Published in final edited form as:

J Comp Neurol. 2012 December 15; 520(18): 4294–4311. doi:10.1002/cne.23213.

Stat3 Defines Three Populations of Müller Glia and Is Required for Initiating Maximal Müller Glia Proliferation in the Regenerating Zebrafish Retina

Craig M. Nelson*, Ryne A. Gorsuch*, Travis J. Bailey, Kristin M. Ackerman, Sean C. Kassen, and David R. Hyde¹

Department of Biological Sciences and the Center for Zebrafish Research, Galvin Life Science Building, University of Notre Dame, Notre Dame, IN 46556 USA

Abstract

We analyzed the role of Stat3, Ascl1a, and Lin28a in Müller glia reentry into the cell cycle following damage to the zebrafish retina. Immunohistochemical analysis was employed to determine the temporal and spatial expression of Stat3 and Ascl1a proteins following rod and cone photoreceptor cell apoptosis. Stat3 expression was observed in all Müller glia, while Ascl1a expression was restricted to only the mitotic Müller glia. Knockdown of Stat3 protein expression did not affect photoreceptor apoptosis, but significantly reduced, without abolishing, the number of proliferating Ascl1a-positive Müller glia. Knockdown of Ascl1a protein also did not change the extent of photoreceptor apoptosis, but did yield significantly fewer Müller glia that reentered the cell cycle relative to the *stat3* morphant and significantly decreased the number and intensity of Stat3 expressing Müller glia. Finally, introduction of *lin28a* morpholinos resulted in decreased Müller glia expression of Stat3 and Ascl1a, significantly reducing the number of proliferating Müller glia. Thus, there are three populations of Müller glia in the light-damaged zebrafish retina: 1) Stat3-expressing Ascl1a-nonexpressing nonproliferating (quiescent) Müller glia, 2) Stat3-dependent Ascl1a-dependent proliferating Müller glia, 3) Stat3-independent Ascl1a-dependent proliferating Müller glia. While Ascl1a and Lin28a are required for Müller glia proliferation, Stat3 is necessary for the maximal number of Müller glia to proliferate during regeneration of the damaged zebrafish retina.

¹**Corresponding author:** David R. Hyde, Ph.D., Department of Biological Sciences, 027 Galvin Life Science Building, University of Notre Dame, Notre Dame, IN, 46556. Telephone: 574-631-8054. Fax: 574-631-7413. dhyde@nd.edu.

*both authors contributed equally to this work

Email addresses:

Craig M. Nelson craig.m.nelson.124@nd.edu

Ryne A. Gorsuch ryne.a.gorsuch.1@nd.edu

Travis J. Bailey tbailey@nd.edu

Kristin M. Ackerman kristin.m.ackerman.13@nd.edu

Sean C. Kassen sean.c.kassen.1@nd.edu

David R. Hyde dhyde@nd.edu

CONFLICT OF INTEREST STATEMENT

None of the authors associated with this work have any known or potential conflict of interest, including financial, personal, or other relationships that would either inappropriately influence or appear to influence this work.

ROLE OF AUTHORS

All the authors of this work had full access to all the data and take responsibility for the integrity of the data and the accuracy of its analysis. Study concept and design: CMN, RAG, SCK, DRH. Acquisition of data: CMN, RAG, TJB, KMA. Analysis and interpretation of data: CMN, RAG, TJB, DRH. Drafting of the manuscript: CMN, RAG, DRH. Critical revision of the manuscript for important intellectual content: CMN, RAG, TJB, DRH. Statistical analysis: CMN, RAG, TJB. Obtained funding: DRH. Study supervisor: DRH.

Keywords

Ascl1; Lin28a; adult stem cell; neuronal progenitor cell; retinal regeneration

INTRODUCTION

The adult zebrafish possesses the remarkable ability to regenerate any neuronal cell type that is lost during retinal damage, which makes it an excellent model system to study the mechanisms required for vertebrate retinal regeneration. Multiple damage paradigms elicit this regeneration response, including constant intense light (Vihtelic and Hyde, 2000; Vihtelic et al., 2006; Kassen et al., 2007; Bernardos et al., 2007), laser injury (Wu et al., 2001), neurotoxin injection (Fimbel et al., 2007; Sherpa et al., 2008), retinal puncture (Fausett and Goldman, 2006), surgical removal of retinal tissue (Cameron, 2000), heat lesion (Raymond et al., 2006), and exposure of *nitroreductase*-expressing transgenic animals exposed to metronidazole (Montgomery et al., 2010). In each of these damage models, different neuronal cell types are lost and only those cell types are regenerated. For example, constant intense light treatment of dark-adapted *albino* zebrafish causes rod and cone photoreceptor cell apoptosis and only photoreceptors are regenerated (Vihtelic and Hyde, 2000; Vihtelic et al., 2006; Kassen et al., 2007; Bernardos et al., 2007). The source of regeneration in all of these damage models is the Müller glia, which dedifferentiate and reenter the cell cycle to yield transiently amplifying multipotent neuronal progenitor cells that migrate to the damaged retinal layer and differentiate into the missing neurons (Yurco and Cameron, 2005; Fausett and Goldman, 2006; Bernardos et al., 2007; Kassen et al., 2007; Fimbel et al., 2007; Thummel et al., 2008).

Several microarray experiments identified genes that significantly change in expression during retinal regeneration (Cameron et al., 2005; Kassen et al., 2007; Craig et al., 2008; Qin et al., 2009; Morris et al., 2011). Some of these genes were subsequently shown to play important roles in neuronal regeneration, including PCNA, Pax6a, Pax6b, Lin28a, let-7 miRNA, Mdka, Mdkb, Hspd1, Mps1, Apobec2a, Apobec2b, HB-EGF, and Ascl1a (Thummel et al., 2008; Fausett et al., 2008; Calinescu et al., 2009; Qin et al., 2009; Thummel et al., 2010; Ramachandran et al., 2010; Ramachandran et al., 2011; Powell et al., 2012; Wan et al., 2012). The Ascl1a protein is a member of the basic helix-loop-helix (bHLH) family of transcription factors. *In situ* hybridization of the puncture-damaged adult zebrafish eye suggested that *ascl1a* expression increased in the Müller glia within hours following eye puncture (Fausett et al., 2008). Treatment of punctured retinas with morpholinos targeted to the *ascl1a* mRNA resulted in decreased numbers of proliferating Müller glia (Fausett et al., 2008), which suggested that Ascl1a plays a critical role in regeneration. It was subsequently shown that Ascl1a was necessary for expression of the pluripotency factor Lin-28 (Ramachandran et al., 2010). Lin-28, which is also required for Müller glia proliferation in the puncture-damaged retina, regulates expression of the *let-7* miRNA, which represses expression of *ascl1a*, *pax6b*, *lin-28*, and *hspd1* (Ramachandran et al., 2010).

An additional molecule that may be involved in retinal regeneration is the transcription factor Stat3 (Signal transducer and activator of transcription 3). Stat3 plays critical roles in a variety of different systems, including stem cell maintenance (Zhou et al., 2007) and tissue development (Levy and Lee, 2002). Stat3 was shown to stimulate both the Notch signaling pathway in promoting neuronal stem cell renewal (Androutsellis-Theotokis et al., 2006; Nagao et al., 2007) and the bone morphogenic protein-Smad pathway to drive astroglialogenesis (Fukada and Taga, 2006; Fukada et al., 2007). Stat3 signaling pathways are activated when an extracellular ligand binds a transmembrane receptor, followed by the Jak

protein phosphorylating tyrosine residues on the bound receptor (Aaronson and Horvath, 2002). Binding of Stat3 to the phosphorylated receptor results in Stat3 phosphorylation and its dimerization (O'Shea et al., 2002). The phosphorylated Stat3 dimer enters the nucleus and binds DNA sequences to regulate gene transcription (Aaronson and Horvath, 2002; O'Shea et al., 2002; Rawlings et al., 2004).

The *stat3* and other *jak/stat* signaling pathway genes were identified in a microarray study of the light-damaged zebrafish retina (Kassen et al., 2007). The expression of *stat3* mRNA and protein is induced within the first 16 hours of the light treatment and both the native and phosphorylated Stat3 proteins increase in expression through the first 68 hours of constant light (Kassen et al., 2007). While Stat3 is required for the CNTF-induced Müller glia proliferation in the undamaged retina (Kassen et al., 2009), its role during regeneration of the damaged zebrafish retina remains unclear. Thus, the purpose of this work is to determine the role of Stat3 during Müller glia proliferation in the damaged zebrafish retina and the relationship of Stat3 function to the *Ascl1a* and *Lin28a* proteins.

MATERIALS AND METHODS

Zebrafish maintenance

All zebrafish lines, *albino* and *albino Tg[*gfap:EGFP*]*nt11** (Kassen et al., 2007), were maintained in the Center for Zebrafish Research at the University of Notre Dame Freimann Life Science Center. Adult zebrafish used for these studies were between 6-12 months old, were between 2-4 cm in length, and were maintained under a standard light-dark cycle at 28.5°C (Westerfield, 1993). All experimental protocols were approved by the animal care and use committee at the University of Notre Dame and are in compliance with the ARVO statement for the use of animals in vision research.

Retinal damage paradigms

Rod and cone cell death was induced by constant intense light according to established protocols (Vihtelic and Hyde, 2000; Vihtelic et al., 2006). Briefly, adult fish were dark adapted for 14 days, then transferred to clear polycarbonate tanks and placed in constant intense light (15,000-20,000 lux) for up to 3 days. Fish were euthanized by anesthetic overdose of 0.2% 2-phenoxyethanol in system water.

