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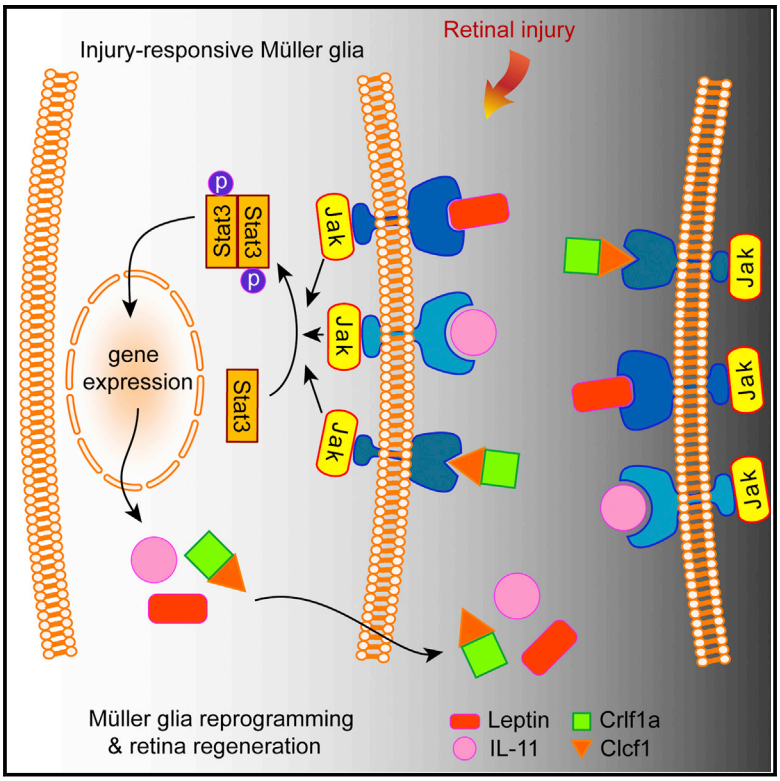
Leptin and IL-6 Family Cytokines Synergize to Stimulate Müller Glia Reprogramming and Retina Regeneration

Graphical Abstract



Authors

Xiao-Feng Zhao, Jin Wan, ..., Martin G. MyersJr., Daniel Goldman

Correspondence

neuroman@umich.edu

In Brief

In zebrafish, Müller glia respond to retinal injury by undergoing a reprogramming event that allows them to divide and generate progenitors for retinal repair. Zhao et al. now show that Jak/pStat3 signaling is necessary for progenitor formation. They report that pStat3 signaling is activated by cytokines expressed by injury-responsive Müller glia. They find that Leptin and IL-11 are induced in the injured retina, necessary for retina regeneration, and sufficient for stimulating Müller glia reprogramming in the uninjured retina.

Highlights

Jak/Stat signaling stimulates MG reprogramming in the injured retina

Jak/Stat signaling is synergistically activated by Leptin and IL-6-type cytokines

Cytokines act in an autocrine/paracrine fashion to stimulate MG reprogramming

Cytokines are sufficient to stimulate MG reprogramming and proliferation

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Leptin and IL-6 Family Cytokines Synergize to Stimulate Müller Glia Reprogramming and Retina Regeneration

Xiao-Feng Zhao,[1,2] Jin Wan,[1,2] Curtis Powell,[1,2] Rajesh Ramachandran,[1,2,4] Martin G. Myers, Jr.,[3] and Daniel Goldman[1,2,*]
[1]Molecular and Behavioral Neuroscience Institute
[2]Department of Biological Chemistry
[3]Molecular and Integrative Physiology
University of Michigan, Ann Arbor, MI 48109, USA
[4]Present address: Indian Institute of Science Education and Research, Mohali 140306, India
*Correspondence: neuroman@umich.edu
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SUMMARY

Unlike mammals, zebrafish can regenerate a damaged retina. This remarkable regenerative response is mediated by Müller glia (MG) that undergo a reprogramming event that drives their proliferation and the generation of multipotent progenitors for retinal repair. The mechanisms that drive MG reprogramming are poorly understood. Here, we report that Leptin and Gp130-coupled receptors, acting via a Jak/Stat signaling pathway, stimulate MG reprogramming and progenitor formation in the injured retina. Importantly, we find that *ascl1a* gene expression, which drives MG reprogramming in fish and mammals, is regulated in a Jak/Stat-dependent manner and requires consensus Stat-binding sites for injury-dependent activation. Finally, we identify cytokines that are induced by retinal injury and exhibit a remarkable synergy in their ability to activate Jak/Stat signaling and MG reprogramming in the uninjured retina. Our study not only furthers our understanding of retina regeneration in zebrafish but also suggests new strategies for awakening retina regeneration in mammals.

INTRODUCTION

Because of their robust regenerative powers, zebrafish have become an ideal model system for studying retina regeneration. Following retinal injury, Müller glia (MG) are reprogrammed and acquire progenitor characteristics that allow them to regenerate all major retinal cell types (Bernardos et al., 2007; Fausett and Goldman, 2006; Fimbel et al., 2007; Ramachandran et al., 2010a, 2010b). A key event in MG reprogramming is the activation of *ascl1a* gene expression (Fausett et al., 2008). This gene encodes a nodal transcription factor that impacts reprogramming genes and signaling cascades, affecting almost all aspects of retina regeneration (Lenkowski et al., 2013; Nelson et al., 2012, 2013; Powell et al., 2012; Ramachandran et al., 2010a, 2011, 2012; Wan et al., 2012). Importantly, ASCL1 also controls MG reprogramming in the postnatal mouse retina (Pollak et al., 2013).

The mechanisms by which injury signals are conveyed to the MG genome to activate reprogramming genes such as *ascl1a* are not well understood. However, one candidate is Stat3, a signal transducer and activator of transcription whose activity and nuclear localization is controlled by growth factors and cytokines (Cao et al., 1996; Grandis et al., 1998; Hirano et al., 2000; Levy and Darnell, 2002; Vogt and Hart, 2011). Importantly, Stat3 expression is induced in the injured retina (Kassen et al., 2007, 2009; Nelson et al., 2012). However, this expression is detected in all retinal layers, and in the inner nuclear layer (INL) both quiescent and proliferating MG express Stat3 (Kassen et al., 2007; Nelson et al., 2012). This expression pattern, along with the assumption that injury-induced Stat3 expression reflects that of activated p-Stat3 (Kassen et al., 2007), has led to models assigning different roles for injury-induced Stat3 in quiescent MG, MG stem cells, and MG-derived progenitors (Gorsuch and Hyde, 2013; Nelson et al., 2012, 2013). However, it remains unknown whether total Stat3 is a true indicator of p-Stat3 in the injured retina, and whether endogenous cytokines acting via Jak/Stat signaling stimulate MG reprogramming and retina regeneration following retinal injury.

Here, we report that unlike total Stat3 expression, activated p-Stat3 signaling is restricted to a subset of MG that reprogram and proliferate in response to retinal injury. We show that Jak/Stat signaling directly controls reprogramming genes such as *ascl1a* and identify endogenous cytokines that act via Leptin and Gp130-coupled receptors that stimulate Jak/Stat signaling and progenitor formation in the injured retina. Importantly, we show that Leptin and interleukin-6 (IL-6)-like cytokines are sufficient for stimulating MG proliferation in the uninjured retina and that they exhibit a remarkable synergy in their action. The local release of cytokines by injury-responsive MG and the synergistic action of these cytokines may be critical for their effectiveness in stimulating MG reprogramming and progenitor formation.

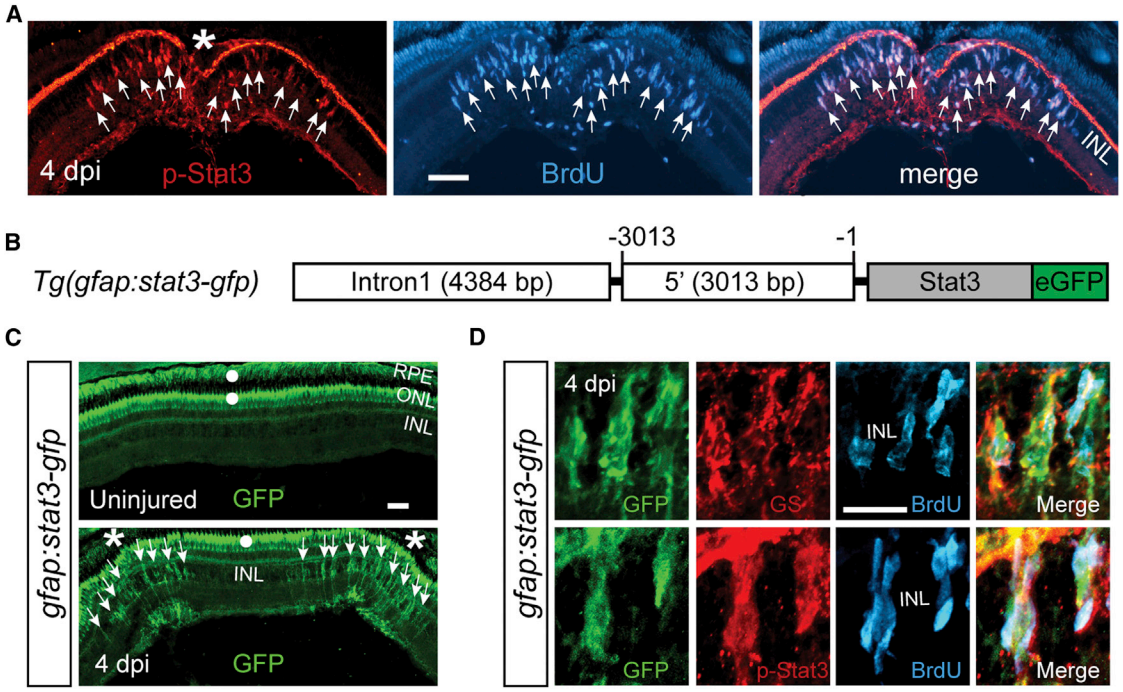

Figure 1. The Jak/Stat3 Signaling Pathway Is Activated following Retinal Injury

(A) Immunofluorescence on retinal sections shows activated p-Stat3 expression in $BrdU^+$ MG-derived progenitors that are localized to the injury site at 4 dpi.

