm linear length of the lesion using the cell counter in ImageJ (National Institutes of Health, Bethesda, MD). We counted cells in the lesioned area of the central retina in an area within 400 μm of the optic nerve head along the nasotemporal axis. We analyzed 8 to 16 sections per fish in sections at least 24 μm apart. PCNA-positive nuclei spanning the outer plexiform layer were included in the outer nuclear layer (ONL). We determined the lesioned area by increased expression of BLBP or GFP in transgenic *gfap:EGFP* fish, in combination with loss of cone nuclei. In morpholino experiments, we counted PCNA-positive nuclei in a 300 μm linear length of 2–4 sections per fish in peripheral regions of the dorsal retina marked with the morpholino lissamine tag, avoiding areas that were damaged by electroporation. To determine the extent of cone regeneration, we counted cone nuclei whose profiles extended more than 50% outside of the outer limiting membrane (OLM), which was visualized by GFP expression in *gfap:EGFP* fish or by ZO-1 immunoreactivity.

Western Blots

We immediately froze isolated retinas from dark-adapted fish on dry ice and then homogenized them in cold lysis buffer (50 μL/retina; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 7.0, 0.1% Triton X-100) with complete mini protease inhibitor cocktail (Roche). We triturated retinas with a 200 μL micropipette tip, incubated them on ice for 30 min, and triturated again (~20 times). We centrifuged samples at 13,000 rpm for 12 min at 4°C, ran protein gels under reducing and denaturing conditions, and performed Western blots using standard protocols. For densitometric analysis, we digitized the film (AlphaEase FC 6.0, Alpha Innotech), subtracted an image of the light box alone from each blot image, and used the

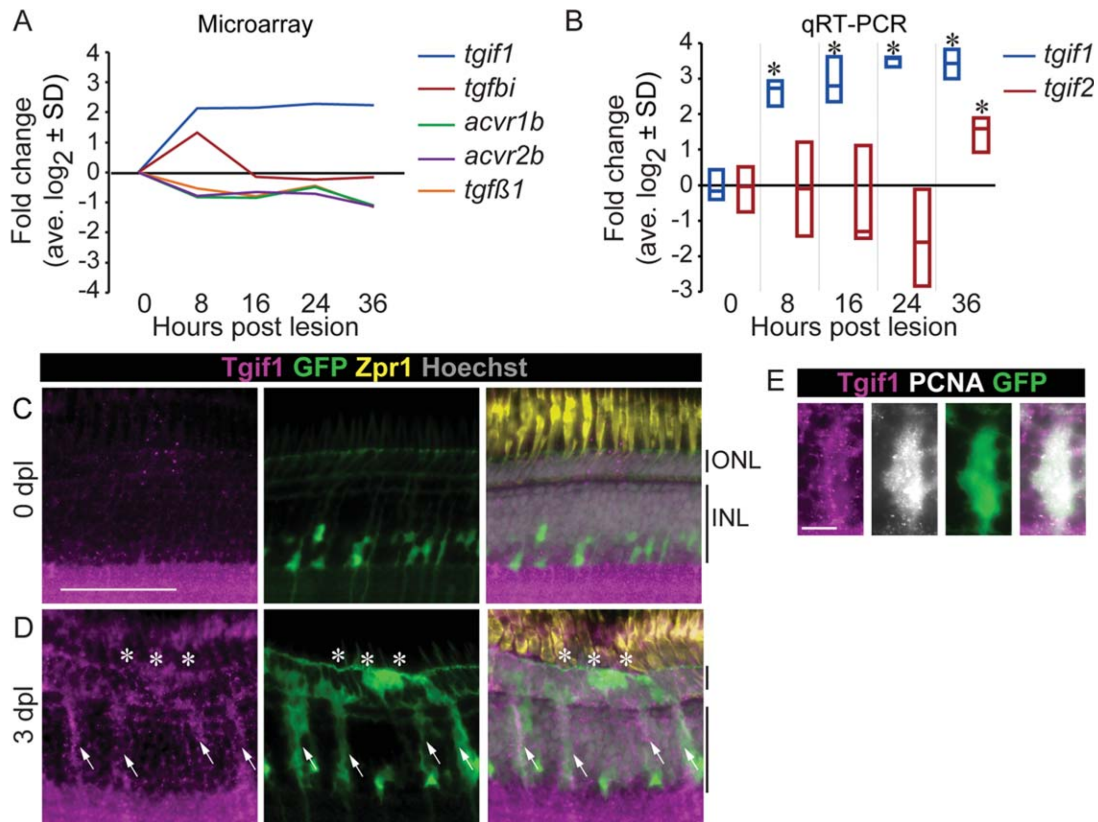


FIGURE 1: Smad2/3 signaling is downregulated and the corepressor Tgif1 upregulated after photoreceptors destruction. (A) Changes in expression of Smad2/3 signaling pathway in isolated Müller glia after acute light lesion, compared with uninjured retina, by microarray analysis (GEO GSE14495; Qin et al., 2009). (B) Changes in expression of *tgif1* and *tgif2* in isolated Müller glia measured by qRT-PCR. * $P<0.05$ relative to 0 hpl, Student t-test. Tgif1 immunoreactivity in an undamaged retina (C) and at 3 dpl (D) in *gfap:EGFP* fish that have GFP in the Müller glia and progenitors (arrows = Müller glia; * = lesion). (E) Colocalization of Tgif1 and PCNA in GFP-positive Müller glia in *gfap:EGFP* fish at 3 dpl. Scale bars = 50 μm in (C,D); 10 μm in (E). ONL = outer nuclear layer; INL = inner nuclear layer; hpl = hours post lesion; dpl = days post lesion.

gel analysis tool in ImageJ. For presentation of blot images (Fig. 2, S2), the color levels were changed uniformly to the image in Photoshop. Antibodies are provided in Supporting Information Table S2.

Real-Time Reverse Transcription PCR (qRT-PCR)

For transcriptional analysis of Müller glia, we isolated the Müller glia from *gfap:EGFP* fish using fluorescence-activated cell sorting (Qin et al., 2009). For whole retina analysis, we pooled retinas from individual fish in TRIzol (75 μL/retina) immediately after dissection. We isolated total RNA according to the manufacturer's instructions and resuspended RNA in 20 μL of RNAse-free water. We treated DNase and extracted the RNA with phenol-chloroform. We generated cDNA with the Superscript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen). For qRT-PCR, we supplemented iQ Sybr Green Supermix (BioRad) with 10 nM ROX and ran samples on a 7500 Fast Real-Time PCR System using Sequence Detection Software v.1.3.1 (Applied Biosystems). Primers are provided in Supporting Information Table S3.