Inner retinal cell death was achieved by intravitreal injection of ouabain at a final concentration of 2 μ M (Fimbel et al., 2007). Before each intravitreal injection, the approximate vitreal volume was calculated based on the difference between the volume of the entire eye globe minus the volume of the lens (calculated using digital calipers). A small incision was made in the posterior cornea adjacent to the lens with a double-edged sapphire microknife (World Precision Instruments, Sarasota, FL) and the appropriate volume (0.2-0.5 μ l) of a freshly prepared ouabain solution (MP Biomedicals, Solon, OH) was injected using a blunt-end 33 gauge Hamilton (Reno, NV) syringe.

Morpholino-mediated knockdown in adult zebrafish retinas

The electroporation of morpholinos was performed as previously described with minor alterations (Thummel et al., 2008). Prior to constant intense light-treatment, dark-adapted adult *albino* zebrafish eyes were intravitreally injected and electroporated with lissamine-labeled standard control morpholino (MO), anti-*stat3* MO (5'-CAGATAAATCGTCCTCCACGGAAC-3'), anti-*ascl1a* MO1 (Fausett et al., 2008) or anti-*lin28a* MO2 (Ramachandran et al., 2010). All morpholinos were purchased from Gene Tools (Philomath, OR).

Quantitative Real-Time PCR

The dorsal half of retinas from light-damaged adult *albino* zebrafish were isolated following 0, 5, 10, 15 or 20 hours of constant light treatment and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Dorsal retinas were used because we previously demonstrated that the light-induced cell death was primarily restricted to the dorsal retina (Vihtelic et al., 2006; Thomas et al., 2012). For each time point, 4 dorsal retinas were pooled to create one biological sample and experiments were run in triplicate. Total cDNA was reverse transcribed using random hexamers and the SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA). Gene-specific primers and methods used were previously described (Kassen et al., 2007): *stat3* forward (5'-GAGGAGGCGTTTGGCAA-3'), *stat3* reverse (5'-TGTGT CAGGGA ACTCAGTGTCTG-3'), *ascl1a* (previously termed *Ash[a]*) forward (5'-GCCAGACGGAACGAGAGAGA 3'), *ascl1a* reverse (5'-AGGGTTGCAAAGCCGTTG-3'), 18S forward (5'-TCGGCTACCACATCCAAGGAAGGCAGC-3'), and 18S reverse (5'-TTGCTGGAATTACCGCGGCTGCTGGCA-3'). The protocols for sample preparation and run conditions described in the ABI 7500 user manual were followed (Applied Biosystems, Foster City, CA). Briefly, 10 μ l SYBR Green master mix (Applied Biosystems) was combined with the various primer pairs (1.2 μ l of each primer at 10 μ M each) and 7.6 μ l cDNA (1:50 or 1:100 dilutions of first strand reaction products) for a total volume of 20 μ l. The reactions were heated for 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Dissociation curve analysis verified that single products were produced with each set of the primer pairs used in these experiments. For each gene examined by qRT-PCR during the light-treatment time course, serial dilutions of cDNA from each of the time points were run in triplicate and the median CT value was normalized against the 18S rRNA CT. The comparative CT method was used for data analysis using 0 hours of light control animals as the reference time point and the 18S rRNA as the reference gene to generate a log₂ difference in gene expression levels (Johnson et al., 2000; Vong et al., 2003).

Immunohistochemistry

Eyes were enucleated and fixed in 4% paraformaldehyde/1x PBS (pH 7.4) overnight at 4°C. After fixing overnight, eyes were washed in 5% sucrose/1x PBS at room temperature, cryoprotected in 30% sucrose/1x PBS overnight at 4°C and then maintained in 2:1 Tissue Freezing Media (TFM; Triangle Biomedical Sciences, Durham, NC): 30% sucrose/1x PBS overnight at 4°C. Eyes were then embedded in 100% TFM and frozen at -80°C until cryosectioning at a thickness of 14-16 μ m. Cryosections were dried at 50°C for a minimum of one hour and stored at -80°C.

Slides were warmed at 50°C for 20 minutes, rehydrated in 1x PBS for 20 minutes and then subjected to antigen retrieval by boiling in 10 mM sodium citrate (pH 6.0) with 0.1% Tween-20 for 15 minutes and then cooled at room temperature (over ~40 minutes). Slides were then washed in 1x PBS and incubated in blocking buffer (1x PBS/4% normal goat serum/0.4% Triton-X-100/2% DMSO) for 1 hour at room temperature prior to incubating overnight at 4°C in primary antibody diluted in blocking buffer. The primary antibodies used for these studies included mouse anti-PCNA monoclonal antibody (1:1000, clone PC10, Sigma-Aldrich, St. Louis, MO), rabbit anti-Stat3 polyclonal antibody (1:100, Kassen et al, 2007), rabbit anti-Ascl1 polyclonal antibody (1:25, Sigma-Aldrich, St. Louis, MO), and mouse anti-GFP monoclonal antibody (1:1000, clone 3E6, Invitrogen, Carlsbad, CA). Following overnight incubation with primary antibody, slides were washed with 1x PBS/0.05% Tween-20 and then incubated for 1 hour at room temperature with secondary antibodies diluted 1:500 in 1x PBS/0.05% Tween-20. The secondary antibodies used for

these studies included goat anti-primary IgG conjugated to Alexa Fluor-488, -594 or -647 (Invitrogen, Carlsbad, CA). Nuclei were stained with TO-PRO-3 (Invitrogen, Carlsbad, CA) at a dilution of 1:1000 in 1x PBS/0.05% Tween-20. Following incubation with secondary antibodies, slides were washed again in 1x PBS/0.05% Tween-20 and a final time in 1x PBS before mounting with No. 1.5 glass coverslips and VECTASHIELD (Vector Laboratories, Burlingame, CA). All antibodies used in this study are described in greater detail in Table 1.

TUNEL was performed using the ApoAlert DNA Fragmentation Assay kit (Clontech, Mountain View, CA) and performed as described previously (Bailey et al., 2010). Biotinylated-dNTPs were detected using Alexa Fluor 488-conjugated Streptavidin (Invitrogen) diluted 1:200 in 1x PBS for 20 minutes at room temperature and the slides were mounted with glass coverslips and VECTASHIELD (Vector Laboratories).

Primary antibody characterization

The mouse monoclonal anti-Actin antibody (A3853, clone AC40; Sigma-Aldrich) was raised against an *in vitro*-synthesized peptide corresponding to the conserved C-terminal pan-actin epitope (SGPSIVHRKCF) fused to a MAP backbone. The antibody detects a single 42 kDa molecular weight protein on immunoblots (manufacturer's data sheet) and detects the same molecular weight protein on our immunoblots as other commercially available Actin antibodies (data not shown).

The rabbit polyclonal anti-Ascl1a antibody (HPA029217; Sigma-Aldrich) was raised against an *in vitro*-synthesized peptide corresponding to amino acids 156-238 of human the ASCL1 protein (MSKVETLRSAYEYIRALQQLLDEHDAVSAAFQAGVLSPTISPNYSNDLNSMAGSPVSS YSSDEGSYDPLSPPEEQELLDFTNWF). The antibody detects a single protein of approximately 26 kDa molecular weight on immunoblots (manufacturer's data sheet). We confirmed the specificity of this antibody by injecting an anti-*asc11a* morpholino into embryos and detecting significantly reduced expression of the 26 kDa protein in the morphant relative to the wild-type embryo lysate on immunoblots (Fig. 6H). We also confirmed that the antibody staining of the Müller glia in the regenerating retina was weaker in intensity and fewer in number in the *asc11a* morphant relative to the control retina (Fig. 6, panels D-F).

Rabbit mouse monoclonal anti-GFP (green fluorescent protein) antibody (1:1,500, A11120, clone 3E6; Invitrogen) was raised against GFP isolated directly from jellyfish *Aequorea victoria*. The specificity of the antibody was confirmed by an Elisa assay (manufacturer's datasheet). The anti-GFP antibody staining reproduced the EGFP fluorescence expression pattern exactly in the Tg(*gfap:EGFP*)^{nt11} transgenic fish and did not label retinal cryosections from wild-type AB strain zebrafish (data not shown).

The mouse monoclonal anti-PCNA antibody (P8825, clone PC10; Sigma-Aldrich) was raised against a recombinant rat PCNA-Protein A fusion protein. The antibody immunoprecipitated PCNA from cell extracts, bound PCNA in competition assays, and showed immunofluorescence staining that was identical to other commercially available PCNA antibodies (Waseem and Lane, 1990). This antibody produced identical labeling in cryosections as the rabbit polyclonal anti-PCNA antibody in the present study and as described in previous studies (Vihtelic and Hyde, 2000; Raymond et al., 2006).

The rabbit polyclonal anti-Stat3 antibody was previously described (Kassen et al., 2007, 2009). This antibody detects the correct sized protein on immunoblots, the intensity of the protein signal was reduced in *stat3* morphant embryos relative to controls on immunoblots (Fig. 6G), and the immunostaining of the retinal Müller glia and ganglion cells was reduced

in intensity and number in the light-damaged *stat3* morphant retina relative to the control retinas (Fig. 6, panels A-C).