(B) A schematic of the *gfap:stat3-gfp* transgene construct shows the fusion gene, *stat3-gfp*, under control of the *gfap* promoter regulatory elements.

(C) In *gfap:stat3-gfp* transgenic fish, Stat3-GFP fusion protein expression is undetectable in MG of the uninjured eye and is restricted to MG-derived progenitors at the injury site at 4 dpi. White dots indicate autofluorescence unique to the green channel (see Figure S2G).

(D) Confocal images show colocalization of Stat3-GFP with GS^+/p-$Stat3^+$/$BrdU^+$ MG-derived progenitors at 4 dpi.

In (A) and (C), the asterisk marks the injury site (needle poke) and arrows point to MG-derived progenitors. Scale bars, 50 μm (A and C) and 20 μm (D). INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; dpi, days postinjury. See also Figures S1 and S2.

RESULTS

Stat3 Signaling Is Restricted to MG Progenitors in the Injured Retina

In the injured retina, Stat3 activation is assumed to reflect total Stat3 expression (Kassen et al., 2007; Nelson et al., 2012). To test this idea, we took advantage of an antibody that specifically detects activated, phosphorylated Stat3 (p-Stat3) (Yamashita et al., 2002). Although p-Stat3 stained processes in the outer plexiform and ganglion cell layers in the uninjured retina, there was no labeling of cells in the INL where MG cell bodies reside (Figure S1A). However, following retinal injury with a needle poke, we observed p-Stat3 staining in the INL that was restricted to bromodeoxyuridine-positive ($BrdU^+$) cells at the injury site (Figures 1A and S1A). We previously demonstrated that essentially all of the proliferating cells in the INL following retinal injury are MG-derived progenitors (Fausett and Goldman, 2006). Furthermore, we show here that although microglia migrate to the injury site, they do not contribute to the proliferating cell population (Figure S1B). Importantly, we repeated these experiments using a light-damage model of photoreceptor death in which only about 50% of the MG reprogram and proliferate (Figures S1C and S1D; Nelson et al., 2012). In this model, both quiescent and proliferating MG show increased Stat3 expression, and it has been assumed that this reflects activated p-Stat3 expression (Kassen et al., 2007; Nelson et al., 2012); however, we found that p-Stat3 was restricted to the proliferating population of MG (Figure S1D), suggesting that Jak/Stat signaling is also restricted to these cells.

To further explore Stat3 activation in the injured retina, we created *gfap:stat3-gfp* transgenic fish, in which the MG-specific *gfap* promoter drives transgene expression (Figure 1B; Bernardos and Raymond, 2006). During development, the Stat3-GFP fusion protein was restricted to the nervous system (Figure S2A), and in the retina it was localized to glutamine synthetase-positive (GS^+) MG (Figure S2B). In the uninjured adult retina, we detected *stat3-gfp* mRNA (Figure S2C) that was localized to GS^+ MG cell bodies and processes in the INL (Figure S2D), but we were unable to detect any Stat3-GFP protein in this layer (Figure 1C, top panel). The green signal noted in the outer nuclear layer (ONL) and retinal pigment epithelium (RPE) (white dots in Figure 1C) is autofluorescence that is unique to the green channel (see Figure S2G). Interestingly, *Stat3* mRNA has been reported in neuronal processes where, in response to injury, it is locally translated (Ben-Yaakov et al., 2012). Whether it serves a similar function in MG remains unknown. The detection of *stat3-gfp* mRNA, but not protein, indicates that the Stat3 may be unstable in the uninjured retina. Remarkably, following retinal injury with a

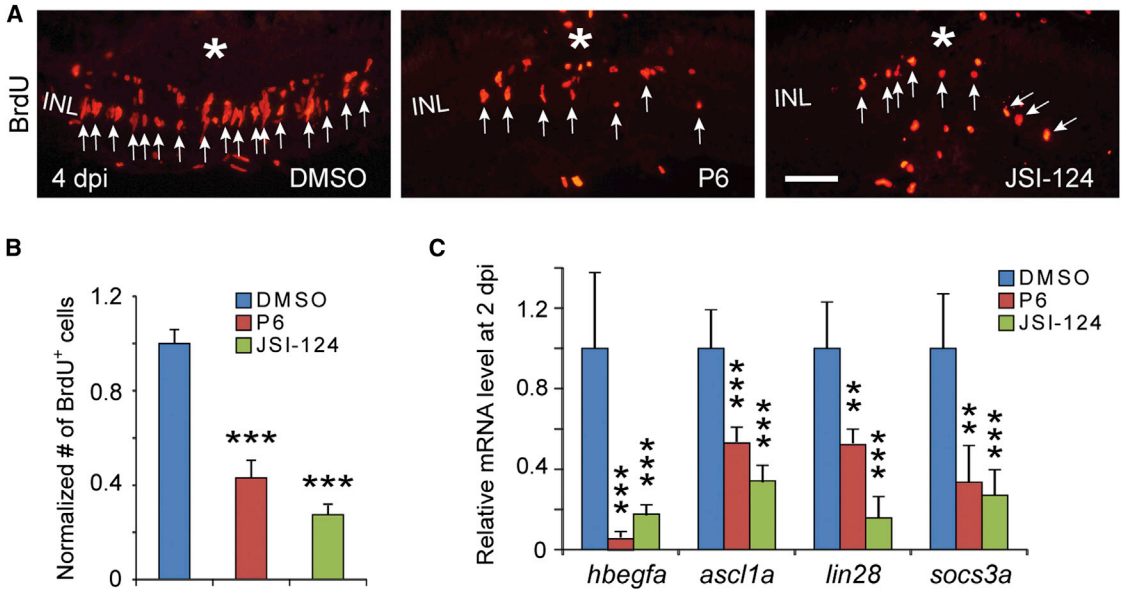

Figure 2. The Jak/Stat3 Signaling Pathway Is Necessary for Retina Regeneration

(A) BrdU immunofluorescence shows that the Jak inhibitors P6 and JSI-124 suppress progenitor formation at 4 dpi. The asterisk marks the injury site (needle poke) and arrows point to MG-derived progenitors.

(B) Quantification of BrdU$^+$ progenitors in (A). ***$p < 0.001$, $n = 4$.

(C) qPCR shows that the Jak inhibitors P6 and JSI-124 inhibit reprogramming gene induction at 2 dpi; **$p < 0.01$, ***$p < 0.001$; $n = 4$, 4, and 5 for DMSO, P6, and JSI-124, respectively.

Error bars, SD. Scale bars, 20 μm (A). See also Figure S3.

needle poke, *stat3-gfp* mRNA levels remained unchanged (Figure S2C), but Stat3-GFP protein increased locally at the injury site in cells exhibiting a typical MG morphology (Figure 1C, bottom panel). Importantly, Stat3-GFP specifically accumulated in BrdU$^+$ and p-Stat3$^+$ MG-derived progenitors localized to the injury site (Figures 1D, S2E, and S2F).

The above data suggested that Jak/Stat3 signaling regulates progenitor formation in the injured retina. Indeed, the Jak/Stat signaling inhibitors JSI-124 and P6 (Blaskovich et al., 2003; Pedranzini et al., 2006) suppressed the generation of MG-derived progenitors (Figures 2A and 2B). Furthermore, by inhibiting Jak/Stat from either 0–2 days postinjury (dpi) or 2–4 dpi, we found that Jak/Stat signaling impacts both the formation of progenitors, which is just beginning at 2 dpi (Fausett and Goldman, 2006), and their later expansion (Figures S3A–S3D). Importantly, Jak inhibitors did not stimulate apoptosis (Figures S3E and S3F).

We next investigated whether Jak/Stat signaling was necessary for injury-dependent Stat3-GFP stabilization. For this analysis, we treated *gfap:stat3-gfp* transgenic fish with JSI-124 and assayed GFP immunofluorescence in the injured retina. Consistent with the idea that Stat3-GFP stabilization reflects activated p-Stat3 expression, JSI-124 treatment dramatically reduced Stat3-GFP expression in the injured retina (Figures S3G and S3H). Whether Jak-mediated stabilization is a direct consequence of Stat3 phosphorylation or phosphorylation of other proteins remains unknown.

Finally, because *socs3* gene expression is often used as a sensitive readout of Stat3 activation (Liang et al., 2012), we characterized its expression in the injured retina and observed injury-dependent *socs3a* and *socs3b* induction within 3 hr postinjury (3 hpi; Figure S3I). This induction occurred before the first detection of p-Stat3 at 2 dpi (Figure S1A) and likely reflects the increased sensitivity of PCR compared with immunofluorescence. Regardless, our data suggest that Stat signaling is activated specifically in MG at the injury site.

Jak/Stat Signaling Regulates MG Reprogramming

The above data suggest that Jak/Stat signaling regulates the generation of MG-derived progenitors. However, prior to progenitor formation, MG reprogram their genome to acquire properties of a retinal stem cell (Fausett et al., 2008; Kassen et al., 2007; Nagashima et al., 2013; Ramachandran et al., 2010a, 2011, 2012). This reprogramming is characterized by the very rapid activation of genes such as *hbegfa*, *ascl1a*, *lin28*, *stat3*, and *socs3a* (Figure S3G; Fausett et al., 2008; Kassen et al., 2007; Ramachandran et al., 2010a; Wan et al., 2012). To investigate whether Jak/Stat signaling also regulated the expression of reprogramming genes, we injured retinas with and without Jak/Stat inhibition and assayed reprogramming gene expression 2 days later, when reprogrammed MG are mostly quiescent (Fausett and Goldman, 2006). These experiments showed that injury-dependent induction of reprogramming genes was suppressed by Jak/Stat inhibitors (Figure 2C).