Imaging and Statistical Analysis

All images were taken on an AxioImager epifluorescent compound microscope with Apotome using Zeiss AxioVision V4.8.0.0. We used Adobe PhotoShop to adjust the brightness and contrast of images equally to the entire image unless otherwise noted. We examined a minimum of three fish for each histological analysis. We generated box plots (indicating 75 and 25% quantiles, median, and outliers outside of whiskers) and performed all statistical analyses using JMP 9.0 (SAS Institute, Inc.).

Results

Tgif1 is Specifically Expressed in Activated Müller Glia After Photoreceptor Destruction

In the adult zebrafish retina, exposure to intense light destroys photoreceptors and leads to rapid upregulation of *tgif1* transcription in Müller glia from 8 through 36-h post lesion (hpl) (Qin et al., 2009). Transcription levels of several other members of the Smad2/3 signaling pathway are also differentially regulated within 8 hpl (Fig. 1A): activin receptors, *acvr1b* and *acvr2b*, and the *tgfβ1* ligand are downregulated, and conversely, the TGFβ target gene *tgfβ-induced* (*tgfbi*) is upregulated at 8 hpl, then downregulated by 16 hpl. We used the cDNA generated from isolated Müller glia in our earlier transcriptional analysis (Qin et al., 2009) to confirm the

increased expression of *tgif1* with qRT-PCR (analysis of variance (ANOVA) F-ratio = 9.72, $P < 0.05$; Fig. 1B). Because the mammalian *Tgif2* is believed to compensate for *Tgif1* in *Tgif1* mouse knock-outs (Shen and Walsh, 2005), we analyzed expression of the putative zebrafish *tgif2* gene (Ensembl CABZ01084922.1–201, Zv9). *tgif2* expression increased in Müller glia but not until 36 hpl (ANOVA F-ratio = 3.53, $P = 0.054$; Fig. 1B). The overall pattern of transcriptional changes in members of the Smad2/3 signaling pathway suggests that TGFβ signaling initially is upregulated in the Müller glia after light lesion, but then is quickly suppressed.

In undamaged retinas, Tgif1 protein (Fig. 1C) and transcripts (Supp. Info. Fig. S1A) are expressed in the ganglion cell layer, more weakly in the inner nuclear layer (INL) and ONL, and in the ciliary marginal zone (Supp. Info. Fig. S1B), which contains retinal stem cells and progenitors in the adult zebrafish retina. Immunostaining showed a subset of amacrine cells expressing higher levels of Tgif1 (Fig. 1C, Supp. Info. S1C–E). At 3 dpl, when Müller glia-derived progenitor cells are migrating from the INL into the ONL to regenerate photoreceptors (Bernardos et al., 2007), cells expressing *tgif1* spanned the INL and ONL in the lesion area (Fig. 1D, Supp. Info. S1A). By 5 dpl, *tgif1* expression decreased, and by 14 dpl, expression further reduced to near control levels in the INL where the Müller glia reside (Supp. Info. Fig. S1A). The cells expressing higher levels of *tgif1* at 3 dpl are proliferating Müller glial-derived progenitors, as Tgif1 colocalized with PCNA and the Müller glia GFP reporter in *gfap:EGFP* fish (arrows Figs. 1D,E).

tgif1$^{-/-}$ Fish Upregulate TGIF1 Expression in Response to Photoreceptor Damage

The N-terminus of Tgif1 contains domains that repress retinoic acid signaling (Bartholin et al., 2006), and the C-terminus binds to pSmad2/3 and recruits histone deacetylases (HDACs) to repress TGFβ signaling (Fig. 2A; Powers et al., 2010; Wotton et al., 1999). Because TGFβ signaling regulates Müller glia and retinal progenitor proliferation, we focused on the potential importance of Tgif1 repression of TGFβ signaling during photoreceptor regeneration with a genetic functional analysis. We used TILLING (Draper et al., 2004) to generate a mutation in the *tgif1* gene which introduces a stop codon (Y143X) and produces a truncated protein (~18 kDa, Figs. 2A,B). *tgif1*$^{+/+}$ fish expressed only the full-length Tgif1 protein (~35 kDa, Fig. 2B).

We performed co-immunoprecipitation experiments *in vitro* and determined that the truncated protein can bind to pSmad2 (Supp. Info. Fig. S2A). Due to the loss of the HDAC binding domain in the truncated protein (Wotton et al., 2001), we predict that the Y143X mutation cannot recruit HDACs to repress TGFβ target genes. In Western