Immunoblot Analysis

Total protein lysates were obtained by pooling 10 adult dorsal retinas for each treatment and homogenizing in protein extraction buffer (1x PBS/1% Triton-X-100/protease inhibitors, Roche Applied Science, Indianapolis, IN) using a polypropylene micro pestle and incubated on ice for 1 hour. Lysates were then centrifuged briefly to remove Triton-X-100-insoluble debris and the supernatant was collected and stored at -80°C . 10 μg of lysate was combined with Novex 4x tris-glycine-SDS sample buffer and 10x sample reducing agent (Invitrogen). Samples were heated at 95°C for 5 minutes and then electrophoresed through Novex 4%-12% tris-glycine gels (Invitrogen). Proteins were transferred to Hybond-P PVDF membrane (GE Healthcare, Piscataway, NJ) and then blocked for 1 hour at room temperature blocking buffer (1x PBS/0.1% Tween-20/5% nonfat dry milk). Membranes were incubated in rabbit anti-Stat3 polyclonal antibody (1:5000, Kassen et al., 2007) or mouse anti-Actin monoclonal antibody (1:10000, clone AC-40, Sigma-Aldrich) diluted in blocking buffer overnight at 4°C . Membranes were washed three times for 5 minutes, then once for 15 minutes in 1x PBS/0.1% Tween-20 before incubating with ECL HRP-conjugated secondary antibodies (1:10,000, GE Healthcare) diluted in blocking buffer. Membranes were washed again and then developed using the ECL Prime kit (GE Healthcare).

For embryos, total protein lysates were obtained by pooling ~30 deyolked 44 hpf embryos for each treatment and homogenizing as described above. Western blot was carried out as described above using rabbit anti-Ascl1 polyclonal antibody (1:2500, Sigma-Aldrich). Exposed film was imaged using a Carestream Gel Logic 2200 Pro imaging station (Carestream Health, Inc., Rochester, NY). Actin was used as a loading control for all blots.

Confocal Imaging and Statistical Analysis

Confocal imaging was performed with a Leica TCS SP5 laser scanning confocal microscope. Low-intensity signals were enhanced and background signals were reduced in representative images using the levels function in Adobe Photoshop (Adobe Systems, San Jose, CA). Levels were adjusted identically to all layers within a panel and to all panels in a figure. Red-green color scheme images were converted to magenta-green using Adobe Photoshop as described previously (Montgomery et al., 2010).

To maintain consistency between eyes and experimental groups, only retinal sections encompassing or immediately adjacent to, the optic nerve were utilized. Quantification of all markers was performed on either 4 μm (Figs. 7 and 8) or 5 μm thick (Figs. 1, 2, 6, and 9) confocal z-stacks that began at the surface of the 14 μm sections closest to the coverslip. All images were acquired across a 350 μm region of the central/dorsal retina that consistently received the same degree of damage and reliably resulted in comparable subsequent high levels of Müller glia proliferation (Thomas et al., 2012). Retinas from ten different individual fish were examined for every control and experimental group at each timepoint in this study. Only INL cells that unequivocally expressed the indicated markers were counted as being positive for that marker. Statistical significance was determined between control and experimental groups of all experiments using a two-tailed Student's t-test, in which p-values less than 0.05, 0.01, and 0.005 were considered significant, highly significant, and very highly significant, respectively.

RESULTS

Light damage induces Stat3 expression in all Müller glia, but Ascl1a in only the subset of proliferating Müller glia

To determine the spatial expression of the Stat3 and Ascl1a proteins, we immunolocalized both proteins independently using rabbit anti-Stat3 and anti-Ascl1 polyclonal antisera in dark-adapted *albino* Tg(*gfap:EGFP*) adult zebrafish after either 0 or 36 hours of constant intense light. The *albino* Tg(*gfap:EGFP*) line expresses EGFP in all Müller glia (Kassen et al, 2007). While Stat3 expression is not detected in the undamaged retina (Fig. 1, panels A and C), it is present after 36 hours of constant light in all the Müller glia (Fig. 1, panels D-F). We quantified the number of EGFP- and Stat3-positive INL cells at 0 (Fig. 2A, blue bars) and 36 hours of constant light (Fig. 2A, red bars). At 0 hours, we detected only a minimal number of Stat3-expressing INL cells (0.9 ± 1.5) compared with 50.6 ± 10.4 EGFP-positive Müller glia (Fig. 2A). At 36 hours of constant light, we identified 46.6 ± 11.5 Stat3-positive INL cells and 47.1 ± 10.1 EGFP-positive Müller glia (Fig. 2A), with Stat3-positive cells coexpressing EGFP. These findings concur that all Müller glia express increased levels of Stat3 at 36 hours of constant light relative to the 0 hour undamaged retina.

We did not detect significant Ascl1a expression in the undamaged retina (Fig. 3, panels A and C), but again observed increased Ascl1a protein in the Müller glia after 36 hours of constant light (Fig. 3, panels D and F). Unlike Stat3 however, Ascl1a was present in only a subset of the EGFP-positive Müller glia (Fig. 3, panels D and F, arrows). In the undamaged control retinal sections, we counted 51.2 ± 9.9 EGFP-positive Müller glia and 0.4 ± 0.7 Ascl1a-positive INL cells (Fig. 2B). Following 36 hours of constant light treatment, we detected 45.6 ± 8.9 EGFP-positive Müller glia and only 28.2 ± 8.9 Ascl1a-positive cells (Fig. 2B), of which 26.6 ± 7.5 cells coexpressed Ascl1a and EGFP (Fig. 2B). This indicates that only a subset of Müller glia (approximately 60%) expressed Ascl1a in the light-damaged retina.

To examine the expression of Stat3 and Ascl1a in relation to proliferating Müller glia after 36 hours of constant light, we immunolabeled either Stat3 or Ascl1a and PCNA on retinal cryosections. The PCNA-positive proliferating cells were a subset of the Stat3-expressing Müller glia (Fig. 4, panels A-C), consistent with previous experiments that demonstrated that only a subset of the Müller glia reentered the cell cycle in the light-damaged retina (Kassen et al., 2007). In contrast, the PCNA-expressing cells colabeled with Ascl1a after 36 hours of constant light (Fig. 4, panels D-F). Overall, these results suggest the increased expression of Ascl1a is restricted to the subset of proliferating Stat3-positive Müller glia.

We next examined the temporal expression of Stat3 relative to Ascl1a. We used quantitative real-time PCR (qRT-PCR) to determine when the expression of *stat3* increases relative to the increased *ascl1a* expression. Dark-adapted *albino* adult zebrafish were exposed to constant intense light for 0, 5, 10, 15, and 20 hours. While *stat3* expression increased significantly within the first 5 hours of constant light treatment, *ascl1a* expression did not increase significantly until 5-10 hours after starting the light treatment (Fig. 5). This data suggests that retinal damage induces *stat3* expression in the retina prior to *ascl1a* expression in the subset of Müller glia that will reenter the cell cycle.

Stat3 and Ascl1a are neither required for photoreceptor cell death nor neuroprotection

To determine if either Stat3 or Ascl1a expression affected photoreceptor cell death in the light-damaged retina, we intravitreally injected and electroporated either the standard control morpholino (which is not complementary to any known sequence in the zebrafish genome) or the anti-*stat3* or anti-*ascl1a* morpholinos into dark-adapted *albino* retinas immediately prior to starting 36 hours of constant light treatment.

Stat3 immunolocalized to Müller glia and ganglion cells after 36 hours of light treatment in both uninjected control and standard control morphant retinas (Fig. 6, panels A and B, respectively). In contrast, only minimal levels of Stat3 protein were detected in Müller glia and ganglion cells in *stat3* morphant retinas (Fig. 6C). We also confirmed the specificity of both the knockdown and the Stat3 antibodies by isolating retinal homogenates after 36 hours of constant light exposure and analyzing them on immunoblots with the rabbit anti-Stat3 polyclonal antiserum (Kassen et al., 2007). Stat3 protein expression was efficiently knocked down by anti-*stat3* morpholinos relative to the uninjected and standard control morpholinos (Fig. 6G).

Ascl1a immunolocalized to the Müller glial nuclei after 36 hours of light treatment in both uninjected controls and standard control morphant retinas (Fig. 6, panels D and E, respectively). Ascl1 protein expression was dramatically reduced in the Müller glia of *ascl1a* morphant retinas (Fig. 6F), suggesting that either the Ascl1b paralog is not significantly expressed in the retina or the anti-Ascl1 polyclonal antiserum does not detect the Ascl1b protein. However, Ascl1 also immunolocalized to cells in the vitreal space, even in the *ascl1a* morphant (Fig. 6F, bracket). These cells likely represent invasive blood cells that entered the eye following the corneal incision, morpholino injection, and/or morpholino electroporation. The increased expression of Ascl1 in these cells relative to the knocked down Ascl1a expression in the Müller glia suggests that these vitreal cells are unaffected by *ascl1a* morpholinos. Therefore, these cells either entered the retina after the electroporation occurred or are expressing the cross-reacting Ascl1b protein, neither of which is affected by the *ascl1a* morpholino.

Because the Ascl1-expressing vitreal cells could not be removed, which would complicate the immunoblot analysis of Ascl1a knockdown in the light-damaged retina, we isolated protein homogenates from embryos injected at the 1-2 cell stage with either nothing, the standard control morpholino or the *ascl1a* morpholino and harvested at 44 hours post fertilization. Immunoblots of these homogenates confirmed that Ascl1a protein was present in both the controls, but dramatically reduced in amount in the *ascl1a* morphant (Fig. 6H). These results confirmed the efficacy of the Ascl1a knockdown and the specificity of the anti-Ascl1 polyclonal antiserum.