To determine whether Jak/Stat signaling directly impinged on reprogramming genes, we focused on the *ascl1a* promoter, since Ascl1a drives MG reprogramming in both zebrafish and mice (Fausett et al., 2008; Pollak et al., 2013; Ramachandran et al., 2010a, 2011). For this analysis, we took advantage of

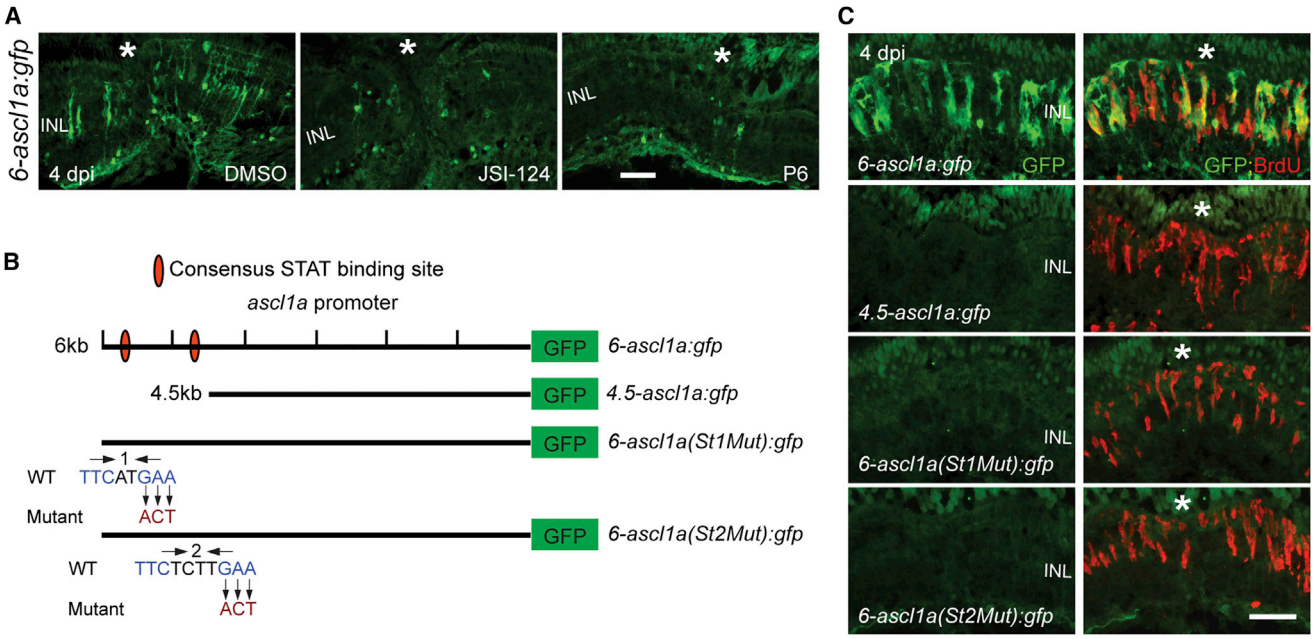

Figure 3. Jak/Stat3 Signaling Mediates Injury-Dependent Induction of the Reprogramming Gene *ascl1a*

(A) GFP immunofluorescence in *6-ascl1a:gfp* fish shows that the Jak inhibitors P6 and JSI-124, applied at the time of retinal injury, inhibit injury-dependent transgene induction.

(B) Diagram of the *ascl1a* promoter constructs used to generate transgenic lines.

(C) GFP immunofluorescence shows that a distal 1.5 kb fragment of the *ascl1a* promoter is required for injury-dependent transgene expression and that both consensus Stat3 sites located in this promoter fragment are necessary for this expression. $BrdU^+$ cells indicate the injury site and a normal regenerative response. The asterisk marks the injury site (needle poke). Scale bar, 50 μm.

See also Figure S4.

6-ascl1a:gfp fish, which harbor a 6 kb *ascl1a* promoter fragment that restricts GFP expression to reprogrammed MG and MG-derived progenitors (Wan et al., 2012). Jak/Stat inhibitors suppressed injury-dependent GFP expression in these fish (Figure 3A). A variety of 5′ promoter deletions allowed us to narrow in on a distal 1.5 kb promoter fragment that was necessary for injury-dependent *ascl1a* promoter activation (Figures 3B, 3C, S4A, and S4B). Within this fragment, we identified two consensus Stat3-binding sites (Ehret et al., 2001) whose mutation prevented injury-dependent *ascl1a* promoter activation (Figures 3B, 3C, S4C, and S4D). BrdU labeling indicated a normal injury response in these fish (Figures 3C, S4C, and S4D). Thus, Jak/Stat signaling stimulates MG reprogramming by activating genes necessary for this process.

IL-6 Family Cytokines Drive MG Progenitor Formation

Because cytokines are often increased in response to tissue damage and stimulate Jak/Stat signaling, we were interested in identifying those that may activate Jak/Stat signaling following retinal injury. A clue to their nature comes from the observation that ciliary neurotrophic factor (CNTF) can stimulate a small amount of MG proliferation in the uninjured retina (Faillace et al., 2002; Kassen et al., 2009). However, a *cntf* gene remains unidentified in the zebrafish genome, making Cntf an unlikely candidate for mediating injury-dependent MG reprogramming and retina regeneration.

CNTF is a member of a family of IL-6-like cytokines whose receptors share the common signaling subunit Gp130 (Hirano et al., 2000). Consistent with a role for Gp130-coupled receptors in regulating MG reprogramming, we detected a low basal expression of *gp130* mRNA in the uninjured retina that was increased in MG-derived progenitors following injury (Figures 4A, 4B, and S5A). Interestingly, a variety of IL-6 family member genes are induced in MG-derived progenitors shortly after injury, including *m17* (also referred to as *lif*), *clcf1*, *crlf1a*, *il-11a*, *il-11b*, and their related receptors (Figures 4A, 4B, S5A, and S5B). This local expression in MG-derived progenitors may indicate that IL-6 family members act in both an autocrine and paracrine fashion to stimulate MG reprogramming and progenitor formation.

To directly test whether IL-6 family cytokines regulated injury-dependent MG reprogramming, we knocked down their common signaling component (Gp130) using a lissamine-tagged morpholino-modified antisense oligonucleotide (MO) whose effectiveness was confirmed in zebrafish embryos overexpressing a Gp130-GFP fusion protein (Figures S5C–S5E). In the adult retina, Gp130 knockdown reduced the generation of $BrdU^+$ progenitors assayed at 4 dpi (Figures 4C and 4D) and inhibited injury-dependent induction of reprogramming genes *hbegfa*, *ascl1a*, *lin28*, and *socs3a* assayed at 2 dpi (Figure 4E), a time when most reprogrammed MG have not yet begun to divide (Fausett and Goldman, 2006). Together, these data suggest

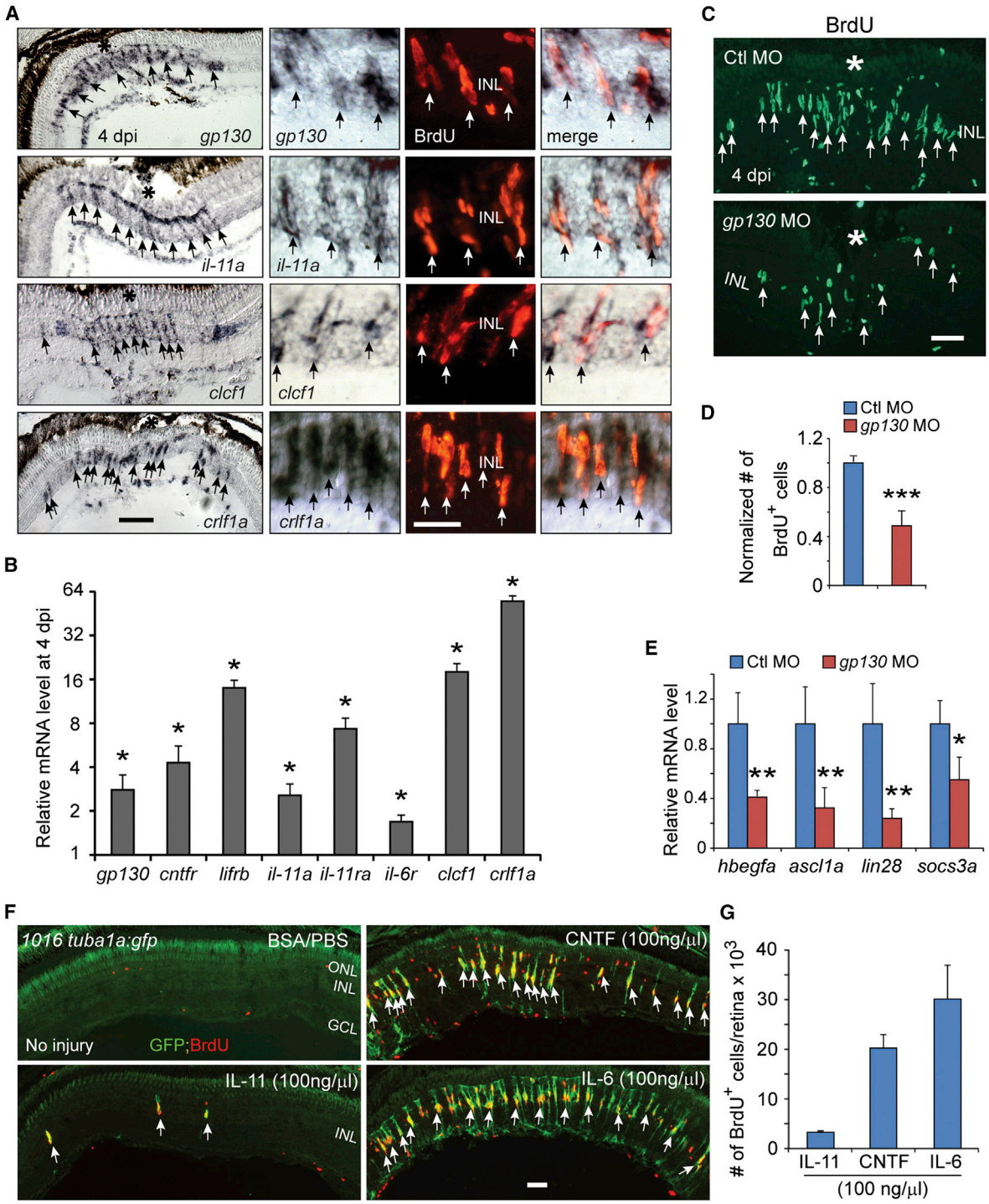

Figure 4. IL-6 Family Cytokines Signaling through Gp130 Are Necessary and Sufficient for Retina Regeneration

(A) In situ hybridization and immunofluorescence show that *gp130*, *il-11a*, *crlf1a*, and *clcf1* are expressed in $BrdU^+$ MG-derived progenitors localized to the injury site.