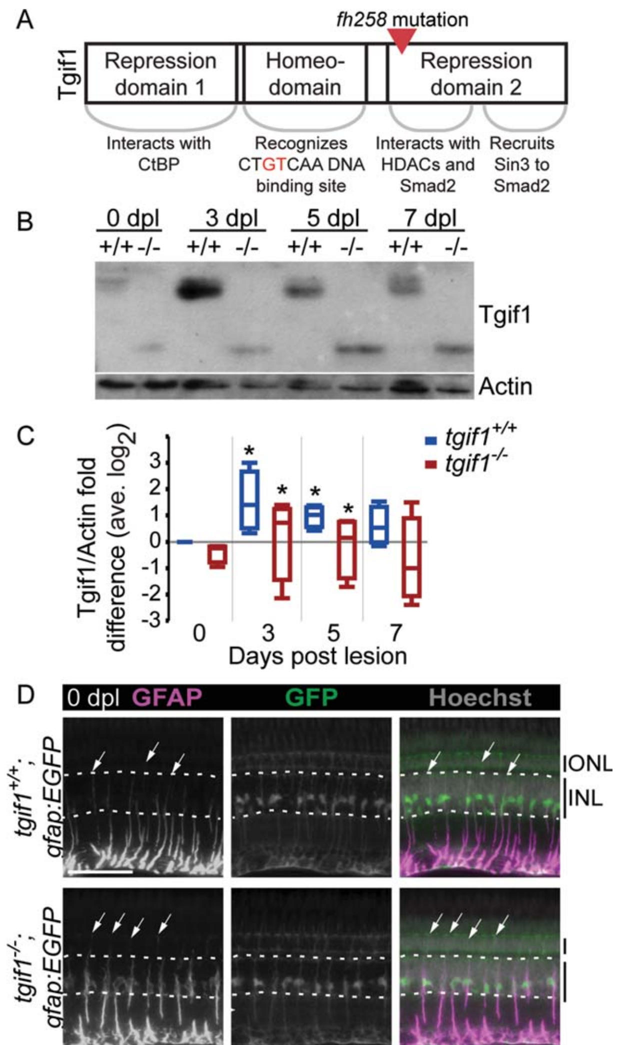


FIGURE 2: Fish homozygous for the *fh258* allele of *tgif1* express a truncated Tgif1 and GFAP localization in Müller glia is altered. (A) Schematic of the Tgif1 protein and the point mutation in the *fh258* allele. (B) Representative Tgif1 Western blot of whole retina extract from *tgif1*$^{+/+}$ and *tgif1*$^{-/-}$ fish. (C) Densitometric analysis of Tgif1 protein in control and light lesioned retinas from *tgif1*$^{+/+}$ and *tgif1*$^{-/-}$ fish normalized to actin and relative to 0 dpl *tgif1*$^{+/+}$ (retinas pooled from two fish, $n = 4$ replicates). * $P < 0.05$ relative to 0 dpl of the same genotype, Student t-test. (D) GFAP immunolocalization (first column) in the unlesioned retina of *tgif1*$^{+/+}$*;gfap:EGFP* (top) and *tgif1*$^{-/-}$*;gfap:EGFP* (bottom) fish expressing GFP only in the Müller glia. We used the same exposure time and did not alter the GFAP images postcapture. Arrows = top of GFAP distribution in individual Müller glia; dotted white lines delineate the base and top of the INL. Abbreviations as in Fig. 1. Scale bar = 50μm.

blot analysis, both *tgif1*$^{+/+}$ and *tgif1*$^{-/-}$ retinas significantly increased Tgif1 expression at 3 and 5 dpl relative to unlesioned retinas and decreased expression by 7 dpl (Figs. 2B,C). We did not make direct comparisons between the levels of the full-length and truncated proteins due to potential differences in how they present in Western blot. Immunocytochemistry showed that at 3 dpl in *tgif1*$^{-/-}$ fish, Tgif1 protein

localized to the Müller glia-derived progeny (supp. Info. Fig. S2B).

tgif1$^{-/-}$ *Fish Have a Normal Number of Müller Glia that Exhibit Features of Gliosis*

Because TGFβ signaling limits Müller glia proliferation during development (Close et al., 2005), we asked if *tgif1*$^{-/-}$ fish had fewer Müller glia than *tgif1*$^{+/+}$ fish. We found no difference between the number of Müller glia in adult whole-mount retinas of *tgif1*$^{-/-}$*;gfap:EGFP* and *tgif1*$^{+/+}$*;gfap:EGFP* fish (Supp. Info. Fig. S2C). GFAP is an intermediate filament that is upregulated in both mammalian and fish Müller glia in reaction to retinal injury and is a component of retinal glial scars (Bernardos et al., 2007; Bringmann et al., 2009; Robel et al., 2011). Normally, GFAP localizes more strongly to Müller glia basal processes (Fig. 2D, top). We examined GFAP distribution in Müller glia of *tgif1*$^{-/-}$*;gfap:EGFP* fish to determine if they exhibited characteristics of gliosis. GFAP protein is distributed more apically along the radial process in *tgif1*$^{-/-}$ Müller glia relative to wild-type siblings (arrows, Fig. 2D, $n = 3$), which suggests that Müller glia in *tgif1*$^{-/-}$ fish are in a more reactive state. GFAP expression also increases in response to TGFβ signaling (Robel et al., 2011), so we predicted it may be more highly expressed in *tgif1*$^{-/-}$ fish due to derepression of TGFβ signaling. Unlesioned retinas from *tgif1*$^{-/-}$ fish tend to have higher levels of GFAP by Western blot analysis, but the difference is nonsignificant (Supp. Info. Fig. S2D, One-way Kruskal-Wallis Rank Sum test $P = 0.15$, $n = 7$).

tgif1$^{-/-}$ *Fish Have Decreased Proliferation After Photoreceptor Destruction*

To compare the amount of proliferation in *tgif1*$^{+/+}$ and *tgif1*$^{-/-}$ fish, we first analyzed the level of PCNA in whole retinas by Western blot (two-way ANOVA F-ratio = 5.13, $P = 0.0011$; Fig. 3A). At 3 dpl, *tgif1*$^{-/-}$ retinas have significantly less PCNA than *tgif1*$^{+/+}$ retinas (Fig. 3A). We then counted PCNA immunolabeled nuclei in the lesioned area in cryosections (Figs. 3B–D). Although there was a significant difference among the genotypes across time points (two-way ANOVA F-ratio = 4.29, $P < 0.05$ and F-ratio = 20.09, $P < 0.0001$ in the INL and ONL, respectively), only the number of PCNA-positive nuclei in the ONL was significantly reduced in *tgif1*$^{-/-}$ fish compared with *tgif1*$^{+/+}$ fish at 3 and 5 dpl (Figs. 3C,D). The proliferating cells in the ONL at 3 and 5 dpl are likely cone progenitors (Bernardos et al., 2007). Our analysis of proliferation indicates that fewer neurogenic progenitors are generated by injury-induced Müller glia in the retinas of *tgif1*$^{-/-}$ fish compared with *tgif1*$^{+/+}$ fish.