To determine if either Stat3, Ascl1a, or Lin28a were necessary for either photoreceptor cell death or neuroprotection, we either did nothing (uninjected control) or intravitreally injected and electroporated the standard control morpholino (S.C. MO), the *stat3* morpholino (*stat3* MO), *ascl1a* morpholino (*ascl1a* MO), or the *lin28a* morpholino (*lin28a* MO) into dark-adapted adult albino zebrafish and then exposed the fish to constant intense light for 16 hours. We then labeled the retinal sections for TUNEL-positive cells as an indication of photoreceptor cell death in the outer nuclear layer (Fig. 7, panels A-E, green). We quantified the number of TUNEL-positive cells in retinal cryosections across 350 μ m of the central-dorsal ONL. The uninjected and standard control morphant retinas had an average of 50.0 ± 8.0 and 46.3 ± 14.1 TUNEL-positive nuclei. Similarly, the *stat3*, *ascl1a*, and *lin28a* morphant retinas possessed 41.4 ± 21.3 , 42.0 ± 20.2 , and 40.8 ± 5.2 TUNEL-positive nuclei in the ONL, respectively. Because knockdown of either Stat3, Ascl1a, or Lin28a did not statistically alter the number of TUNEL-positive cells relative to the control retinas, they are not required for either photoreceptor apoptosis or photoreceptor neuroprotection of light-induced photoreceptor cell death.

A subset of Müller glia require Stat3 for proliferation, which is regulated by Ascl1a and Lin28a

To determine the role of Stat3 and Ascl1a on Müller glia proliferation during regeneration of the light-damaged retina, we compared Stat3 and PCNA expression in uninjected and

morphant (standard control, *stat3*, *ascl1a*, and *lin28a*) retinas of dark-adapted adult *albino* fish exposed to constant intense light for 36 hours. Uninjected control retinas contained 60.8 ± 2.7 Stat3-positive cells and 43.9 ± 5.3 PCNA-positive cells, of which 41.1 ± 5.2 were also Stat3-positive (Fig. 8, panels A, B; Fig. 8K, blue bars; Table 2). Thus, 19.7 ± 3.1 Stat3-positive cells were identified that did not express PCNA (Fig. 8, panels A, B, arrowheads; Fig. 8K; Table 2). This is consistent with our previous results that Stat3 is expressed in all Müller glia and only a subset of these cells is PCNA-positive. Retinas electroporated with the standard control morpholino (Fig. 8, panels C, D; Fig. 8K, red bars; Table 2) did not result in any statistically significant change in any of these cell counts relative to the uninjected controls. However, knockdown of Stat3 resulted in significantly fewer numbers of PCNA-positive INL nuclei, Stat3-positive cells, and Stat3/PCNA coexpressing cells (Fig. 8, panels E, F; Fig. 8K, green bars; Table 2) relative to both controls. Surprisingly, knockdown of Stat3 yielded significantly more PCNA-positive Stat3-negative INL cells (Fig. 8, panels E and F, open arrows; Fig. 8K; Table 2) compared to the controls. Knockdown of either *Ascl1a* or *Lin28a*, a putative downstream regulatory target of *Ascl1a* (Ramachandran et al., 2010), resulted in significantly fewer PCNA-positive cells relative to either the controls or the *stat3* morphant (Fig. 8, panels G-J; Fig. 8K, purple and yellow bars, respectively; Table 2). Knockdown of either *Ascl1a* or *Lin28a* also yielded significantly fewer Stat3-positive cells relative to either of the controls (Fig. 8, panels G-J; Fig. 8K purple and yellow bars, respectively; Table 2). These data suggest that after 36 hours of light, *Ascl1a* and *Lin28a* are required for Stat3 expression in the maximal number of Müller glia. However, only a subset of Müller glia requires Stat3 expression for proliferation.

Stat3 and Lin28a are both required for the maximal number of Ascl1a-expressing and proliferating Müller glia of the light-damaged retina

To test whether the knockdown of either Stat3 or Lin28a affected the expression of *Ascl1a* in Müller glia, we compared *Ascl1a* and PCNA expression in uninjected and morphant (standard control, *stat3*, *ascl1a*, and *lin28a*) retinas of dark-adapted adult *albino* fish exposed to constant intense light for 36 hours. Uninjected control retinas (Fig. 9, panels A, B; Fig. 9K, blue bars; Table 3) contained 45.6 ± 4.1 *Ascl1a*-positive cells and 47.1 ± 3.7 PCNA-positive cells, of which 44.6 ± 3.9 were also *Ascl1a*-positive (Fig. 9, panels A, B, arrows; Fig. 9K; Table 3). Similar numbers of cells were observed in the standard control morphant retinas (Fig. 9, panels C, D; Fig. 9K, red bars; Table 3). Knockdown of *Ascl1a* expression resulted in significantly fewer *Ascl1a*-positive cells, PCNA-positive cells, and *Ascl1a*/PCNA-colabeled cells (Fig. 9, panels G, H; Fig. 9K, purple bars; Table 3) relative to both control groups. Knockdown of Stat3 expression resulted in significantly fewer PCNA-positive cells, *Ascl1a*-positive cells, and *Ascl1a*/PCNA-colabeled cells (Fig. 9, panels E, F; Fig. 9K, green bars; Table 3) relative to both control groups, but significantly more than found in the *ascl1a* morphant (Fig. 9, panels G, H; Fig. 9K, purple bars; Table 3). Knockdown of *Lin28a* expression yielded a similar reduced number of either PCNA-positive or *Ascl1a*-positive cells compared to the *ascl1a* morphant (Fig. 9, panels I, J; Fig. 9K, yellow bars; Table 3). These data confirm that *Ascl1a* and *Lin28a* expression are both required for Müller glia proliferation and Stat3 is necessary for a subset of the Müller glia to proliferate in the light-damaged retina. Furthermore, *Ascl1a* expression is dependent on *Lin28* expression, while Stat3 is required for maximal *Ascl1a* expression in the Müller glia.

Stat3, Ascl1a, and Lin28a are required for Müller glia proliferation in the ouabain-damaged retina

To determine if Stat3, *Ascl1a*, and *Lin28a* are also required for Müller glia proliferation subsequent to loss of inner retinal neurons, we intravitreally-injected a low concentration of ouabain to kill ganglion, amacrine, and bipolar cells, without significantly damaging photoreceptors (Fimbel et al., 2007). We intravitreally injected and electroporated either

nothing (uninjected control) or morpholinos (standard control, *stat3*, *ascl1a*, and *lin28a*) immediately prior to injecting a final vitreal concentration of 2 μ M ouabain into adult zebrafish. The control and morphant retinas were collected 3 days post ouabain injection and labeled for PCNA to detect proliferating cells (Fig. 10, panels A-E, green).

We quantified the number of PCNA-positive INL cells across a 350 μ m region of the central-dorsal retina (Fig. 11). Both controls, one lacking any morpholino or electroporation and the other electroporated with the standard control morpholino, contained 34.8 ± 11.7 and 33.7 ± 10.0 PCNA-positive INL nuclei (Fig. 10, panels A, B; Fig. 11, blue and red bars, respectively). The *stat3*, *ascl1a*, and *lin28a* morphant retinas contained significantly fewer PCNA-positive cells relative to the standard control morphant (23.3 ± 10.0 , 15.0 ± 11.0 , and 16.1 ± 9.2 PCNA-positive INL nuclei, respectively; Fig. 11, green, purple, and yellow bars, respectively). While the *stat3* morphant did not contain significantly more PCNA-positive cells than either the *ascl1a* or *lin28a* morphants, the trend of more PCNA-positive cells in the *stat3* morphant was similar to that observed in the light-damaged retina. These results demonstrate that Stat3, Ascl1a, and Lin28a are required for maximal Müller glia proliferation in response to ganglion cell and INL cell loss without significant photoreceptor cell death. This demonstrates a broad requirement for these three proteins in the reentry of the Müller glia into the cell cycle, which is required for regeneration of any neuronal cell type in the damaged retina. This suggests that later signaling events designate the cell-type specific regeneration that is observed following damage to diverse populations of cells in the adult zebrafish retina.

DISCUSSION

We analyzed the roles of Stat3 and Ascl1a proteins independently and relative to each other during Müller glia proliferation at the start of neuronal regeneration in the damaged adult zebrafish retina. We showed that Stat3 is expressed in all Müller glia, while Ascl1a is expressed in the proliferating Müller glia. Furthermore, *stat3* mRNA expression increases significantly in the light-damaged retina prior to *ascl1a* expression. Taken together, it appears that Stat3 expression in all the Müller glia precedes Ascl1a expression in the dedifferentiated/proliferating Müller glia. We also demonstrated that Stat3 is required for both the maximal expression of Ascl1a expression and maximal Müller glia proliferation. Additionally, we confirmed a previous study that Ascl1a is required for retinal regeneration (Fausett et al., 2008) and demonstrated that Ascl1a is necessary for maximal Stat3 expression after 36 hours of constant light treatment. Finally, we found that Lin28a, which was previously described as functioning downstream of Ascl1a (Ramachandran et al., 2010), is required for Ascl1a and Stat3 expression in the regenerating retina. Taken together, these findings suggest that Stat3, Ascl1a, and Lin28a are all required for the maximal Müller glia reentry into the cell cycle at the beginning of the regeneration response in the light-damaged adult zebrafish retina and likely involves two populations of proliferating Müller glia, one that is Stat3-dependent and the other is Stat3-independent.