(B) qPCR quantifies *il-6* family gene induction in MG-derived progenitors (FACS purified from *1016tuba1a:gfp* fish retinas at 4 dpi) relative to MG from uninjured retina (FACS purified from uninjured *gfap:gfp* fish retinas). *$p < 0.05$, $n = 3$.

(C and D) Gp130 knockdown inhibits the generation of $BrdU^+$ MG-derived progenitors at 4 dpi. Control (Ctl) or *gp130*-targeting MOs were electroporated into the retina at the time of injury and the fish received an i.p. injection of BrdU 3 hr before sacrifice on 4 dpi. ***$p < 0.001$, $n = 4$.

(E) qPCR showing Gp130 knockdown inhibits injury-dependent induction of reprogramming genes at 2 dpi. *$p < 0.05$, **$p < 0.01$, $n = 4$.

(legend continued on next page)

that IL-6 family cytokines are increased in the injured retina and may contribute to MG reprogramming and proliferation.

We next investigated whether IL-6 family cytokines were able to stimulate MG reprogramming in the uninjured retina. For this analysis, recombinant mammalian IL-6, IL-11, or CNTF was intravitreally injected once daily for 3 days into the uninjured eye of *1016 tuba1a:gfp* transgenic fish. On the 4th day, fish received an i.p. injection of BrdU 3 hr before sacrifice. In these fish, GFP reports MG reprogramming and BrdU reports MG proliferation (Fausett and Goldman, 2006; Fausett et al., 2008; Ramachandran et al., 2010a, 2011, 2012; Wan et al., 2012). Using this strategy, we previously showed that heparin-binding EGF-like growth factor (HB-EGF) can stimulate MG reprogramming and proliferation in the uninjured retina (Wan et al., 2012). However, Nelson et al. (2013) raised concerns that using a needle to puncture the cornea and injecting a 2 μl volume might cause retinal injury and result in an MG response independent of HB-EGF. Therefore, we felt it was important to address these concerns before testing the cytokines described above.

Nelson et al. (2013) suggested that gaining access to the intravitreal space by cutting the cornea with a sapphire blade would cause less trauma than a needle puncture, and that delivery of fluid volumes below 2 μl would also result in less intraocular pressure, so we compared these variables. When we made an incision with a sapphire blade, we delivered HB-EGF or vehicle with a blunt 33-gauge needle as previously suggested (Nelson et al., 2013). We found that delivery of 0.5–2 μl volumes of HB-EGF, regardless of the delivery method used, stimulated MG proliferation to a similar extent (Figures S6A–S6D). Importantly, intravitreal injection of vehicle caused no MG proliferation. Because the vehicle control (Figure S6C) and certain cytokines (see below) did not stimulate MG proliferation in our study, any noted effects on MG proliferation are significant. However, the observation that HB-EGF did not stimulate MG proliferation in the study by Nelson et al., (2013) is puzzling. Perhaps the location of HB-EGF delivery influenced the results. To test this possibility, we compared responses to HB-EGF delivery above and below the lens. Interestingly, only when HB-EGF was delivered below the lens did we observe a robust proliferative response (Figures S6E and S6F). These studies demonstrate that injecting drugs beneath the lens using either a sapphire blade or a beveled needle to gain access to the intravitreal space, along with injection volumes ranging from 0.5 to 2 μl, is appropriate for investigating the effects of substances on MG proliferation.

We next investigated whether IL-6 family members that are induced during retina regeneration are sufficient to stimulate MG proliferation in the uninjured retina. For these studies, we used CNTF as a positive control, since it is known to stimulate a small amount of MG proliferation (Faillace et al., 2002; Kassen et al., 2009). Interestingly, both CNTF and IL-6 stimulated MG proliferation, whereas IL-11 was barely effective (Figures 4F and 4G). The green fluorescence noted above the ONL in the top left-hand panel of Figure 4F is autofluorescence that is unique to the green channel (Figure S7K). Notable in the CNTF-treated retina were $BrdU^+/GFP^-$ cells that, based on their location in the ONL, likely represent proliferating rod progenitors (Stenkamp, 2011).

Although CNTF and IL-6 stimulated MG proliferation in the uninjured retina, a zebrafish *cntf* gene remains unidentified and *il-6* mRNA was undetectable in the injured retina (Figure S5A). However, MG and MG-derived progenitors do express RNAs encoding receptors for these IL-6 family cytokines (Figures 4B, S5A, and S5B). Furthermore, genes encoding the alternative Cntf receptor ligand, Clcf1/Crlf1a (Elson et al., 2000), are highly induced in the injured retina (Figures 4A, 4B, and S5A). Therefore, Clcf1/Crlf1a may be responsible for Cntf receptor activation in the injured retina. Although *il-6* mRNA was not detected in the injured retina, it is possible that IL-6 is present in the blood and released from damaged vessels following retinal puncture. This IL-6 may then act via MG-resident IL-6 receptors to stimulate MG reprogramming and proliferation. However, this possibility remains untested. The stimulation of MG proliferation by CNTF and IL-6 in the uninjured retina is consistent with the idea that MG are capable of responding to injury signals mediated by IL-6 family members.

Leptin Signaling Stimulates MG Reprogramming and Proliferation

Although IL-11 had little effect on MG proliferation in the uninjured retina (Figures 4F and 4G), we were intrigued by the robust induction of *il-11* mRNA following retinal injury (Figures 4A, 4B, and S5A) and wondered whether it may synergize with other cytokines. Because Gp130 knockdown incompletely suppressed MG reprogramming in the injured retina (Figures 4C–4E), we suspected that IL-11 may synergize with cytokines acting independently of Gp130. A search for cytokines that met this criterion identified the mammalian *Leptin* homologs *lepa* and *lepb*, which are increased within 1 hpi and restricted to MG-derived progenitors at 4 dpi (Figures 5A, 5B, and S7A). Although *lepa* and *lepb* mRNAs are rapidly induced following injury, they only begin to return to preinjury levels around 8 dpi. It is not clear why these mRNAs persist for so long or even whether the protein is still being expressed; however, this expression may reflect an incompletely recovered retina.

Importantly, leptin receptor (*lepr*) RNA is detected in MG-derived progenitors (Figures 5B and S7A), and Lepr knockdown using a previously verified *lepr*-targeting MO (Liu et al., 2012) decreased progenitor formation and suppressed the injury-dependent induction of reprogramming genes (Figures 5C–5E). Surprisingly, the effects of Gp130 and Lepr knockdown were not additive (Figures S7B–S7D) and this was also reflected in

(F) Intravitreal injection of recombinant mammalian IL-6-like cytokines into the uninjured eye of *1016tuba1a:gfp* fish stimulates GFP expression and BrdU incorporation in MG throughout the retina's INL. Intravitreal injection of PBS/BSA did not stimulate GFP expression or BrdU incorporation. The green fluorescence above the ONL in the top left-hand panel is autofluorescence unique to the green channel (see Figure S7K).

(G) Quantification of $BrdU^+$ cells following intravitreal injection of recombinant mammalian IL-6-like cytokines (n = 3).

Error bars, SD. In (A) and (C), the asterisks mark the injury site (needle poke). In (A), (C), and (F), arrows point to MG-derived progenitors. Scale bars, 20 μm (A and C) and 50 μm (F). GCL, ganglion cell layer. Primers are listed in Table S1. See also Figures S5 and S6.

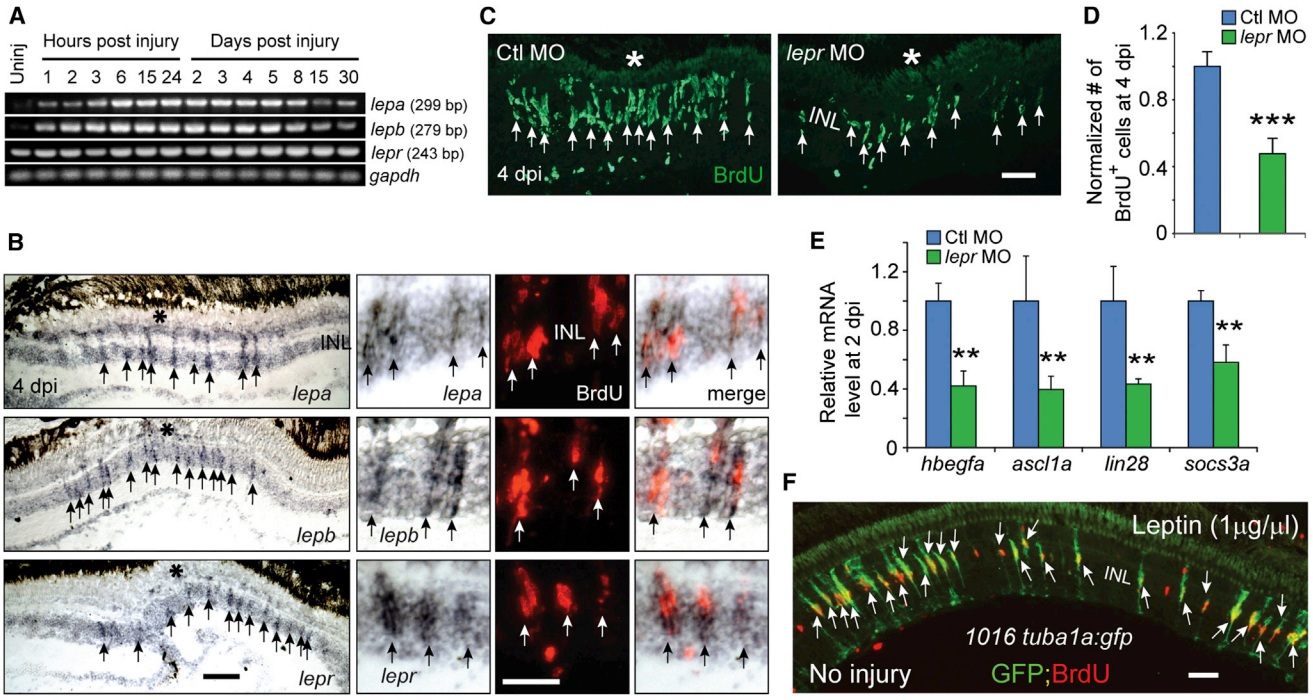

Figure 5. Leptin Signaling Is Necessary and Sufficient for Retina Regeneration

(A) RT-PCR analysis of mRNAs (from whole retina) encoding Leptin and Lepr at various times after retinal injury.