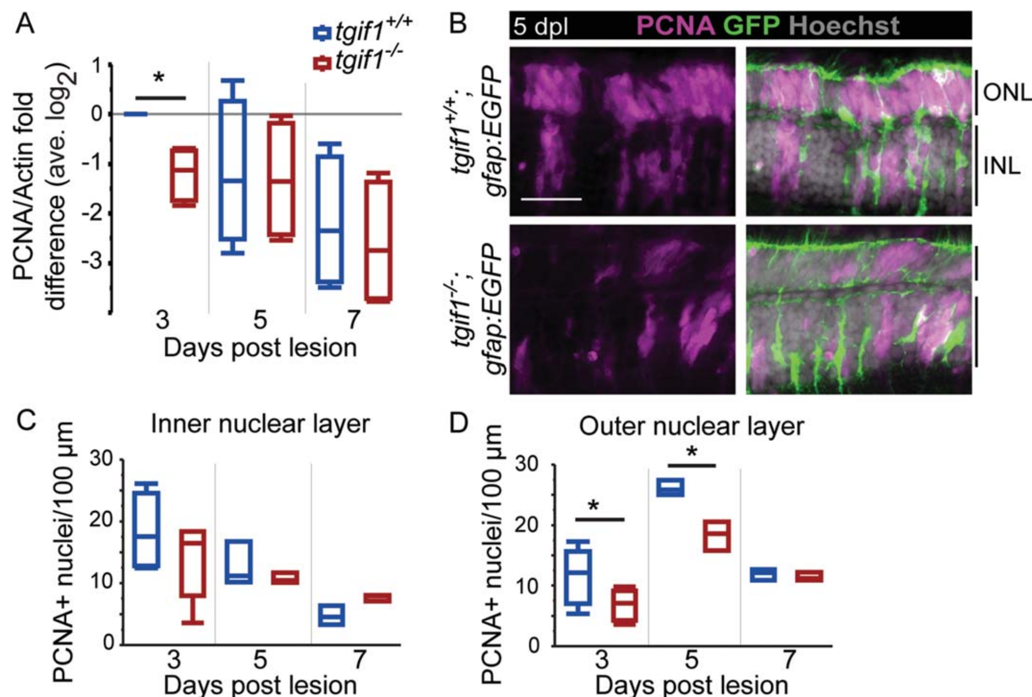


FIGURE 3: Proliferation is reduced in *tgif1*$^{-/-}$ fish compared with *tgif1*$^{+/+}$ fish. (A) Densitometric analysis of PCNA in Western blots normalized to actin and relative to 3 dpl *tgif1*$^{+/+}$ (retinas pooled from two fish, $n = 4$ replicates). (B) Representative images of PCNA immunoreactivity at 5 dpl in *tgif1*$^{+/+}$ and *tgif1*$^{-/-}$ fish with the *gfap:EGFP* transgene. The average number of PCNA-positive nuclei in the INL (C) and ONL (D) in *tgif1*$^{+/+}$ and *tgif1*$^{-/-}$ fish; 8–17 sections per fish; $n = 5$ at 3 dpl; $n = 3$ at 5 and 7 dpl. Scale bars = 25 µm. * $P < 0.05$, Student t-test comparing genotypes at the same lesion interval. Abbreviations as in Fig. 1.

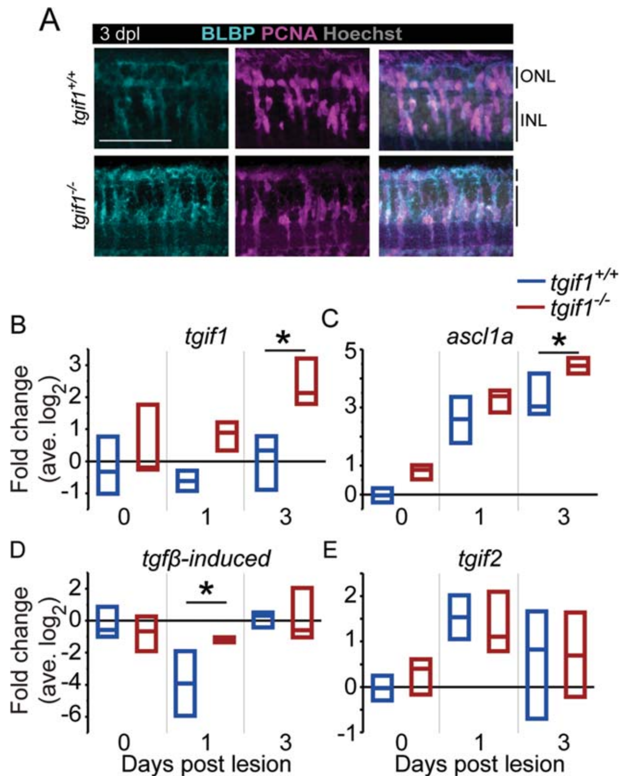


FIGURE 4: After acute light lesion, Müller glia in *tgif1*$^{-/-}$ fish express a dedifferentiation marker similar to *tgif1*$^{+/+}$ but misregulate Smad2/3 target genes. (A) BLBP and PCNA immunoreactivity in *tgif1*$^{+/+}$ and *tgif1*$^{-/-}$ fish at 3 dpl in the lesioned area, as determined by the absence of cone nuclei. (B–E) qRT-PCR of whole retina extracts (retinas pooled from individual fish, $n = 3$ replicates). We normalized gene expression to *gpia* and calculated fold change relative to the average *tgif1*$^{+/+}$ 0 dpl level. * $P < 0.05$, Student t-test comparing genotypes at the same lesion interval. Abbreviations as in Fig. 1.

Müller Glia are Activated After Acute Light Lesion, but Smad2/3 Targets are Misregulated in tgif1$^{-/-}$ Retinas

At 1 dpl, the same number of Müller glia were activated in *tgif1*$^{+/+}$*;gfap:nGFP* and *tgif1*$^{-/-}$*;gfap:nGFP* retinas (Supp. Info. Fig. S2E). BLBP is a marker of neural stem cells and radial glia in the brain (Götz and Barde, 2005) and increases in adult zebrafish Müller glia by 1 hpl (Nagashima, Barthel, Raymond, submitted). In both *tgif1*$^{+/+}$ and *tgif1*$^{-/-}$ retinas, BLBP expression increased in the lesioned area at 3 dpl where there is proliferation (Fig. 4A). Therefore, Müller glia in *tgif1*$^{-/-}$ fish are activated and re-express the retinal progenitor markers BLBP and Pax6 (data not shown) in response to acute light lesion.