Because there is a strong correlation between the amount of cell death and the subsequent numbers of proliferating Müller glia in the damaged retina (Montgomery et al., 2010; Thomas et al., 2012), we assayed neuronal cell death using TUNEL in all control and experimental morphants. We did not observe any significant differences in the number of TUNEL-positive cells in the light-damaged *stat3*, *ascl1a*, or *lin28a* morphants relative to the controls (Fig. 7), which demonstrates that they are not involved in either neuroprotection or cell death. This and the confirmation of protein knockdown (Fig. 6) confirms that the changes in Müller glia proliferation in these morphants is due to the reduced expression of the target proteins and not due to changes in cell death.

We confirmed the efficacy of the *stat3* and *ascl1a* morpholinos by both immunoblots and immunohistochemistry (Fig. 6). These experiments also confirmed that both the anti-Stat3 and anti-Ascl1 polyclonal antisera properly recognized the Stat3 and Ascl1a proteins, respectively. However, the detection of Ascl1-positive cells in the vitreous of electroporated eyes (Fig. 6F) was due to either the expression of Ascl1b and/or the presence of non-electroporated blood cells following removal of the cornea, injection of morpholinos, and/or electroporation of the eye. Because of these contaminating cells, we tested the specificity of the *ascl1a* morpholino (Fausett et al., 2008) using protein lysates from 44 hpf *ascl1a* morphant embryos (Fig. 6H), which confirmed the knockdown of the 25 kDa Ascl1a protein relative to the controls.

We initiated this study by examining the role of Stat3 in Müller glia reentry into the cell cycle at the start of retinal regeneration for two major reasons. First, we previously demonstrated that the light-damaged retina induces *stat3* mRNA expression and significantly increased levels of the Stat3 and phosphorylated Stat3 protein (Kassen et al., 2007). Second, we had shown that Stat3 was required for the CNTF-dependent induction of Müller glia proliferation in the undamaged retina (Kassen et al., 2009). It is important to note that multiple CNTF injections were required to induce a small number of Müller glia to reenter the cell cycle in the undamaged retina, thus the role of Stat3 in the CNTF-induced Müller glia proliferation may not be the same as its role in Müller glia proliferation in the light-damaged retina. Additionally, CNTF activates mammalian retinal progenitor cells to maintain an undifferentiated state and inhibits rod photoreceptor differentiation during development (Kirsch et al., 1998; Rhee et al., 2004; Zhang et al., 2005b). Finally, Stat3 has been reported to play a significant role in regulating neuronal stem cell renewal and precursor cell proliferation in the developing central nervous system (Zhang et al., 2005a; Androutsellis-Theotakis et al., 2006; Nagao et al., 2007).

We demonstrated that Stat3 is required by a subset of Müller glia for reentry into the cell cycle in the light-damaged retina (Fig. 8). The remaining PCNA-positive Müller glia in the *stat3* morphant retinas could have resulted from two different possibilities. First, the morpholino may not be 100% effective and the electroporation technique may not be 100% efficient, resulting in some Müller glia containing insufficient copies of morpholino. However, this is unlikely based on the observation that electroporation of the anti-*pcna* morpholino completely abolished PCNA expression in the Müller glia of the light-damaged zebrafish retina (Thummel et al., 2008) and the very low number of Stat3-positive cells observed in the *stat3* morphant. Alternatively, a subset of Müller glia may possess the ability to proliferate in a Stat3-independent manner. The significant number of Stat3-negative and PCNA-positive INL cells (Fig. 8K) is consistent with the latter scenario.

The moderate, but statistically significant, reduction in the number of Ascl1a-positive Müller glia in *stat3* morphant retinas suggested that Stat3 is necessary for maximal expression of Ascl1a (Fig. 9K). However, because there are significantly more Ascl1a-positive cells than Stat3-expressing cells in the *stat3* morphant (compare Fig. 8K and 9K), Ascl1a is not simply downstream of Stat3 in a signaling cascade. The mechanism by which Stat3 regulates the number of Ascl1a-expressing Müller glia is unknown.

Knockdown of Ascl1a prior to the onset of light treatment also resulted in a significant reduction in the number of Stat3-positive and PCNA-positive Müller glia. This demonstrated that Ascl1a is necessary for both maximal Stat3 expression and Müller glia proliferation. However, the number of Stat3-expressing cells in the *ascl1a* morphant is greater than the number of Ascl1a-positive cells in the *ascl1a* morphant (Figs. 8K and 9K, respectively), which suggests that a population of Stat3-expressing Müller glia are not dependent on Ascl1a expression. This likely represents the Stat3-positive non-proliferating

Müller glia, which do not express *Ascl1a*. Furthermore, the very low number of *Ascl1a*-positive/PCNA-negative and *Ascl1a*-negative/PCNA-positive cells in both controls and all three morphants is consistent with our conclusion that proliferating Müller glia express *Ascl1a* and *Ascl1a*-positive Müller glia express PCNA.

Electroporation of dark-adapted retinas with *lin28a* morpholinos significantly reduced the number of PCNA-positive and Stat3-positive cells after constant light treatment, suggesting that *Lin28a* may be required for Müller glia proliferation by regulating the number of Stat3-positive cells. Surprisingly, the *lin28a* morphant retinas also resulted in significantly fewer *Ascl1a*-positive Müller glia. This result was unexpected because it was previously reported that *Lin28a* acted downstream of *Ascl1a* in puncture damaged retinas (Ramachandran et al., 2010; Wan et al., 2012). We attribute this discrepancy to: i) the previous studies failing to quantify the number of Müller glia expressing *Ascl1a* protein, ii) the increased numbers of *Ascl1*-expressing cells in the vitreous, which are likely to also be present in the puncture-damaged eye, or iii) potential changes in the expression of *ascl1a* and *lin28* in the circumferential marginal zone. Regardless, our study demonstrated that *Lin28a* functions either upstream of *Ascl1a* or in a feedback regulatory loop.

Finally, we tested for a common requirement of Stat3 and *Ascl1a* for the initiation of Müller glia proliferation during regeneration of inner retinal neurons following ouabain damage. Intravitreal injection of ouabain results in the apoptosis of ganglion and INL cells without damaging photoreceptors (Fimbel et al., 2007). Knockdown of either Stat3, *Ascl1a*, or *Lin28a* expression significantly reduced the number of PCNA-positive INL cells at 3 dpi compared to controls (Figs. 10 and 11). This showed that a similar mechanism for initiating the reentry of Müller glia into the cell cycle is utilized in response to the death of diverse classes of retinal neurons. Therefore, the early events in the regeneration response may be due to a signaling cascade that is general and not specific to the cell type that is damaged.

Based on these data, we propose a model for the initiation of photoreceptor cell regeneration that involves dying photoreceptors differentially imparting unknown death signals that activate Stat3 and *Ascl1a* expression. Because an antibody that recognizes the zebrafish *Lin28a* protein does not exist, we are unable to either confirm the knockdown or assess the direct effect of retinal damage on *Lin28a* protein expression, although *Lin28a* appears to be necessary for both Stat3 and *Ascl1a* expression. This model suggests that there are three different populations of Müller glia. One population is Stat3-positive and *Ascl1a*-negative and does not proliferate (Fig. 12). A second population (Primary Proliferating Müller glia, Fig. 12) is activated by the damage signal produced by dying neurons. This signal activates *Lin28a* and *Ascl1a*, which drives these cells to reenter the cell cycle. *Ascl1a* also activates Stat3 expression in the Primary Proliferating Müller glia, which produces a signal that activates *Ascl1a* and *Lin28a* in nearby Secondary Proliferating Müller glia (the third population of Müller glia, Fig. 12) and induces these cells to reenter the cell cycle. This model is consistent with the knockdown of either *Ascl1a* or *Lin28a* significantly reducing the expression of Stat3 and suppressing Müller glia proliferation. Additionally, it accounts for the moderate, but statistically significant, number of *Ascl1a*-positive proliferating Müller glia in *stat3* morphants relative to either the *ascl1a* or *lin28a* morphants. Heparin binding-EGF may act as the signal between the Primary and Secondary Proliferating Müller glia (Wan et al., 2012). Alternatively, we recently identified tumor necrosis factor alpha (TNF α) as being expressed in Müller glia and being required to induce the maximal number of Müller glia that reenter the cell cycle (Nelson and Hyde, unpublished data). This supports the model involving two different populations of Müller glia that proliferate sequentially during retinal regeneration.

While this is an attractive model for a variety of reasons, it fails to account for how Stat3 expression is induced in the subset of non-proliferating Müller glia and why these cells fail to reenter the cell cycle. It is possible that the absence of Lin28a and Ascl1a in these Stat3-positive Müller glia prevents their proliferation. Regardless, these quiescent Müller glia may function to preserve some structural integrity to the retina during a regeneration response.