(B) In situ hybridization and BrdU immunofluorescence shows that *lepa*, *lepb*, and *lepr* RNAs are increased in BrdU$^+$ MG-derived progenitors at the injury site. The asterisk marks the injury site (needle poke).

(C and D) MO-mediated knockdown of Lepr inhibits the generation of BrdU$^+$ MG-derived progenitors at 4 dpi. ***$p < 0.001$, n = 4.

(E) qPCR shows that Lepr knockdown suppresses injury-dependent induction of reprogramming genes at 2 dpi. **$p < 0.01$, n = 4.

(F) Intravitreal injection of recombinant human Leptin into the uninjured eye of *1016tuba1a:gfp* fish stimulates GFP expression and BrdU incorporation in MG throughout the retina's INL.

Error bars, SD. In (B) and (C), arrows point to MG-derived progenitors. Scale bars, 20 μm (B and C) and 50 μm (E). Primers are listed in Table S1. See also Figure S7.

Stat3-GFP expression (Figures S7E and S7F). This suggests that both Leptin and Gp130 signaling pathways must be stimulated in the same cell in order to achieve sufficient Stat3 activation for MG to reprogram and proliferate. Consistent with this idea, we found that almost all BrdU$^+$ progenitors express both *gp130* and *lepr* mRNAs (Figure S7G), suggesting that MG-derived progenitors are a relatively homogeneous population.

We next investigated whether Leptin could stimulate MG reprogramming and proliferation in the uninjured retina. For these experiments, Leptin (1 μg/μl) was intravitreally injected once daily for 3 days into the uninjured eye of *1016 tuba1a:gfp* transgenic fish. On the 4th day, the fish received an i.p. injection of BrdU 3 hr before they were sacrificed. Interestingly, Leptin stimulated a remarkable amount of MG reprogramming (GFP expression) and proliferation (BrdU incorporation) (Figure 5F). However, these effects required a high dose (1 μg/μl) and when lower amounts were used, a very meager response was noted (Figures 6A, 6B, S7H, and S7I).

Leptin and IL-6 Family Cytokines Synergize with Each Other to Stimulate MG Reprogramming and Proliferation

The relative ineffectiveness of Leptin and IL-11 in stimulating MG reprogramming at low doses (Leptin at 62.5 ng/μl and IL-11 at 25 ng/μl; Figure 6A) prompted us to investigate whether they exhibit synergy in their action. For these experiments, uninjured retinas of *1016 tuba1a:gfp* transgenic fish received daily intravitreal injections of Leptin (62.5 ng/μl) and IL-11 (25 ng/μl) as described above. This combination of cytokine injection resulted in a remarkable synergy that stimulated widespread MG reprogramming (GFP expression) and proliferation (BrdU incorporation) (Figures 6A and 6B). However, when lower amounts of Leptin and IL-11 were used, only small responses were noted (Figure S7J). The synergy noted between Leptin and IL-11 may reflect a general feature of Leptin and IL-6 family cytokines, since Leptin also exhibited synergy with CNTF (Figures 6A and 6B). Importantly, knockdown of Gp130 or Lepr suppressed the synergistic effect of Leptin/IL-11 on MG reprogramming and proliferation (Figures 6E and 6F), suggesting that they act via their cognate receptors. Furthermore, like injury-dependent MG reprogramming (Fausett et al., 2008; Ramachandran et al., 2010a, 2011), cytokine-mediated reprogramming in the uninjured retina was dependent on Ascl1a expression (Figures 6C and 6D).

We next investigated whether the synergistic actions of Leptin and IL-6 family cytokines on MG reprogramming and proliferation were reflected in Jak/Stat signaling. For this analysis,

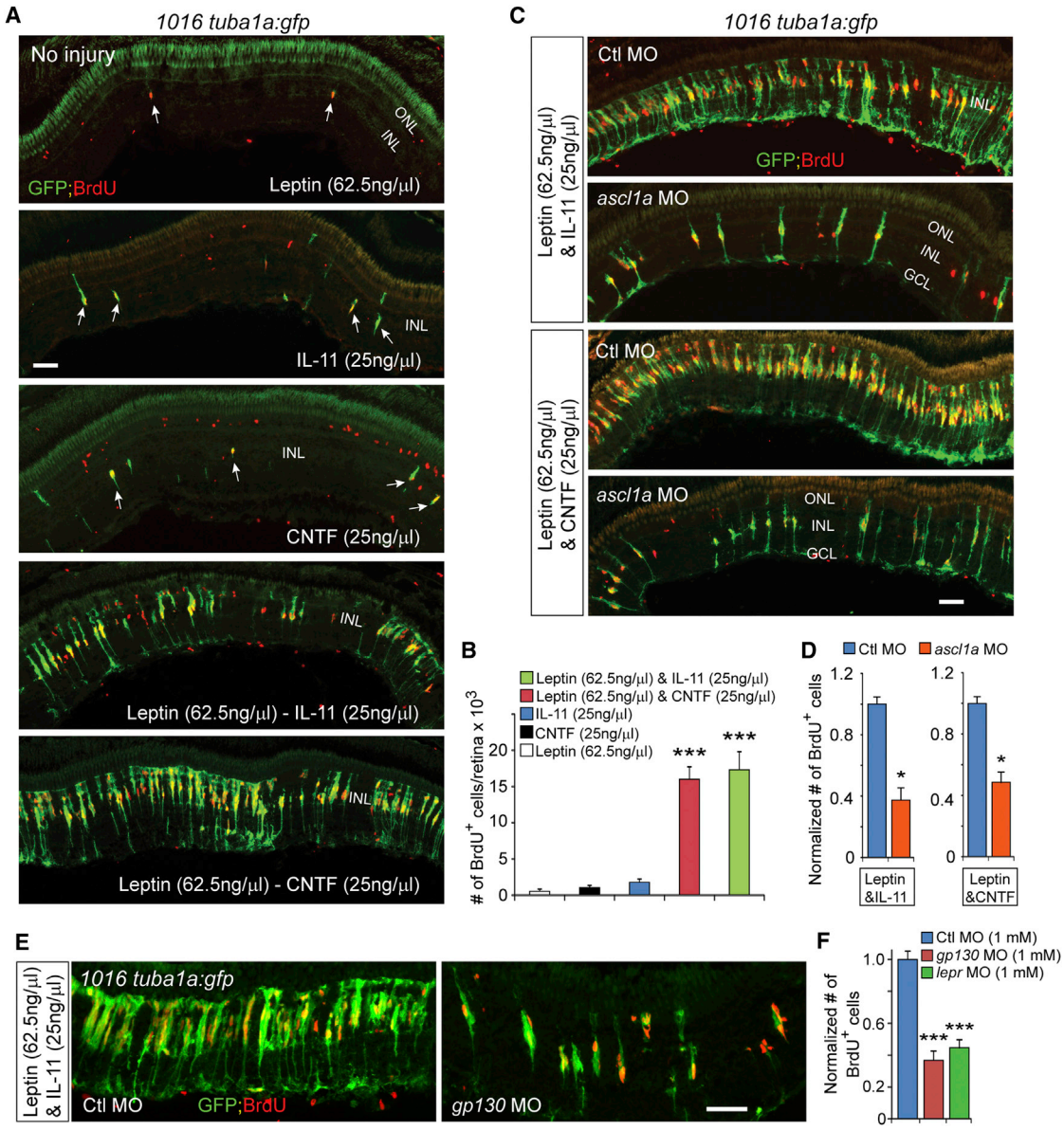

Figure 6. Leptin Synergizes with IL-11 and CNTF to Stimulate MG Reprogramming and Proliferation in the Uninjured Retina

(A) GFP and BrdU immunofluorescence shows that Leptin synergized with IL-11 and CNTF to stimulate GFP expression and MG proliferation in the uninjured retina *1016tuba1a:gfp* fish, whereas Leptin, IL-11, or CNTF alone had little effect. Arrows point to MG-derived progenitors in the top three panels.

(B) Quantification of the effects of cytokines on MG proliferation when delivered individually or in combination to the uninjured retina. ***$p < 0.001$ (combination versus individual), n = 4 per group.

(C and D) MO-mediated Ascl1a knockdown inhibits the synergistic effects of Leptin/IL-11 or Leptin/CNTF on GFP induction and MG proliferation. *$p < 0.05$, n = 3.

(E and F) Knockdown of Gp130 or Lepr inhibits the synergistic effects of Leptin/IL-11 on proliferation. ***$p < 0.001$, n = 4.

Error bars, SD. Scale bars, 50 μm (A, C, and E).

uninjured *gfap:stat3-gfp* transgenic fish, which report Stat3 activation (Figures 1 and S2), received an intravitreal injection of Leptin, CNTF, and IL-11 individually or in combination. As expected, the synergistic action of cytokines was reflected in both Stat3 activation (GFP) and progenitor formation (BrdU) (Figures 7A–7C). We further confirmed this synergy by western blot analysis of endogenous p-Stat3 in whole retinal extracts from uninjured eyes that received intravitreal injections of increasing amounts of CNTF or Leptin with and without CNTF coinjection (Figure 7D). Together, these data indicate that cytokines in the injured retina synergize with each other to stimulate MG reprogramming and proliferation by activating a Jak/Stat signaling cascade, and that these cytokines are sufficient to drive MG proliferation in the uninjured retina.