Because Müller glia in *tgif1*$^{-/-}$*;gfap:EGFP* fish upregulated retinal progenitor markers, indicative of dedifferentiation, we used qRT-PCR to examine the expression of genes that are regulated by TGFβ signaling and important in neural stem cells or Müller glia-mediated retina regeneration: *tgif1* (Liu et al., 2011; Qin et al., 2009), *tgfbi* (Thapa et al., 2007), and *ascl1a* (Fausett et al., 2008; Liu et al., 2011). As controls, we examined the expression of several genes that are not regulated by Smad2/3 but are critical for retinal neuron regeneration: *dkk1b* (Ramachandran et al., 2011); *hb-egfa* (Wan et al., 2012); *pax6a* and *pax6b* (Thummel et al., 2010); and *six3b* (Fig. 5). Compared with *tgif1*$^{+/+}$ siblings, *tgif1*$^{-/-}$ fish have significantly higher levels of Smad2/3 targets after lesion (Figs. 4B–D): *tgif1* (two-way ANOVA F-ratio = 4.48, $P < 0.05$), *ascl1a* (two-way ANOVA F-ratio = 26.28, $P < 0.001$), and *tgfbi* (two-way ANOVA F-ratio = 4.61, $P < 0.05$). These data confirm that Smad2/3-mediated TGFβ signaling is increased in *tgif1*$^{-/-}$ fish during photoreceptor regeneration.

In contrast, we detected no significant difference between *tgif1*$^{+/+}$ and *tgif1*$^{-/-}$ retinas in the levels of several non-Smad2/3-regulated genes (Fig. 4E, Supp. Info. Fig. S3): *hb-egfa* (two-way ANOVA F-ratio = 1.44, $P = 0.28$), *pax6a* (two-way ANOVA F-ratio = 0.94, $P = 0.49$), *pax6b* (two-way ANOVA F-ratio = 0.73, $P = 0.62$), *six3b* (two-way ANOVA F-ratio = 1.04, $P = 0.44$), and *tgif2* (two-way ANOVA F-ratio = 0.61, $P = 0.70$). These data suggest that *tgif2* is not upregulated to compensate for the defective Tgif1 protein in *tgif1*$^{-/-}$ fish. Previous studies showed that whole retina qRT-PCR analysis can detect changes in expression in Müller glia of *ascl1a*, *dkk1b*, *hb-egfa*, *pax6a*, and *pax6b* (Ramachandran et al., 2010; Ramachandran et al., 2011; Wan et al., 2012). We also saw no difference

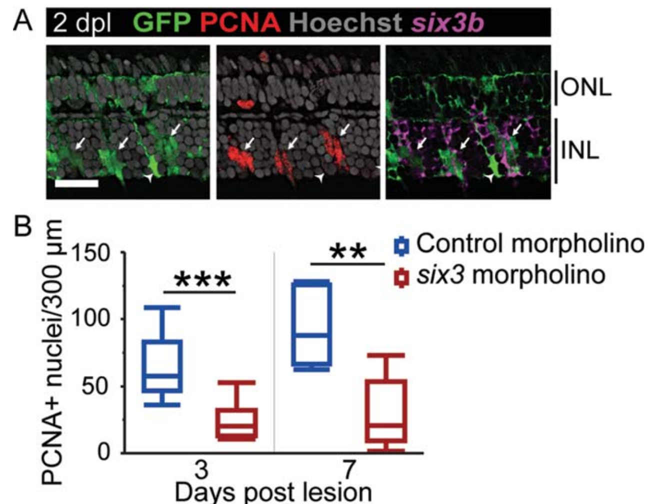


FIGURE 5: Repression of the Smad2/3 corepressor *six3* inhibits proliferation after photoreceptor lesion. (A) *six3b in situ* hybridization and PCNA immunoreactivity in GFP-positive Müller glia and their progeny (arrows) at 2 dpl in *gfap:EGFP* fish. The arrowhead indicates a PCNA-negative Müller glial cell that is not expressing *six3b*. (B) The number of PCNA-positive nuclei at 3 and 7 dpl after electroporation of morpholinos targeting *six3a/b*. $n = 11$ for control morpholino, $n = 10$ for *six3* morpholino at 3 dpl, and $n = 5$ at 7 dpl. Scale bar = 20 µm. ** $P < 0.01$; *** $P < 0.0001$, Student t-test comparing genotypes at the same lesion interval. Abbreviations as in Fig. 1.

in levels of *six3b* between *tgif1*$^{-/-}$ and wild-type siblings, however, it is possible that changes in *six3b* transcript levels in the Müller glia could not be detected in whole retina extracts because they are masked by high levels of expression in retinal neurons (Supp. Info. Fig. S3E). Interestingly, undamaged retinas of *tgif1*$^{-/-}$ fish express significantly lower levels of the Wnt/βcatenin inhibitor *dkk1b* than wild-type siblings (two-way ANOVA F-ratio = 9.82, $P < 0.001$; Supp. Info. Fig. S3A), indicating that other signaling pathways in addition to TGFβ are altered in the retinas of *tgif1*$^{-/-}$ fish.

Concurrent Inhibition of Two six3-Related Genes Reduces Proliferation After Light Lesion

The TGFβ corepressor *six3b* is also rapidly upregulated in the Müller glia of light-lesioned adult zebrafish (Qin et al., 2009). Zebrafish have three *six3*-related genes that are differentially expressed in the unlesioned adult retina (Supp. Info. Fig. S4): *six3a*, *six3b*, and *six7* (Inbal et al., 2007). We confirmed that only *six3b*, not *six3a* or *six7*, is upregulated in the lesion area (Supp. Info. Fig. S4) and that *six3b* colocalizes with GFP-positive, PCNA-positive Müller glia, and their neurogenic progenitors (Fig. 5A). We did not detect a regeneration defect in preliminary experiments using zebrafish homozygous for a *six3b* null allele, perhaps due to compensation by *six3a* as in early development (Inbal et al., 2007). Therefore, we used morpholinos that inhibit translation of both *six3a* and *six3b* paralogs (Ando et al., 2005) to ask whether Six3 is important for proliferation during retinal regeneration (Fig. 5B). We found a significant difference in PCNA-positive nuclei among the groups (ANOVA F-ratio = 14.77, $P < 0.0001$) and between control and *six3* morpholino at 3 dpl (Student t-test $P < 0.0001$) and 7 dpl (Student t-test $P < 0.01$). As with our *tgif1* results, perturbation of *six3* function significantly reduced proliferation of neurogenic progenitors after light lesion.