An additional apparent discrepancy in our model is the increased expression of *stat3* mRNA prior to *ascl1a* mRNA (Fig. 5) even though our model places Ascl1a upstream of Stat3 in the initial proliferating Müller glia (Fig. 12). This qRT-PCR data simply suggests that the initial increase in both *ascl1a* and *stat3* is due to the generation of a signal that is rapidly produced after initiating the constant intense light treatment. This qRT-PCR data could represent changes in *stat3* and *ascl1a* expression throughout the retina, including the circumferential marginal zone (CMZ). In this case, potentially increased CMZ expression could misrepresent the changes in *stat3* and *ascl1a* expression in the damaged region.

Alternatively, the initial increase in *stat3* expression could be regulated by Ascl1a protein that is already present in the Müller glia. However, neither Ascl1a protein or *ascl1a* mRNA expression was detected in the undamaged retina (Fig. 3A; Ramachandran et al., 2010; Ramachandran et al., 2011). Because knockdown of Stat3 expression does not completely block the increased Ascl1a expression, it is unlikely that Stat3 activates the initial expression of *ascl1a*. Thus, another signal likely activates the expression of *stat3* and *ascl1a* shortly after starting the constant light treatment. In this regard, we recently found that tumor necrosis factor alpha (TNF α) is rapidly expressed in photoreceptors in the light-damaged retina, is required for both Stat3 and Ascl1a expression in the Müller glia, and is necessary for maximal Müller glia proliferation (Nelson and Hyde, unpublished data). We believe that TNF α expression in the damaged photoreceptors activates a signaling cascade in the Müller glia that stimulates *stat3* and *ascl1a* expression, which leads to Müller glia dedifferentiation and reentry into the cell cycle.

Recently, expression of *heparin-binding epidermal-like growth factor* (*hb-egf*) mRNA was shown to be induced in Müller glia at the site of a retinal puncture (Wan et al., 2012). Morpholino-mediated knockdown of HB-EGF expression significantly reduced the number of proliferating Müller glia, but did not abolish all of the proliferating Müller glia (Wan et al., 2012). Furthermore, intravitreal injection of recombinant soluble human HB-EGF into an undamaged eye was sufficient to induce Müller glia proliferation (Wan et al., 2012). The expression of HB-EGF in Müller glia and its requirement for inducing the maximal number of proliferating Müller glia is consistent with HB-EGF serving as our proposed amplification signal from the Primary Müller glia to the Secondary Müller glia (Fig. 12). We are currently testing the potential role of HB-EGF in this proposed model.

The identification of the regulatory steps required for zebrafish retinal regeneration may reveal why the mammalian Müller glia possess only a limited ability to proliferate in the damaged retina (Ooto et al., 2004), and could identify approaches to induce Müller glial cell proliferation and subsequent regeneration in the damaged or diseased human retina.

Acknowledgments

The authors would like to thank the Freimann Life Science Center staff for the excellent care and maintenance of the zebrafish and to previous and present members of the Hyde lab for helpful discussion. 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.

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.

LITERATURE CITED

- Aaronson DS, Horvath CM. A road map for those who don't know JAK-STAT. *Science*. 2002; 296:1653–1655. [PubMed: 12040185]
- Androutsellis-Theotokis A, Leker RR, Soldner F, Hoepfner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD. Notch signalling regulates stem cell numbers *in vitro* and *in vivo*. *Nature*. 2006; 442:823–826. [PubMed: 16799564]
- Bailey TJ, Fossum SL, Fimbel SM, Montgomery JE, Hyde DR. The inhibitor of phagocytosis, O-phospho-L-serine, suppresses Müller glia proliferation and cone cell regeneration in the light-damaged zebrafish retina. *Exp Eye Res*. 2010; 91:601–612. [PubMed: 20696157]
- Bernardos RL, Barthel LK, Meyers JR, Raymond PA. Late-Stage Neuronal Progenitors in the Retina Are Radial Müller Glia That Function as Retinal Stem Cells. *J Neurosci*. 2007; 27:7028–7040. [PubMed: 17596452]
- 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]
- 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]
- Cameron DA. Cellular proliferation and neurogenesis in the injured retina of adult zebrafish. *Vis Neurosci*. 2000; 17:789–797. [PubMed: 11153658]
- 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]
- Fausett BV, Goldman D. A role for alpha1 tubulin-expressing Müller 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]
- Fukuda S, Taga T. Roles of BMP in the development of the central nervous system. *Clin Calcium*. 2006; 16:781–785. [PubMed: 16679619]
- Fukuda S, Abematsu M, Mori H, Yanagisawa M, Kagawa T, Nakashima K, Yoshimura A, Taga T. Potentiation of astrogliogenesis by STAT3-mediated activation of bone morphogenetic protein-Smad signaling in neural stem cells. *Mol Cell Biol*. 2007; 27:4931–4937. [PubMed: 17452461]
- Johnson MR, Wang K, Smith JB, Heslin MJ, Diasio RB. Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. *Anal Biochem*. 2000; 278:175–184. [PubMed: 10660460]
- 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, Campochiaro LA, Harding MJ, Bennett NA, Hyde DR. CNTF induces photoreceptor neuroprotection and Müller glial cell proliferation through two different signaling pathways in the adult zebrafish retina. *Exp Eye Res*. 2009; 88:1051–1064. [PubMed: 19450453]
- Kirsch M, Schulz-Key S, Wiese A, Fuhrmann S, Hofmann H. Ciliary neurotrophic factor blocks rod photoreceptor differentiation from postmitotic precursor cells in vitro. *Cell Tissue Res*. 1998; 291:207–216. [PubMed: 9426308]
- Levy DE, Lee CK. What does Stat3 do? *J Clin Invest*. 2002; 109:1143–1148. [PubMed: 11994402]
- 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]

- Nagao M, Sugimori M, Nakafuku M. Cross talk between notch and growth factor/cytokine signaling pathways in neural stem cells. *Mol Cell Biol.* 2007; 27:3982–3994. [PubMed: 17371842]
- Ooto S, Akagi T, Kageyama R, Akita J, Mandai M, Honda Y, Takahashi M. Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. *Proc Natl Acad Sci U S A.* 2004; 101:13654–13659. [PubMed: 15353594]
- O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell.* 2002; 109(Suppl):S121–131. [PubMed: 11983158]
- Powell C, Elsaiedi F, Goldman D. Injury-dependent Müller 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]
- Ramachandran R, Fausett BV, Goldman D. Ascl1a regulates Müller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. *Nat Cell Bio.* 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]
- Rawlings JS, Rennebeck G, Harrison SM, Xi R, Harrison DA. Two *Drosophila* suppressors of cytokine signaling (SOCS) differentially regulate JAK and EGFR pathway activities. *BMC Cell Biol.* 2004; 5:38. [PubMed: 15488148]
- Raymond PA, Barthel LK, Bernardos RL, Perkowski JJ. Molecular characterization of retinal stem cells and their niches in adult zebrafish. *BMC Cell Biol.* 2006; 6:36.
- Rhee KD, Goureau O, Chen S, Yang XJ. Cytokine-induced activation of signal transducer and activator of transcription in photoreceptor precursors regulates rod differentiation in the developing mouse retina. *J Neurosci.* 2004; 24:9779–9788. [PubMed: 15525763]
- 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]
- Thomas JL, Nelson CM, Luo X, Hyde DR, Thummel R. Characterization of multiple light damage paradigms reveals regional differences in photoreceptor loss. *Exp Eye Res.* 2012; 97:105–116. [PubMed: 22425727]
- Thummel R, Kassen SC, Montgomery JE, Enright JM, Hyde DR. Inhibition of Müller glial cell division blocks regeneration of the light-damaged zebrafish retina. *Dev Neurobiol.* 2008; 68:392–408. [PubMed: 18161852]
- 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]
- 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]
- Vihtelic TS, Soverly JE, Kassen SC, Hyde DR. Retinal regional differences in photoreceptor cell death and regeneration in light-lesioned albino zebrafish. *Exp Eye Res.* 2006; 82:558–575. [PubMed: 16199033]
- Vong QP, Chan KM, Cheng CH. Quantification of common carp (*Cyprinus carpio*) IGF-I and IGF-II mRNA by real-time PCR: differential regulation of expression by GH. *J Endocrinol.* 2003; 178:513–521. [PubMed: 12967342]
- Wan J, Ramachandran R, Goldman D. HB-EGF is necessary and sufficient for Müller glia dedifferentiation and retina regeneration. *Dev Cell.* 2012; 22:334–347. [PubMed: 22340497]
- Waseem NH, Lane DP. Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. *J Cell Sci.* 1990; 96:121–129. [PubMed: 1695635]
- Westerfield, M. *The Zebrafish Book; A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio)*. University of Oregon Press; 1993.

- Wu DM, Schneiderman T, Burgett J, Gokhale P, Barthel L, Raymond PA. Cones regenerate from retinal stem cells sequestered in the inner nuclear layer of adult goldfish retina. *Invest Ophthalmol Vis Sci*. 2001; 42:2114–2124.
- Yurco P, Cameron DA. Responses of Müller glia to retinal injury in adult zebrafish. *Vision Res*. 2005; 45:991–1002. [PubMed: 15695184]
- Zhang SS, Liu MG, Kano A, Zhang C, Fu XY, Barnstable CJ. STAT3 activation in response to growth factors or cytokines participates in retina precursor proliferation. *Exp Eye Res*. 2005a; 81:103–115. [PubMed: 15978261]
- Zhang CW, Lu Q, You SW, Zhi Y, Yip HK, Wu W, So KF, Cui Q. CNTF and BDNF have similar effects on retinal ganglion cell survival but differential effects on nitric oxide synthase expression soon after optic nerve injury. *Invest Ophthalmol Vis Sci*. 2005b; 46:1497–1503. [PubMed: 15790921]
- Zhou Q, Chipperfield H, Melton DA, Wong WH. A gene regulatory network in mouse embryonic stem cells. *Proc Natl Acad Sci U S A*. 2007; 104:16438–16443. [PubMed: 17940043]

Figure 1.