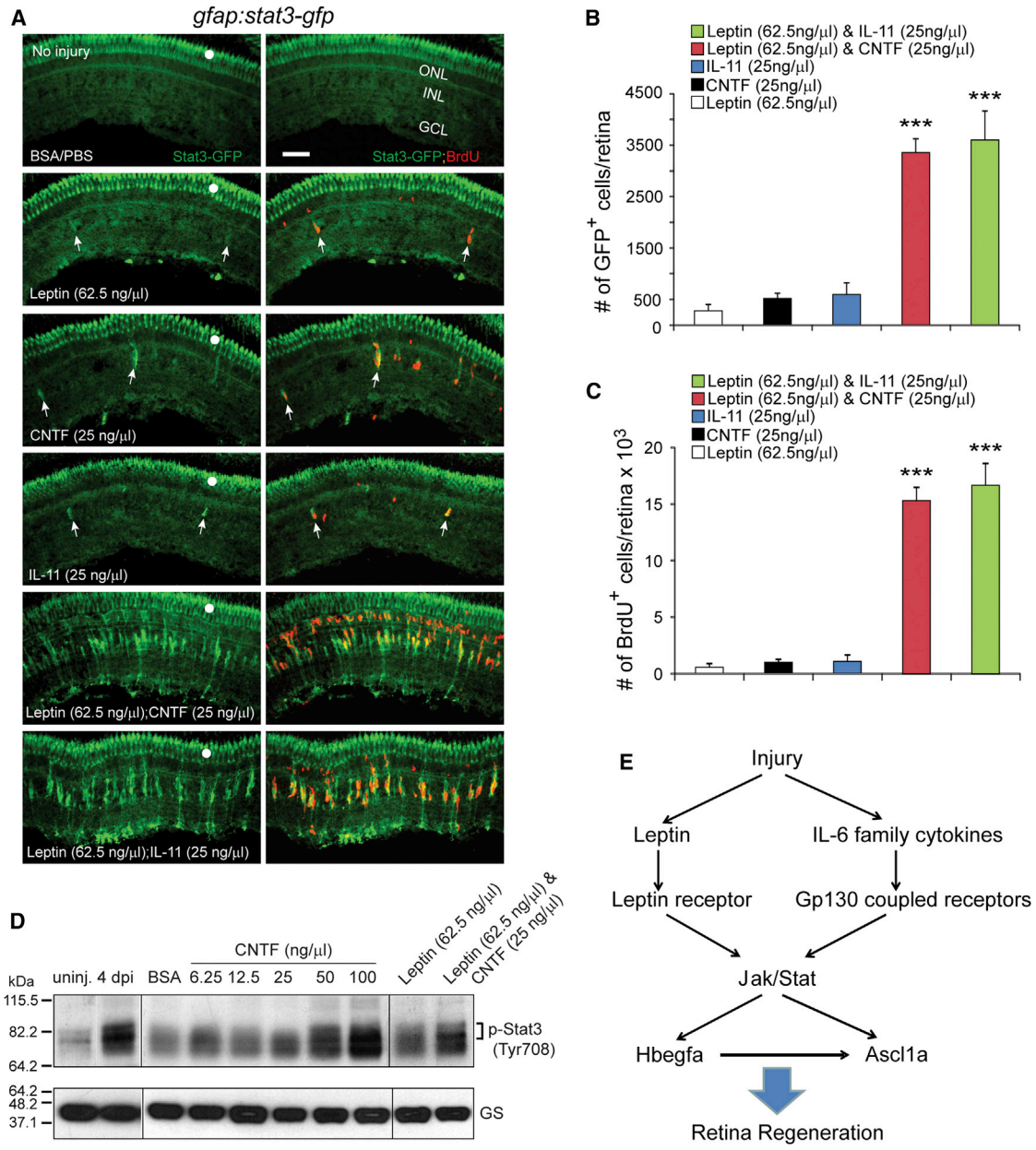

Figure 7. Leptin Synergizes with IL-11 and CNTF to Stimulate Jak/Stat3 Signaling in MG-Derived Progenitors

(A) Intravitreal injection of Leptin/CNTF, or Leptin/IL-11 into the eye of *gfap:stat3-gfp* fish stimulated Stat3-GFP expression and BrdU incorporation throughout the uninjured retina's INL, whereas Leptin, CNTF, or IL-11 alone had little effect. Note that GFP reports activated p-Stat3 expression (Figures 1 and S2). White dots indicate autofluorescence unique to the green channel (see Figure S7K). The arrows point to MG-derived progenitors in the top four panels.

(B) Quantification of Stat3-GFP$^+$ cells in (A). ***$p < 0.001$ (combination versus individual), n = 4.

(C) Quantification of BrdU$^+$ cells in (A). ***$p < 0.001$ (combination versus individual), n = 4.

(D) Western blot shows that retinal injury or intravitreal injection of cytokines into an uninjured eye increases p-Stat3 expression. GS serves as the loading control.

(E) Model showing that Leptin and IL-6 family cytokines synergize to stimulate MG reprogramming and retina regeneration via a Jak/Stat3 signaling pathway, which is essential for activating reprogramming genes such as *hbegf* and *ascl1a*.

Error bars, SD. Scale bars, 50 μm.

DISCUSSION

Our studies identified Leptin and IL-6 family cytokines induced in the injured retina that regulate MG reprogramming and the generation of MG-derived progenitors via a Jak/Stat3 signaling pathway (Figure 7E). We show that this signaling plays an important role in driving MG to acquire progenitor characteristics by stimulating the expression of reprogramming genes such as

ascl1a. By treating uninjured retinas with different combinations of Leptin and IL-6 family members, we found that these cytokines were sufficient to stimulate MG reprogramming and proliferation, and that they acted in a synergistic fashion. Finally, the endogenous expression of these cytokines by injury-responsive MG suggests that the MG themselves may contribute to their own reprogramming and proliferation.

The expression of cytokines by injury-responsive MG provides a convenient mechanism for amplifying a local signal that may initiate the injury response. The cytokine-activated Jak/Stat signaling pathway is well poised to serve as an early responder to injury, since Stat proteins can rapidly transduce information from membrane receptors to the nucleus in the absence of new protein synthesis (Hirano et al., 2000). Indeed, we found that this pathway contributes to the activation of a variety of reprogramming genes (e.g., *ascl1a*, *lin28*, *hbegfa*, and *socs3*) that are induced within hours following retinal injury and well before MG cell division, which begins around 2 dpi (Fausett and Goldman, 2006). Furthermore, by selectively inhibiting Jak/Stat signaling at 2–4 dpi, we were able to show that this pathway is important not only for MG reprogramming but also for progenitor proliferation and amplification.

It was previously suggested that increased Stat3 expression in MG of the injured retina reflects activated p-Stat3 (Kassen et al., 2007). Importantly, this expression was identified in both quiescent and proliferating MG, and two types of proliferating MG (primary responders and secondary responders) were proposed (Gorsuch and Hyde, 2013; Kassen et al., 2007; Nelson et al., 2012). How three types of MG could harbor activated Stat3 yet yield different phenotypes has remained an enigma. In this work, we sought to clarify these issues by specifically assaying for activated p-Stat3 expression in the injured retina. We found that regardless of the type of injury (i.e., mechanical injury to all retinal layers or light-induced injury that was restricted to photoreceptors), activated p-Stat3 was only detected in proliferating MG-derived progenitors. Thus, injury-dependent induction of Stat3 does not reflect activated p-Stat3 as previously proposed (Kassen et al., 2007; Nelson et al., 2012).

The high correlation of p-Stat3 expression with progenitor proliferation suggests that it does not distinguish primary from secondary responding MG, and that this distinction between progenitors may simply represent a temporal sequence of events controlled by similar mechanisms. The idea that Stat3 differentially affects primary and secondary MG was based on the observation that MO-mediated Stat3 knockdown only partially reduced progenitor formation/proliferation in the injured retina (Nelson et al., 2012). We suggest that this may reflect either incomplete Stat3 knockdown allowing residual p-Stat3 signaling or the action of other Stat proteins that collaborate with Stat3 in mediating an injury response.

The restricted expression of activated p-Stat3 to MG-derived progenitors at the injury site suggests that cytokines and/or their receptors exhibit a similar spatial restriction. Since MG reprogramming and proliferation can be induced by cytokines intravitreally injected into eye without retinal injury, these cytokine receptors probably preexist on MG at low levels. The injury-dependent induction of cytokines and their receptors in reprogrammed MG may further amplify their responsiveness to these factors.

The source of cytokines and other factors that contribute to MG reprogramming may be dying cells, invading microglia, vasculature, and the MG themselves. Previous studies have suggested that phagocytosis of dying cells by MG and the release of TNFα from dying cells may represent the initial signals that trigger MG reprogramming and proliferation (Bailey et al., 2010; Nelson et al., 2013). However, an effect of invading microglia or the MG themselves has not been ruled out. Our data are consistent with the idea that MG at the injury site are an important source of cytokines that regulate their own reprogramming and proliferation.

We observed a remarkable synergy in the ability of Leptin and IL-6 family members to stimulate MG proliferation in the uninjured retina. This synergy is reflected in Jak/Stat3 signaling, and although the mechanism underlying this synergy remains unexplained, it may result from the effect these cytokines have on additional signaling molecules that are coupled to the Jak/Stat3 signaling pathway. Importantly, this synergy may be necessary for stimulating MG reprogramming and retina regeneration in mammals.

We used mammalian Leptin and IL-6 family members to stimulate MG reprogramming and proliferation in the uninjured zebrafish retina. These cytokines exhibit limited (~20%–30%) identity to their zebrafish counterparts, which likely contributes to the relatively high concentrations needed to elicit a response (Table S2). Nonetheless, zebrafish and mammalian Leptin and IL-6 family members share a characteristic cytokine fold and other sequence elements that determine their receptor binding specificity (Gorissen et al., 2009; Huising et al., 2006; Prokop et al., 2012; Varela et al., 2012). More important is a consideration of the Leptin and IL-6 family receptors, whose amino acid identity between fish and mammals ranges from 20% (for IL-6R) to 53% (for CntfR). However, this overall homology hides the fact that domains of high conservation exist and that structural conservation may be as important as amino acid identity. Indeed, the cytokine-binding domains of these receptors from zebrafish and mammals share Ig superfamily, fibronectin type-III, and WSXWS domains that are organized in a similar fashion (Huising et al., 2006; Prokop et al., 2012; Varghese et al., 2002). Importantly, and consistent with the idea that these structures allow mammalian cytokines to act in a receptor-specific fashion in zebrafish, we found that knockdown of LepR or Gp130 inhibited the action of mammalian cytokines acting through these receptor components.