Regeneration of Photoreceptors is Impaired in tgif1$^{-/-}$ and tgif1$^{-/-}$; six3b$^{-/-}$ Fish

At 14 dpl, cone regeneration in wild-type fish is largely complete, but rod regeneration is continuing (Raymond et al., 2006). We asked whether the reduced proliferation in fish with impaired Tgif1 or Six3b function has a consequence for cone regeneration by exposing fish to BrdU from 1 through 7 dpl and examining BrdU-labeled nuclei at 14 dpl (Supp. Info. Fig. S5A). *tgif1*$^{+/+}$*;gfap:EGFP* fish had BrdU-labeled photoreceptors in the ONL (arrowheads, Supp. Info. Fig. S5A) and BrdU-labeled Pax6-positive and Crx1-positive neurons in the INL (Supp. Info. Figs. S5B,C). *tgif1*$^{-/-}$*;gfap:EGFP* fish had BrdU-labeled regenerated neurons in the ONL and the INL (arrows and arrowheads, respectively, supp. Info. Fig. S5), but there were fewer BrdU-positive nuclei in the INL, reinforcing our observations of reduced proliferation in *tgif1*$^{-/-}$ fish (Fig. 3). Müller glia in *tgif1*$^{+/+}$ fish had resumed a normal morphology and were labeled with BrdU at 14 dpl (arrows, Supp. Info. Fig. S5A). Interestingly, in *tgif1*$^{-/-}$*;gfap:EGFP* fish occasional Müller glia were still highly GFP-positive with dense processes at the OLM (double arrow, Supp. Info. Fig. S5A). This morphology was never observed in *tgif1*$^{+/+}$*;gfap:EGFP* retinas and is reminiscent of gliotic hypertrophy of mammalian Müller glia, further supporting our hypothesis that normal levels of *tgif1* are critical for regulating neurogenic proliferation of Müller-glia after light lesion.

We predicted that the proliferation defect in *tgif1*$^{-/-}$ fish should result in reduced regeneration of cone photoreceptors. We quantified the number of cone nuclei in the lesioned area, as demarcated by increased BLBP expression in Müller glia (Fig. 6A). Cone nuclei were distinguished from rods by the position of their nuclei apical to the OLM, which we labeled with zonula occludens-1 (ZO-1) (Fig. 6B). There were significantly fewer regenerated cone photoreceptors in *tgif1*$^{-/-}$ fish compared with *tgif1*$^{+/+}$ fish (Student t-test $P < 0.005$; Figs. 6B,C).

Finally, we crossed *tgif1*^{fh258} and *six3b*^{vu87} alleles into the *gfap:EGFP* Müller glia reporter line to test if *tgif1* and *six3b* interact genetically. The cytoarchitecture of unlesioned retinas in adult *tgif1*$^{-/-}$*;six3b*$^{-/-}$*;gfap:EGFP* is normal (Supp. Info. Fig. S6). However, at 14 dpl, *tgif1*$^{-/-}$*;six3b*$^{-/-}$*;gfap:EGFP* fish exhibit an irregular OLM in the lesioned area (arrow, Fig. 6B) and significantly fewer regenerated cones compared with *tgif1*$^{+/+}$ (Student t-test $P < 0.0001$) and *tgif1*$^{-/-}$ fish (Student t-test $P < 0.005$) (Fig. 6C). Therefore, loss of Six3b function enhances the regeneration defect we observed in *tgif1*$^{-/-}$ fish.

Discussion

Adult zebrafish Müller glia have the capacity to self-renew and generate multipotent neuronal progenitors, characteristics typical of neural stem cells, and as such are a potential target for tissue-specific, regenerative stem cell therapies in the eye (Karl and Reh, 2010). To identify therapeutic strategies to promote stem cell properties in human Müller glia, it is necessary to identify both inductive and inhibitory cues (Locker et al., 2009). Our results indicate that Smad2/3-mediated TGFβ signaling acts to inhibit proliferation of neuronal progenitors following photoreceptor destruction in the adult zebrafish retina and may bias the damage response toward gliosis (Fig. 7). The mechanisms by which TGFβ signaling regulates proliferation are likely complex. TGFβ signaling-induced scarring is necessary for heart regeneration in adult zebrafish (Chablais and Jaźwińska, 2012), and similarly,

TGFβ-induced deposition of extracellular matrix is critical for fin regeneration (Jaźwińska et al., 2007). However, studies of the vertebrate central nervous system suggest that the TGFβ signaling pathway normally promotes glial scarring in damaged neural tissues and inhibits cell proliferation (Bringmann et al., 2009; Robel et al., 2011).

TGFβ signaling influences retinal development and disease, but a role in the regulation of stem cells during retinal regeneration has not been explored. Small changes in the levels of Smad2/3 signaling in ESCs changes their transcriptional profile, leading to cell fate changes (Lee et al., 2011). Our results are consistent with this finding in that perturbations in the function of two TGFβ corepressors, *tgif1* and *six3b*, inhibited the proliferative response of Müller glial-derived neuronal progenitors and, more importantly, diminished photoreceptor regeneration following acute light lesion (Fig. 7). The best understood mechanism by which Tgif1 inhibits TGFβ signaling is through binding to pSmad2/3 and then recruiting HDACs (Wotton et al., 1999), and Six3 is a Smad2/3 transcriptional corepressor (Inbal et al., 2007; Powers et al., 2010). Photoreceptor regeneration is not completely inhibited in *tgif1*$^{-/-}$ fish, perhaps because other functions of Tgif1 were not perturbed or because of functional redundancy with other genes that repress TGFβ signaling. Consistent with the later interpretation, we found that disruption of either *six3* or *tgif1* caused reduced proliferation after acute light lesion, and loss-of-function in both had a synergistic, negative effect on cone photoreceptor regeneration. This suggests that they cooperate in the same pathway to block TGFβ signaling in Müller glia and their neurogenic progeny.