Stat3 is expressed in all Müller glia following 36 hours of constant light treatment. Dark-adapted adult *albino* *Tg(gfap:EGFP)* transgenic fish, which express EGFP in all Müller glia, were exposed to constant intense light for either 0 (A-C) or 36 (D-F) hours. At 0 hours, EGFP-positive Müller glia (B and C, green; arrowheads) did not express any detectable levels of Stat3 (A and C, magenta). Following 36 hours of constant intense light, nearly all EGFP-positive Müller glia (green; arrows) expressed Stat3 (D and F, magenta). The area in the dashed box is magnified 2-fold in the inset. INL, inner nuclear layer; ONL, outer nuclear layer. Panel A scale bar is 50 μ m and is the same for panels B-F.

Figure 2.

Quantification of Stat3 and Ascl1a positive Müller glia in the light-damaged retina. A. The number of Stat3-positive cells and EGFP-positive Müller glia in Tg(*gfap:EGFP*) retinas (Fig. 1) were quantified at 0 and 36 hours of light (blue and red bars, respectively). Significantly more Stat3-positive cells were observed following 36 hours of light compared to undamaged retinas ($p < 0.005$, $n = 10$). B. The number of Ascl1a-positive cells and EGFP-positive Müller glia in Tg(*gfap:EGFP*) retinas (Fig. 3) were quantified at 0 and 36 hours of light (blue and red bars, respectively). Significantly more Ascl1a-positive cells were observed following 36 hours of light compared to undamaged retinas ($p < 0.01$, $n = 10$). However, approximately 60% of the EGFP-positive Müller glia expressed Ascl1a.

Figure 3.

Ascl1a is expressed in a subset of Müller glia following 36 hours of constant light treatment. Dark-adapted adult *albino* *Tg(gfap:EGFP)* transgenic fish were exposed to constant intense light for either 0 (A-C) or 36 (D-F) hours. At 0 hours, EGFP-positive Müller glia (B and C, green) did not express any detectable levels of Ascl1a (A and C, magenta; arrowheads). Following 36 hours of constant intense light, a subset of the EGFP-positive Müller glia (green) expressed Ascl1a (D-F, magenta; arrows) and the remaining Müller glia were Ascl1a-negative (D-F; arrowheads). The area in the dashed box is magnified 2-fold in the inset. INL, inner nuclear layer; ONL, outer nuclear layer. Panel A scale bar is 50 μm and is the same for panels B-F.

Figure 4.

Ascl1a is expressed in the proliferating subset of Stat3-positive Müller glia following 36 hours of constant light treatment. Retinas of dark-adapted adult *albino* fish that were light-damaged for 36 hours were immunolabeled for PCNA (B, C, E, F, magenta) and either Stat3 (A and C, green) or Ascl1a (D and F, green). One subset of Stat3-positive Müller glia expressed PCNA (A-C, arrowheads) and the remainder did not (A-C, arrows). In contrast, all of the Ascl1a-positive Müller glia expressed PCNA (D-F, arrowheads). The area in the dashed box is magnified 2-fold in the inset. INL, inner nuclear layer; ONL, outer nuclear layer. Panel A scale bar is 50 μ m and is the same for panels B-F.

Figure 5.

Temporal qRT-PCR gene expression profiles of *stat3* and *ascl1a*. Dark-adapted adult *albino* fish were exposed to constant intense light for 0, 5, 10, 15, and 20 hours and then sacrificed. Total RNA was isolated from the dorsal retinas and converted to cDNA for quantitative real-time PCR (qRT-PCR). The log₂ of the mean expression for *stat3* (squares and solid line; n = 3) and *ascl1a* (diamonds and dashed line; n = 3) is plotted for the qRT-PCR. The *stat3* expression is significantly increased at 5 (p < 0.01), 10 (p < 0.05), and 15 (p < 0.05) hours of light relative to the undamaged control retina. The *ascl1a* expression is significantly increased at only 10 (p < 0.05) hours of light relative to the undamaged control retina.

Figure 6.

Effective and specific morpholino-mediated knockdown of Stat3 and Ascl1a protein expression. Dark-adapted adult *albino* zebrafish were either uninjected (A, D) or intravitreally injected and electroporated with the Standard Control morpholino (B, E), *stat3* morpholino (C), or the *ascl1a* morpholino (F) and then exposed to constant intense light for 36 hours. The retinas were then immunolabeled with either anti-Stat3 (A-C) or anti-Ascl1 (D-F). Stat3 expression was observed in GCL and INL cells in both control retinas (A and B), but not in *stat3* morphant retinas (C). Ascl1a expression was observed in INL cells of both control retinas (D and E), but not in *ascl1a* morphant retinas (F). Ascl1-expressing cells were detected in the vitreous of the *ascl1a* morphant (F, bracket), which possibly corresponded to cells that invaded the vitreous that are either Ascl1a- or Ascl1b-expressing. Uninjected fish (UI), standard control morphants (S.C. MO) or *stat3* morphants (*stat3* MO) were light-damaged for 36 hours and then analyzed for Stat3 expression by immunoblots, using Actin as a loading control (G, top and lower bands, respectively). Stat3 protein expression was diminished in *stat3* morphants compared to both controls (G). Zebrafish embryos were either uninjected (UI) or injected with either standard control morpholinos (S.C. MO) or *ascl1a* morpholinos (*ascl1a* MO) at the 1-4 cell stage and then analyzed 44 hours later for Ascl1a expression, using Actin as a loading control (H, top and lower bands, respectively). Ascl1a protein expression was reduced in the *ascl1a* morphant relative to both controls (H). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; Vit, vitreous. Panel A scale bar is 50 μ m and is the same for panels B-E. Panel F scale bar is 50 μ m.

Figure 7.

Knockdown of Stat3, Ascl1a and Lin28a does not affect cell death in the light-damaged retina. Dark-adapted adult *albino* zebrafish retinas were either uninjected (A) or injected and electroporated with the Standard Control morpholino (B, S.C. MO), *stat3* morpholino (C), the *ascl1a* morpholino (D), or the *lin28a* morpholino (E) and then exposed to constant intense light for 16 hours. Retinal cryosections were then labeled for cell death using TUNEL (A-E, green) and stained with TO-PRO-3 to identify the nuclei (A-E, blue). GCL, ganglion cell layer; INIL, inner nuclear layer; ONL, outer nuclear layer; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Panel A scale bar is 50 μ m and is the same for panels B-E.

Figure 8.

Ascl1a and Lin28a regulate Stat3 expression that identifies Stat3-dependent and Stat3-independent groups of proliferating Müller glia. Dark-adapted adult *albino* zebrafish retinas were either uninjected (A and B) or injected and electroporated with either the Standard Control morpholino (C and D, S.C. MO), *stat3* morpholino (E and F), the *asc11a* morpholino (G and H), or the *lin28a* morpholino (I and J) and then exposed to constant intense light for 36 hours. Retinal cryosections were then immunolabeled for both Stat3 and PCNA (A, C, E, G, and I, green and magenta, respectively) or only Stat3 (B, D, F, H, and J) and cells were quantified across a 350 μ m region of the central-dorsal retina. Both controls contained a subset of INL cells that coexpressed Stat3 and PCNA (A-D, arrows), while the remaining Stat3-positive INL cells did not express PCNA (arrowheads). Stat3-positive INL cells were nearly absent in all three morphants (E-J, green). Several Stat3-negative and PCNA-positive cells were present in the *stat3* morphant (E and F, open arrowheads). The number of Stat3-positive cells, PCNA-positive cells, Stat3- and PCNA-positive cells, Stat3-negative and PCNA-positive cells and Stat3-positive and PCNA-negative cells were quantified for the uninjected, standard control morphant, *stat3* morphant, *asc11a* morphant, and *lin28a* morphant retinas (K; blue bars, red bars, green bars, purple bars, and yellow bars, respectively). The Student's t-test was used to statistically analyze the values for the *stat3* morphant, *asc11a* morphant, and *lin28a* morphant retinas relative to the standard control morphant ($n = 10$; *, $p < 0.05$; ***, $p < 0.001$). The area in the dashed box is magnified in the inset. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Panel A scale bar is 50 μ m and is the same for panels B-J.

Figure 9.