It is interesting that IL-6 family cytokines and p-Stat3 signaling stimulate MG to reprogram and generate progenitors in the zebrafish retina, whereas in birds and mammals these signaling molecules appear to act on MG to stimulate a gliotic response that functions to protect the retina from damage (Fischer et al., 2004a, 2004b; Peterson et al., 2000; Rhee et al., 2013; Xue et al., 2011). Jak/Stat signaling may collaborate with other signaling pathways to stimulate retina regeneration in fish, but these additional pathways may not be regulated in a similar fashion in mammals. In addition, the downstream targets of these pathways may differ between fish and mammals. The identification of these signaling pathways and an understanding

of their mechanisms of action in both fish and mammals may suggest strategies for switching MG from a gliotic to a regenerative response when confronted with a damaged retina. Our data suggest that Jak/Stat signaling may be one component of the regenerative response in fish. In an accompanying paper in this issue of *Cell Reports* (Wan et al., 2014), we report on additional signaling pathways that collaborate with Jak/Stat signaling in the injured fish retina. We speculate that this collaboration is a key element underlying retina regeneration in fish.

EXPERIMENTAL PROCEDURES

Animals and Retinal Injury

The animals used in this study were treated in accordance with the guidelines of the University Committee on Use and Care of Animals at the University of Michigan. Zebrafish were kept at 26–28°C on a 14 hr/10 hr light/dark cycle. *1016tuba1a:gfp*, *ascl1a:gfp*, and *gfap:gfp* transgenic fish have been previously described (Fausett and Goldman, 2006; Kassen et al., 2007; Wan et al., 2012). The fish were anesthetized with tricaine methane sulfonate before injection or injury. Retinal lesions were performed as previously described (Fausett and Goldman, 2006; Ramachandran et al., 2010a). Photoreceptor damage by UV light was performed as previously described (Bernardos et al., 2007). The fish were exposed to UV light for 30 min and then returned to their home tanks. Adult zebrafish of similar age and size were randomly allocated to experimental groups.

Florescence-Activated Cell Sorting

For fluorescence-activated cell sorting (FACS), GFP^+ MG from *gfap:gfp* retinas and GFP^+ MG-derived progenitors from *1016 tuba1a:gfp* retinas at 4 dpi were isolated on a BC Biosciences FACSViDa 3 laser high-speed cell sorter as previously described (Ramachandran et al., 2010a).

Plasmid Construction and Generation of Transgenic Lines

The Tol2 transposon system was used to generate transgenic lines using the Tol2 vector pTAL200R150G (Urasaki et al., 2006). *gfap* regulatory elements (Bernardos and Raymond, 2006) were amplified from zebrafish genomic DNA, and the *stat3* coding sequence was amplified from cDNA and cloned into a Tol2 vector. A distal 1.5 kb promoter of *ascl1a* was deleted from *ascl1a:gfp* (Wan et al., 2012) using restriction enzymes. PCR-mediated site-directed mutagenesis was done as previously described (Ramachandran et al., 2012) to generate *6-ascl1a(St1Mut):gfp* and *6-ascl1a(St2Mut):gfp* constructs. To generate in situ hybridization probes labeled with digoxigenin (DIG; Roche), *gp130*, *clcf1*, *crlf1a*, *lepa*, and *lepb* were cloned into pCS2, and *cntfr* and *lifrb* were cloned into pBSSK. PCR products using T3 and T7 primers were used as a template to generate DIG-labeled in situ hybridization probes for *lepr*, *il-11a*, *il-11ra*, and *il-6r*.

RT-PCR and Quantitative PCR

Total RNA was isolated from retinas using TRIzol (Invitrogen). Oligo(dT) and Superscript II reverse transcriptase (Invitrogen) were used to generate cDNA. PCR reactions used Taq polymerase and gene-specific primers (Table S1). Quantitative PCR (qPCR) was carried out in triplicate with Absolute SYBR Green Fluorescein Master Mix (Thermo Scientific) on an iCycler real-time PCR detection system (BioRad). The ΔΔCt method was used to determine the relative expression of mRNAs in control and injured retinas, normalized to *gapdh* mRNA levels. Primer sequences are list in Table S1.

Inhibitors and Recombinant Protein

The Jak inhibitor P6 (EMD Chemicals) was used at 10 μM and JSI-124 (Indofine Chemical Company) was used at 1 μM. Inhibitors were delivered at the time of retinal injury or were injected intravitreally at the indicated time. Recombinant human Leptin (a gift from Amylin Pharmaceuticals), recombinant rat CNTF, recombinant human IL-6, and recombinant mouse IL-11 (R&D Systems) were reconstituted in PBS with 0.1% BSA, and 0.5–2 μl was injected intravitreally at the indicated concentration (see Table S2 for dissociation constants and estimates of intravitreal concentrations). Intravitreal injection was done through the front of the eye by first making a small incision with either a double-edge sapphire blade (World Precision Instruments) or a 30-gauge beveled needle attached to a Hamilton syringe. If a sapphire blade was used to make the incision, a Hamilton syringe equipped with a blunt 33-gauge needle was used to deliver molecules behind the lens. If a Hamilton syringe equipped with a 30-gauge beveled needle was used to make an incision, recombinant molecules were delivered through this needle. Similar results were obtained regardless of the method used for intravitreal injection. Recombinant proteins were injected once daily for 3 days, and 4 days after the first injection, fish received an intraperitoneal injection of BrdU 3 hr prior to sacrifice. Experimenters remained blind to the material injected into the vitreous until after data analysis.

MO Electroporation

Lissamine-tagged MOs (Gene Tools) were introduced at the time of injury using a Hamilton syringe. MO delivery to cells was accomplished by electroporation as previously described (Fausett et al., 2008). The control MOs, *ascl1a* MO and *lepr* MO (5′-TGAAGACAGACATCATTTCACTTGC-3′), have been previously described (Fausett et al., 2008; Liu et al., 2012). The *gp130* MO sequence is 5′-ACAGCCAATGATGTGAAGTGTCCAT-3′. The amount of control MO was used to match the highest amount of experimental MO for each experiment.

BrdU Labeling and In Situ Hybridization

BrdU labeling was accomplished by injecting 20 μl of BrdU (20 mM) intraperitoneally 3 hr prior to sacrifice. Fish were overdosed with tricaine methane sulfonate and their eyes were dissected, enucleated, fixed, and sectioned as previously described (Fausett and Goldman, 2006). In situ hybridization was performed on retinal sections with DIG-labeled cRNA probes (DIG RNA labeling kit; Roche Diagnostics). Immunofluorescence protocols and antibodies were used as previously described (Ramachandran et al., 2011).

Immunofluorescence and Western Blots

Anti-GFP and 4C4 immunofluorescence were used as previously described (Craig et al., 2010; Fausett and Goldman, 2006). p-Stat3 immunofluorescence was performed using mouse anti-phospho-zebrafish Stat3 (Tyr708) antibody (MBL) at 1:100 dilution. For p-Stat3 epitope retrieval, the slides were boiled in 10 mM citrate buffer (pH 6) for 40 min. For BrdU immunofluorescence, sections were treated with 2N HCl at 37°C for 20 min, rinsed in 0.1 sodium borate (pH 8.5) for 10 min, and then processed according to standard procedures (Fausett and Goldman, 2006). SDS-PAGE and western blots were carried out according to standard protocols. Mouse anti-phospho-zebrafish Stat3 (Tyr708) antibody (MBL) was used at 1:1,000 dilution, and mouse anti-GS antibody (Millipore) was used at 1:3,000 dilution.

TUNEL Assay

An In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science) was used to detect apoptotic cells according to the manufacturer's protocol. Ouabain-treated eyes served as a positive control.

Microscopy and Statistical Analysis

Slides were examined with a Zeiss Axiophot, Observer.Z1 microscope or an Olympus FluoView FV1000 confocal imaging system. Cell counts were determined by counting fluorescently labeled $BrdU^+$ or GFP^+ cells in retinal sections visualized by fluorescent microscopy. All experiments were done in triplicate or more and repeated at least twice. Experimenters were blind to the animal treatments used until after data analysis. ANOVA with a Bonferroni/Dunn post hoc t test was used for multiple comparisons and a two-tailed unpaired Student's t test was used for single comparison (experimental versus control group). When n = 3, the Mann-Whitney test was employed for single comparison between control and experimental groups.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.047.

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REFERENCES

Bailey, T.J., Fossum, S.L., Fimbel, S.M., Montgomery, J.E., and Hyde, D.R. (2010). 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. *91*, 601–612.

Ben-Yaakov, K., Dagan, S.Y., Segal-Ruder, Y., Shalem, O., Vuppalanchi, D., Willis, D.E., Yudin, D., Rishal, I., Rother, F., Bader, M., et al. (2012). Axonal transcription factors signal retrogradely in lesioned peripheral nerve. EMBO J. *31*, 1350–1363.

Bernardos, R.L., and Raymond, P.A. (2006). GFAP transgenic zebrafish. Gene Expr. Patterns *6*, 1007–1013.

Bernardos, R.L., Barthel, L.K., Meyers, J.R., and Raymond, P.A. (2007). Late-stage neuronal progenitors in the retina are radial Müller glia that function as retinal stem cells. J. Neurosci. *27*, 7028–7040.

Blaskovich, M.A., Sun, J., Cantor, A., Turkson, J., Jove, R., and Sebti, S.M. (2003). Discovery of JSI-124 (cucurbitacin I), a selective Janus kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice. Cancer Res. *63*, 1270–1279.

Cao, X., Tay, A., Guy, G.R., and Tan, Y.H. (1996). Activation and association of Stat3 with Src in v-Src-transformed cell lines. Mol. Cell. Biol. *16*, 1595–1603.

Craig, S.E., Thummel, R., Ahmed, H., Vasta, G.R., Hyde, D.R., and Hitchcock, P.F. (2010). The zebrafish galectin Drgal1-l2 is expressed by proliferating Müller glia and photoreceptor progenitors and regulates the regeneration of rod photoreceptors. Invest. Ophthalmol. Vis. Sci. *51*, 3244–3252.

Ehret, G.B., Reichenbach, P., Schindler, U., Horvath, C.M., Fritz, S., Nabholz, M., and Bucher, P. (2001). DNA binding specificity of different STAT proteins. Comparison of in vitro specificity with natural target sites. J. Biol. Chem. *276*, 6675–6688.