Several Smad2/3 targets are rapidly upregulated in Müller glia in response to light damage, including *ascl1a*, *tgfβ-induced*, and *tgif1* (Qin et al., 2009). Expression of the TGFβ ligand activin-βb increases dramatically in the ONL within 12 h after the initiation of a chronic light lesion in adult zebrafish (Craig et al., 2008). This ligand could be secreted by injured photoreceptors or activated microglia in the lesion and could induce the initial increase in Smad2/3 signaling that we observed. Interestingly, both of the corepressors we studied, *tgif1* and *six3b*, maintain high levels of expression through the first few days after light lesion, whereas *tgfβi*, a secreted protein component of the extra-

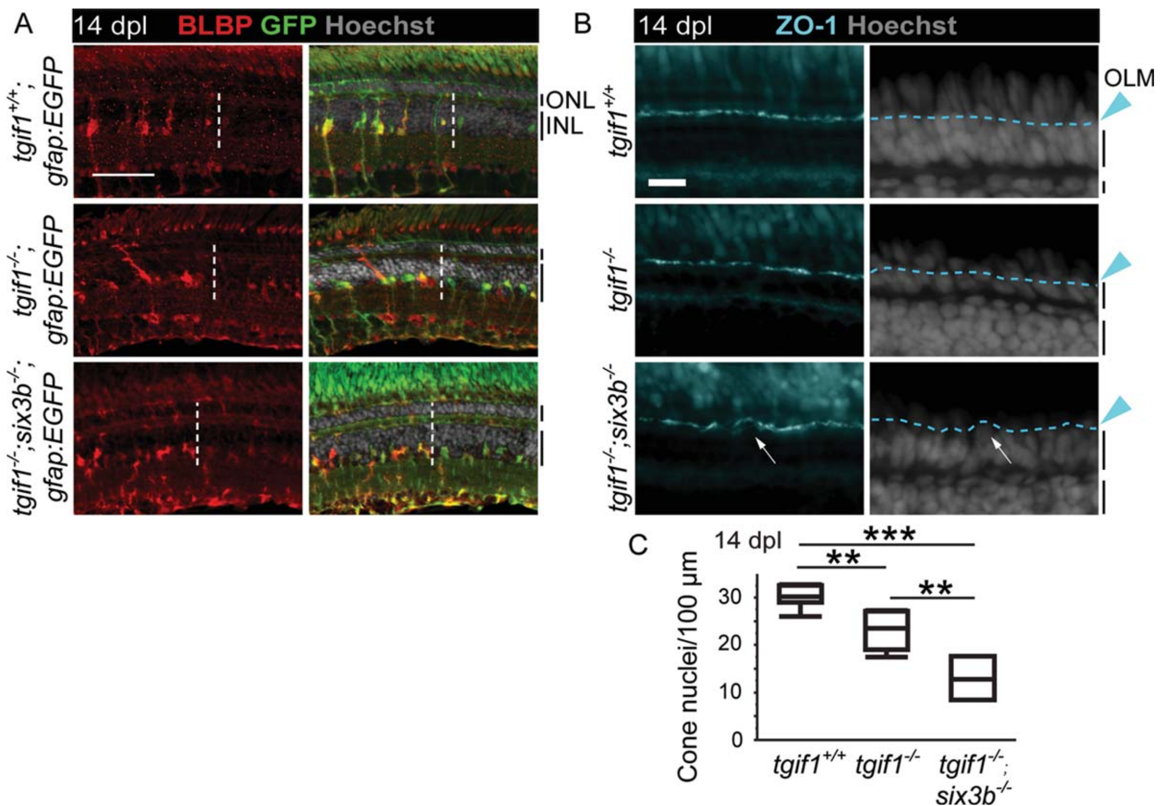
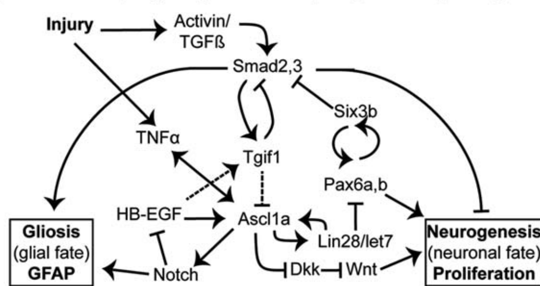


FIGURE 6: Regeneration is significantly impaired in fish homozygous for *tgif1*$^{-/-}$ or both *tgif1*$^{-/-}$ and *six3b*$^{-/-}$ relative to *tgif1*$^{+/+}$ fish. (A) BLBP immunoreactivity at 14 dpl in *tgif1*$^{+/+}$*;gfap:EGFP*, *tgif1*$^{-/-}$*;gfap:EGFP*, and *tgif1*$^{-/-}$*;six3b*$^{-/-}$*;gfap:EGFP* fish expressing GFP in the Müller glia (lesioned area is left of dotted line). (B) ZO-1 immunolocalization at 14 dpl (panels at left) to visualize the OLM (dashed line in panels at right) of *tgif1*$^{+/+}$, *tgif1*$^{-/-}$, and *tgif1*$^{-/-}$*;six3b*$^{-/-}$ fish. (C) The number of photoreceptor nuclei in the regenerated area at 14 dpl. Scale bars = 50 µm in (A), 10 µm in (B). ** $P<0.005$, *** $P<0.0001$, Student t-test pairwise comparisons of genotypes. OLM = outer limiting membrane. Other abbreviations as in Fig. 1.

cellular matrix, is only transiently increased. Therefore, different mechanisms must regulate the transcription of cofactors and downstream targets after the initial increase in Smad2/3 signaling.

A growing body of research suggests that the growth factor EGF plays a critical role in the ability of Müller glia to become mitotically active. Murine Müller glia in retinal explants become mitotically active in response to EGF via signal transduction requiring bone morphogenetic protein/Smad signaling (Ueki and Reh, 2013). In the adult zebrafish retina, heparin-binding EGF (HB-EGF) is necessary in the repair of stab wounds and acts by mediating expression of critical genes such as *pax6b* and *ascl1a* (Wan et al., 2012), although a recent study of photoreceptor regeneration in adult zebrafish indicates that *hb-egfa* is not necessary for Müller glia-derived photoreceptor regeneration (Nelson et al., 2013). We found that retinas of *tgif1*$^{-/-}$ fish had similar levels of *hb-egfa* expression after acute light lesion relative to wild-type fish. This suggests that HB-EGF and canonical TGFβ signaling act in parallel pathways in retinal regeneration. It is also known that the mitotic effect of growth factors on Müller glia and neural stem cells is inhibited by TGFβ signaling both *in vitro* (Aigner and Bogdahn, 2008; Ikeda and Puro, 1995) and *in vivo* during rat retina development (Close et al., 2005). Additionally, EGF signaling increases phosphorylation and stabilization of Tgif1 *in vitro* (Lo et al., 2001), which would further suppress TGFβ signaling and allow for proliferation.