Ascl1a is downstream of Lin28a and required for Müller glial proliferation. Dark-adapted adult *albino* zebrafish retinas were either uninjected (A and B) or injected and electroporated with either the Standard Control morpholino (C and D, S.C. MO), *stat3* morpholino (E and F), the *ascl1a* morpholino (G and H), or the *lin28a* morpholino (I and J) and then exposed to constant intense light for 36 hours. Retinal cryosections were then immunolabeled for both Ascl1a and PCNA (A, C, E, G, and I, green and magenta, respectively) or only Ascl1a (B, D, F, H, and J, green) and cells were quantified across a 350 μ m region of the central-dorsal retina. All PCNA-positive cells in both controls (A and C; magenta) co-expressed Ascl1a (A-D; green, arrows). The *stat3* morphant retinas displayed fewer PCNA-positive INL cells relative to control retinas (E; magenta, arrows) and nearly all colabeled for Ascl1a (E and F; green). Very few PCNA-positive and Ascl1a-expressing INL cells were observed in the *ascl1a* morphant (G and H) and *lin28a* morphant (I and J) retinas. The number of Ascl1a-positive cells, PCNA-positive cells, Ascl1a- and PCNA-positive cells, Stat3-negative and Ascl1a-positive cells and Ascl1a-positive and PCNA-negative cells were quantified for the uninjected, standard control morphant, *stat3* morphant, *ascl1a* morphant, and *lin28a* morphant retinas (K; blue bars, red bars, green bars, purple bars, and yellow bars, respectively). The Student's t-test was used to statistically analyze the values for the *stat3* morphant, *ascl1a* morphant, and *lin28a* morphant retinas relative to the standard control morphant ($n = 10$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). The area in the dashed box is magnified in the inset. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Panel A scale bar is 50 μ m and is the same for panels B-J.

Figure 10.

Stat3, Ascl1a and Lin28a are required for optimal Müller glial proliferation in the ouabain-damaged retina. Adult zebrafish retinas were either uninjected (A) or injected and electroporated with the Standard Control morpholino (B, S.C. MO), *stat3* morpholino (C), the *ascl1a* morpholino (D), or the *lin28a* morpholino (E) and then injected to a final vitreal concentration of 2 μ M. After 3 days, the fish were sacrificed and retinal cryosections were immunolabeled for PCNA and stained with TO-PRO-3 to identify the nuclear layers (A-E, green and blue, respectively). Both control groups possessed more PCNA-positive cells in the INL than the three experimental morphants. INL, inner nuclear layer; ONL, outer nuclear layer. Panel A scale bar is 50 μ m and is the same for panels B-E.

Figure 11.

Quantification of the number of PCNA-positive Müller glia in the light-damaged retina. The number of PCNA-labeled cells were quantified for the uninjected, standard control morphant, *stat3* morphant, *ascl1a* morphant, and *lin28a* morphant retinas at three days after ouabain injection (blue bars, red bars, green bars, purple bars, and yellow bars, respectively). The Student's t-test was used to statistically analyze the values for the *stat3* morphant, *ascl1a* morphant, and *lin28a* morphant retinas relative to the standard control morphant (n=10; *, $p < 0.05$; **, $p < 0.01$).

Figure 12.

Model of Stat3, Ascl1a, and Lin28a in inducing maximal Müller glial proliferation in the light-damaged zebrafish retina. In the light-damaged zebrafish retina, there are three groups of Müller glia (Primary and Secondary Proliferating Müller glia and Quiescent Müller glia). Cell death produces a damage signal to the Primary Proliferating Müller glia (PPMg) and possibly the other two Müller glia groups. This signal activates Lin28a, which activates Ascl1a and induces the PPMg to reenter the cell cycle (in a Stat3-independent manner). Ascl1a then activates Stat3 expression in the PPMg, which is necessary to generate a signal to the Secondary Proliferating Müller glia (SPMg). This signal to the SPMg, which may be HB-EGF (Ramachandran et al., 2012), activates the Lin28a pathway again to drive the SPMg to reenter the cell cycle. Thus, the SPMg proliferation is dependent on Stat3 expression in the PPMg. The Quiescent Müller glia (QMg) express Stat3, but not Ascl1a and do not proliferate. It is unclear if the damage signal induces Stat3 expression in the QMg or if another mechanism induces the Stat3 expression.

Table 1
Antibodies used in this study

Antibody	Immunogen	Manufacturer, Catalog number, Species raised, and Purification method
Actin	Synthetic Peptide: Conserved C-terminal pan-actin epitope fused to MAP backbone (Immunogen sequence: SGPSIVHRKCF)	Sigma, # A3853, Mouse monoclonal (Clone AC40), Affinity Purified IgG2a
Ascll	Synthetic Peptide: Recombinant peptide corresponding to residues 156-238 of human ASCL1 (Immunogen sequence: MSKVETLRSAVEYIRALQQLDEHDAVSA AFQAGVLSPTISPNYSNDLNSMAGSPVSS YSSDEGSYDPLSPREEQLLDFTNWF)	Sigma, # HPA029217, Rabbit polyclonal, Affinity Purified
GFP	GFP isolated directly from jellyfish <i>Aequorea victoria</i>	Invitrogen, # A11120, Mouse monoclonal (Clone 3E6), G-protein Affinity Purified IgG2a
PCNA	Recombinant Fusion Protein: Full length Rat-PCNA fused to Protein A	Sigma, # P8825, Mouse monoclonal (Clone PC10), Ascites Fluid
Stat3	Synthetic Peptide: S-tagged recombinant peptide corresponding to residues 689-806 of zebrafish Stat3 (Immunogen sequence: RPEAHPDTEFPDTGCVTPYLYKTKFICV TPCPSVFMDFPDSELLGNGFPGTNSGNT SDLFPMSPRTLDSLHNEAAEANPGPLE SLTLDMESSDHASPMREGFAASTVSDM DTCRNA)	Hydrex lab, Rabbit polyclonal, Immunopurified using recombinant Stat3 peptide

Table 2

Quantification of Stat3 and PCNA expressing cells

Treatment	Avg # Cells per 350 μm $\pm$ SEM					p-value vs SC MO				
	PCNA+	Stat3+	PCNA+ Stat3+	PCNA only	Stat3 only	PCNA+	Stat3+	PCNA+ Stat3+	PCNA only	Stat3 only
Uninjected	43.9 $\pm$ 5.3	60.8 $\pm$ 2.7	41.1 $\pm$ 5.2	2.8 $\pm$ 0.8	19.7 $\pm$ 3.1					
SC MO	45.7 $\pm$ 3.3	56.3 $\pm$ 1.5	39.2 $\pm$ 3.9	5.0 $\pm$ 1.0	17.1 $\pm$ 4.2					
<i>stat3</i> MO	21.8 $\pm$ 2.5	9.8 $\pm$ 1.6	6.6 $\pm$ 1.6	15.2 $\pm$ 1.3	3.2 $\pm$ 1.2	1.7 $\times 10^{-5}$	3.4 $\times 10^{-2}$	3.6 $\times 10^{-7}$	7.9 $\times 10^{-6}$	5.0 $\times 10^{-3}$
<i>asc/la</i> MO	8.4 $\pm$ 2.2	12.1 $\pm$ 1.6	4.8 $\pm$ 1.8	3.6 $\pm$ 1.0	7.2 $\pm$ 1.7	2.3 $\times 10^{-8}$	3.5 $\times 10^{-16}$	2.0 $\times 10^{-7}$	0.33	4.2 $\times 10^{-2}$
<i>lin28</i> MO	12.4 $\pm$ 1.5	10.0 $\pm$ 0.9	5.8 $\pm$ 1.1	5.6 $\pm$ 2.5	4.2 $\pm$ 0.7	2.4 $\times 10^{-8}$	1.1 $\times 10^{-15}$	1.1 $\times 10^{-7}$	0.78	6.7 $\times 10^{-3}$

Column 1 shows the type of treatment that was performed on the retinas. Columns 2-6 show the average number of cells that were quantified for each group and the standard error of the mean (SEM). Columns 7-11 show the results of the two-tailed Student's t-test of each value against the corresponding value in the Standard Control morphant (SC MO).

Quantification of Ascl1a and PCNA expressing cells

Treatment	Avg # Cells per 350 μ m $\pm$ SEM					p-value vs SC MO				
	PCNA+	AsclII +	PCNA+ AsclII +	PCNA only	AsclII only	PCNA+	AsclII +	PCNA+ AsclII +	PCNA only	AsclII only
Uninjected	47.1 $\pm$ 3.7	45.6 $\pm$ 4.1	44.6 $\pm$ 3.9	2.5 $\pm$ 0.7	1.0 $\pm$ 0.2	0.77	0.91	0.75	0.90	0.14
SC MO	45.5 $\pm$ 3.9	45.0 $\pm$ 3.6	42.9 $\pm$ 3.8	2.6 $\pm$ 0.7	2.1 $\pm$ 0.6					
<i>stat3</i> MO	31.9 $\pm$ 3.8	33.1 $\pm$ 3.7	31.1 $\pm$ 3.9	1.4 $\pm$ 0.7	2.4 $\pm$ 0.6	2.5$\times 10^{-2}$	3.7$\times 10^{-2}$	2.9$\times 10^{-2}$	0.24	0.10
<i>asc/lla</i> MO	7.4 $\pm$ 1.4	6.0 $\pm$ 1.5	5.0 $\pm$ 1.5	2.4 $\pm$ 0.5	1.0 $\pm$ 0.4	2.3$\times 10^{-7}$	9.1$\times 10^{-8}$	2.0$\times 10^{-7}$	0.78	0.13
<i>lin28</i> MO	12.5 $\pm$ 1.3	12.3 $\pm$ 1.2	9.6 $\pm$ 2.0	2.8 $\pm$ 1.1	2.6 $\pm$ 1.6	1.6$\times 10^{-6}$	7.9$\times 10^{-7}$	2.4$\times 10^{-6}$	0.90	0.64

Column 1 shows the type of treatment that was performed on the retinas. Columns 2-6 show the average number of cells that were quantified for each group and the standard error of the mean (SEM). Columns 7-11 show the results of the two-tailed Student's t-test of each value against the corresponding value in the Standard Control morphant (SC MO).