Elson, G.C., Lelièvre, E., Guillet, C., Chevalier, S., Plun-Favreau, H., Froger, J., Suard, I., de Coignac, A.B., Delneste, Y., Bonnefoy, J.Y., et al. (2000). CLF associates with CLC to form a functional heteromeric ligand for the CNTF receptor complex. Nat. Neurosci. *3*, 867–872.

Faillace, M.P., Julian, D., and Korenbrot, J.I. (2002). Mitotic activation of proliferative cells in the inner nuclear layer of the mature fish retina: regulatory signals and molecular markers. J. Comp. Neurol. *451*, 127–141.

Fausett, B.V., and Goldman, D. (2006). A role for alpha1 tubulin-expressing Müller glia in regeneration of the injured zebrafish retina. J. Neurosci. *26*, 6303–6313.

Fausett, B.V., Gumerson, J.D., and Goldman, D. (2008). The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. J. Neurosci. *28*, 1109–1117.

Fimbel, S.M., Montgomery, J.E., Burket, C.T., and Hyde, D.R. (2007). Regeneration of inner retinal neurons after intravitreal injection of ouabain in zebrafish. J. Neurosci. *27*, 1712–1724.

Fischer, A.J., Omar, G., Eubanks, J., McGuire, C.R., Dierks, B.D., and Reh, T.A. (2004a). Different aspects of gliosis in retinal Muller glia can be induced by CNTF, insulin, and FGF2 in the absence of damage. Mol. Vis. *10*, 973–986.

Fischer, A.J., Schmidt, M., Omar, G., and Reh, T.A. (2004b). BMP4 and CNTF are neuroprotective and suppress damage-induced proliferation of Müller glia in the retina. Mol. Cell. Neurosci. *27*, 531–542.

Gorissen, M., Bernier, N.J., Nabuurs, S.B., Flik, G., and Huising, M.O. (2009). Two divergent leptin paralogues in zebrafish (Danio rerio) that originate early in teleostean evolution. J. Endocrinol. *201*, 329–339.

Gorsuch, R.A., and Hyde, D.R. (2013). Regulation of Müller glial dependent neuronal regeneration in the damaged adult zebrafish retina. Exp. Eye Res. *123*, 131–140.

Grandis, J.R., Drenning, S.D., Chakraborty, A., Zhou, M.Y., Zeng, Q., Pitt, A.S., and Tweardy, D.J. (1998). Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor- mediated cell growth In vitro. J. Clin. Invest. *102*, 1385–1392.

Hirano, T., Ishihara, K., and Hibi, M. (2000). Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene *19*, 2548–2556.

Huising, M.O., Kruiswijk, C.P., and Flik, G. (2006). Phylogeny and evolution of class-I helical cytokines. J. Endocrinol. *189*, 1–25.

Kassen, S.C., Ramanan, V., Montgomery, J.E., Burket, C.T., Liu, C.G., Vihtelic, T.S., and Hyde, D.R. (2007). Time course analysis of gene expression during light-induced photoreceptor cell death and regeneration in albino zebrafish. Dev. Neurobiol. *67*, 1009–1031.

Kassen, S.C., Thummel, R., Campochiaro, L.A., Harding, M.J., Bennett, N.A., and Hyde, D.R. (2009). CNTF induces photoreceptor neuroprotection and Müller glial cell proliferation through two different signaling pathways in the adult zebrafish retina. Exp. Eye Res. *88*, 1051–1064.

Lenkowski, J.R., Qin, Z., Sifuentes, C.J., Thummel, R., Soto, C.M., Moens, C.B., and Raymond, P.A. (2013). Retinal regeneration in adult zebrafish requires regulation of TGFβ signaling. Glia *61*, 1687–1697.

Levy, D.E., and Darnell, J.E., Jr. (2002). Stats: transcriptional control and biological impact. Nat. Rev. Mol. Cell Biol. *3*, 651–662.

Liang, J., Wang, D., Renaud, G., Wolfsberg, T.G., Wilson, A.F., and Burgess, S.M. (2012). The stat3/socs3a pathway is a key regulator of hair cell regeneration in zebrafish. [corrected]. J. Neurosci. *32*, 10662–10673.

Liu, Q., Dalman, M., Chen, Y., Akhter, M., Brahmandam, S., Patel, Y., Lowe, J., Thakkar, M., Gregory, A.V., Phelps, D., et al. (2012). Knockdown of leptin A expression dramatically alters zebrafish development. Gen. Comp. Endocrinol. *178*, 562–572.

Nagashima, M., Barthel, L.K., and Raymond, P.A. (2013). A self-renewing division of zebrafish Müller glial cells generates neuronal progenitors that require N-cadherin to regenerate retinal neurons. Development *140*, 4510–4521.

Nelson, C.M., Gorsuch, R.A., Bailey, T.J., Ackerman, K.M., Kassen, S.C., and Hyde, D.R. (2012). Stat3 defines three populations of Müller glia and is required for initiating maximal müller glia proliferation in the regenerating zebrafish retina. J. Comp. Neurol. *520*, 4294–4311.

Nelson, C.M., Ackerman, K.M., O'Hayer, P., Bailey, T.J., Gorsuch, R.A., and Hyde, D.R. (2013). Tumor necrosis factor-alpha is produced by dying retinal neurons and is required for Muller glia proliferation during zebrafish retinal regeneration. J. Neurosci. *33*, 6524–6539.

Pedranzini, L., Dechow, T., Berishaj, M., Comenzo, R., Zhou, P., Azare, J., Bornmann, W., and Bromberg, J. (2006). Pyridone 6, a pan-Janus-activated kinase inhibitor, induces growth inhibition of multiple myeloma cells. Cancer Res. *66*, 9714–9721.

Peterson, W.M., Wang, Q., Tzekova, R., and Wiegand, S.J. (2000). Ciliary neurotrophic factor and stress stimuli activate the Jak-STAT pathway in retinal neurons and glia. J. Neurosci. *20*, 4081–4090.

Pollak, J., Wilken, M.S., Ueki, Y., Cox, K.E., Sullivan, J.M., Taylor, R.J., Levine, E.M., and Reh, T.A. (2013). ASCL1 reprograms mouse Muller glia into neurogenic retinal progenitors. Development *140*, 2619–2631.

Powell, C., Elsaeidi, F., and Goldman, D. (2012). Injury-dependent Müller glia and ganglion cell reprogramming during tissue regeneration requires Apobec2a and Apobec2b. J. Neurosci. *32*, 1096–1109.

Prokop, J.W., Duff, R.J., Ball, H.C., Copeland, D.L., and Londraville, R.L. (2012). Leptin and leptin receptor: analysis of a structure to function relationship in interaction and evolution from humans to fish. Peptides *38*, 326–336.

Ramachandran, R., Fausett, B.V., and Goldman, D. (2010a). Ascl1a regulates Müller glia dedifferentiation and retinal regeneration through a Lin-28-dependent, let-7 microRNA signalling pathway. Nat. Cell Biol. *12*, 1101–1107.

Ramachandran, R., Reifler, A., Parent, J.M., and Goldman, D. (2010b). Conditional gene expression and lineage tracing of tuba1a expressing cells during zebrafish development and retina regeneration. J. Comp. Neurol. *518*, 4196–4212.

Ramachandran, R., Zhao, X.F., and Goldman, D. (2011). Ascl1a/Dkk/beta-catenin signaling pathway is necessary and glycogen synthase kinase-3beta inhibition is sufficient for zebrafish retina regeneration. Proc. Natl. Acad. Sci. USA *108*, 15858–15863.

Ramachandran, R., Zhao, X.F., and Goldman, D. (2012). Insm1a-mediated gene repression is essential for the formation and differentiation of Müller glia-derived progenitors in the injured retina. Nat. Cell Biol. *14*, 1013–1023.

Rhee, K.D., Nusinowitz, S., Chao, K., Yu, F., Bok, D., and Yang, X.J. (2013). CNTF-mediated protection of photoreceptors requires initial activation of the cytokine receptor gp130 in Müller glial cells. Proc. Natl. Acad. Sci. USA *110*, E4520–E4529.

Stenkamp, D.L. (2011). The rod photoreceptor lineage of teleost fish. Prog. Retin. Eye Res. *30*, 395–404.

Urasaki, A., Morvan, G., and Kawakami, K. (2006). Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. Genetics *174*, 639–649.

Varela, M., Dios, S., Novoa, B., and Figueras, A. (2012). Characterisation, expression and ontogeny of interleukin-6 and its receptors in zebrafish (Danio rerio). Dev. Comp. Immunol. *37*, 97–106.

Varghese, J.N., Moritz, R.L., Lou, M.Z., Van Donkelaar, A., Ji, H., Ivancic, N., Branson, K.M., Hall, N.E., and Simpson, R.J. (2002). Structure of the extracellular domains of the human interleukin-6 receptor alpha -chain. Proc. Natl. Acad. Sci. USA *99*, 15959–15964.

Vogt, P.K., and Hart, J.R. (2011). PI3K and STAT3: a new alliance. Cancer Discov. *1*, 481–486.

Wan, J., Ramachandran, R., and Goldman, D. (2012). HB-EGF is necessary and sufficient for Müller glia dedifferentiation and retina regeneration. Dev. Cell *22*, 334–347.

Wan, J., Zhao, X.-F., Vojtek, A., and Goldman, D. (2014). Retinal injury, growth factors, and cytokines converge on β-catenin and pStat3 signaling to stimulate retina regeration. Cell Rep. Published online September 25, 2014. http://dx.doi.org/10.1016/j.celrep.2014.08.048.

Xue, W., Cojocaru, R.I., Dudley, V.J., Brooks, M., Swaroop, A., and Sarthy, V.P. (2011). Ciliary neurotrophic factor induces genes associated with inflammation and gliosis in the retina: a gene profiling study of flow-sorted, Müller cells. PLoS ONE *6*, e20326.

Yamashita, S., Miyagi, C., Carmany-Rampey, A., Shimizu, T., Fujii, R., Schier, A.F., and Hirano, T. (2002). Stat3 controls cell movements during zebrafish gastrulation. Dev. Cell *2*, 363–375.