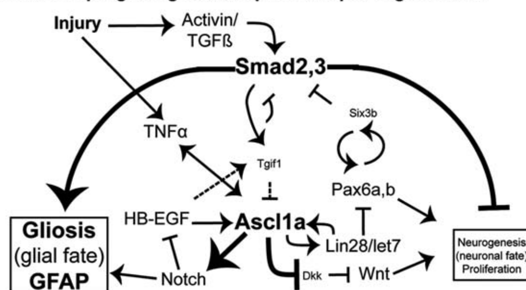


FIGURE 7: Signaling pathways that regulate Müller glial-derived regeneration of retinal neurons. Signaling pathways implicated in Müller glial response to retinal injury and retinal regeneration based on the present work and published studies (Bernardos et al., 2005; Ikeda and Puro, 1995; Inbal et al., 2007; Liu et al., 2006; Liu et al., 2011; Meyers et al., 2012; Nelson et al., 2012, 2013; Ramachandran et al., 2010; Robel et al., 2011; Thummel et al., 2010; Wan et al., 2012). Regeneration requires a dynamically balanced regulation of signals and transcriptional regulators promoting proliferation and differentiation, as well as cell fate choices between a glial fate (gliosis) and a neuronal fate (neurogenesis). See text for details.

Consistent with these antiproliferative effects of TGFβ signaling, we propose that upregulation of the TGFβ corepressors Tgif1 and Six3b promotes the proliferation of Müller glia and Müller glia-derived neurogenic progenitors. The time course of Tgif1 and Six3b expression in the initial response in Müller glia suggests their importance: They reach a maximum level of expression before the initial mitotic division of Müller glia (Fig. 1) and earlier than other genes necessary for retina regeneration reach their maximum expression, such as *ascl1a* (Nelson et al., 2012; Qin et al., 2009; Ramachandran et al., 2010). Carefully controlled regulation of transcription factors and signaling pathways is very important in determining stem cell behavior and progenitor fate (Hsieh, 2012), and TGFβ signaling interacts with transcription factors that have critical roles in retinal development and regeneration: Pax6a and Ascl1a both have Smad2 binding sites (Liu et al., 2011); Six3b can inhibit Smad2/3-mediated signaling (Inbal et al., 2007); and Six3 activates Pax6, which is later necessary for maintenance of Six3 expression in lens development (Liu et al., 2006). Although *tgif1*$^{-/-}$ fish have significantly higher levels of *ascl1a* after light damage, they also have significantly less proliferation and fewer regenerated cone photoreceptors, indicating that *ascl1a* expression alone is not sufficient for retinal regeneration. The expression of *ascl1a* also indirectly inhibits expression of the Wnt signaling pathway inhibitor *dkk1b* (Ramachandran et al., 2011), and our study indicates that *ascl1a* and *dkk1b* expression are both downstream of Tgif1 transcriptional regulation. Consistent with increased *ascl1a* after lesion in *tgif1*$^{-/-}$ mutants, expression of *dkk1b* was reduced (Fig. 7). Wnt signaling is known to regulate aspects of Müller glia dedifferentiation, proliferation, differentiation, and cell survival in the retina of mice (Liu et al., 2013). In acute light lesions of larval zebrafish retina, hyperactivation of Wnt signaling does not increase proliferation of Müller glia (Meyers et al., 2012), but following stab wounds of the adult retina, inhibition of Wnt signaling reduces proliferation (Ramachandran et al., 2011). Further studies are necessary to clarify the role of Wnt signaling in retinal regeneration and the potential differences in lesion paradigms.

The proposition that dynamic and carefully controlled regulation of TGFβ signaling is particularly important for stem cell behavior and proliferation is consistent with methods developed for production of neural retinal precursors from ESCs: ESCs are first treated with LeftyA, a TGFβ signaling inhibitor,

and Dkk1, then activin to upregulate TGFβ signaling (Ikeda et al., 2005). A small molecule inhibitor of TGFβ signaling can even replace Sox2 or cMyc to reprogram fibroblasts to become induced pluripotent stem cells (iPSC) (Ichida et al., 2009). Zebrafish Müller glia-derived progenitors express the pluripotent factors *oct4* and *lin28* following retina injury, suggesting they are similar to iPSCs (Ramachandran et al., 2010). Increased Tgif1 expression is associated with higher levels of proliferation and migration in several cancers as well (Castro et al., 2010; Yeh et al., 2012), and this may indicate the importance of functional separation of TGFβ inhibition of proliferation and induction of epithelial–mesenchymal transition (EMT) (Barrios-Rodiles et al., 2005; Hamid and Brandt, 2009). During Müller glia-based photoreceptor regeneration, dynamic regulation of TGFβ is likely critical in this environment: An initial upregulation of TGFβ signaling would allow for EMT and migration of Müller glia-derived neuronal progenitors, while subsequent inhibition of Smad2/3-mediated signaling by corepressors Tgif1 and Six3b would allow for proliferation of progenitors committed to a neuronal, not glial, fate.

Unlike neurogenesis in the localized and specialized stem cell niches of the adult vertebrate brain, Müller glia are mature differentiated cells embedded within the complex neuronal environment of the retina. After damage, Müller glia must actively repress glial differentiation signals and upregulate a transcriptome that will allow for controlled proliferation and generation of neuronal progenitors. Like development of the retina (Swaroop et al., 2010) and control of stem cells (Hsieh, 2012), this transcriptional program likely consists of a complex gene regulatory network in which the precise levels of many signals are critical for a successful regenerative outcome. An intriguing hypothesis is that an initial increase in TGFβ signaling is important for the damage response (gliosis) in Müller glia, and that subsequent inhibition of TGFβ signaling in adult zebrafish Müller glia distinguishes them from mammalian Müller glia and allows for a neurogenic response to damage (Fig. 7). Both fish and mammalian Müller glia react to retinal injury by upregulating GFAP, Pax6, and BLBP (Bernardos et al., 2007; Joly et al., 2011; Thummel et al., 2010). These are classic signs of gliosis (Joly et al., 2011), and yet adult zebrafish can regenerate lost neurons, whereas adult mammals generate glial scars. As more research is directed at the transcriptional and regulatory processes that allow for retinal regeneration in adult vertebrates, differences in the gene regulatory networks activated in damage responses in mammalian and nonmammalian Müller glia will be revealed. Our studies suggest that down regulation of Smad2/3 signaling in the Müller glia is particularly important for the proliferative, neurogenic, response of Müller glia to light-induced destruction of photoreceptors in the adult zebrafish.

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