Supplemental Information

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

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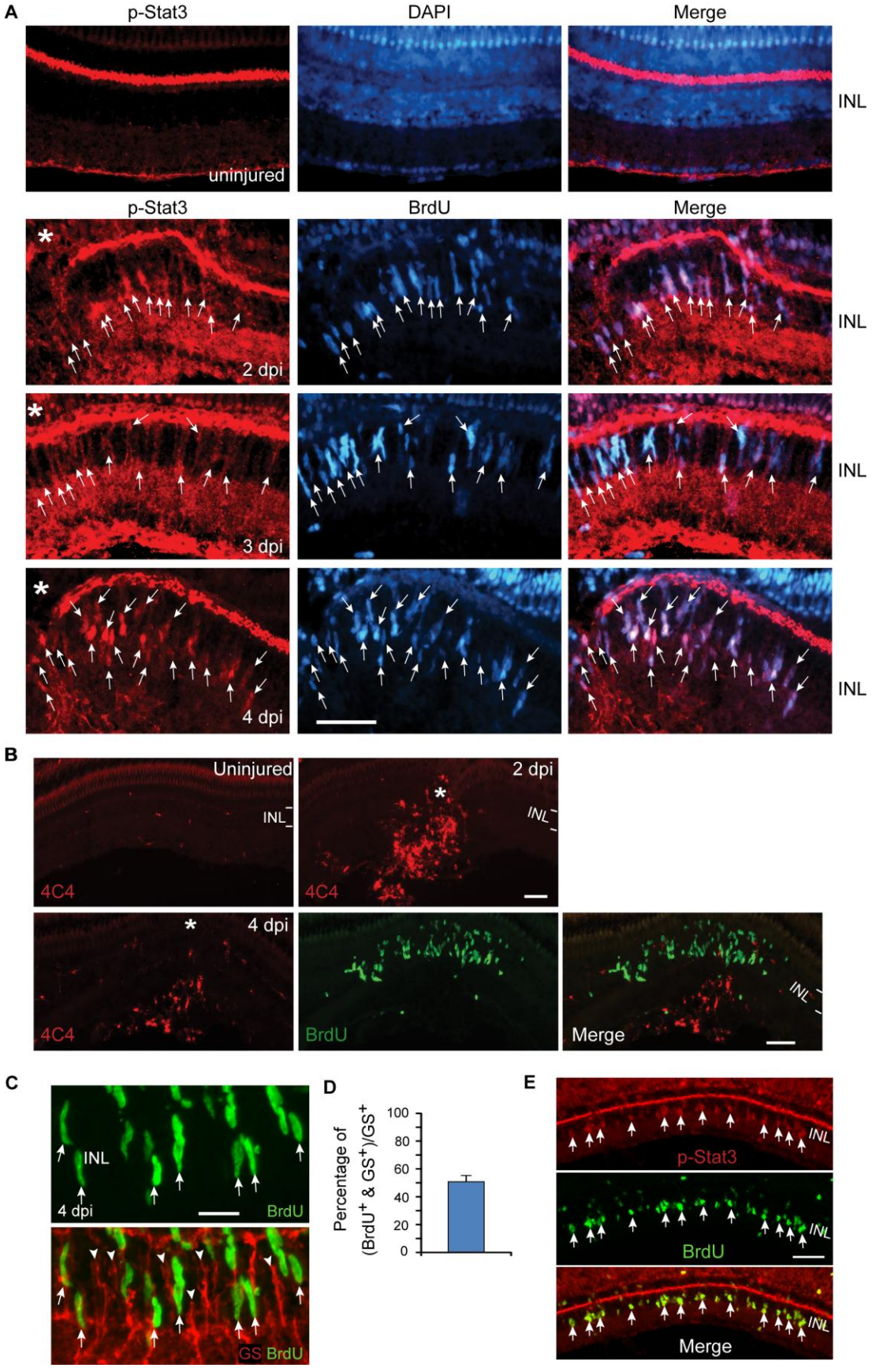

Figure S1. Injury-dependent activation of the Jak/Stat3 signaling pathway. Related to

Figure 1. (A) p-Stat3 (red) and BrdU (blue) immunofluorescence shows p-Stat3 is induced in $BrdU^+$ MG-derived progenitors at the injury site at 2-4 dpi; n=3. Arrows point to $p\text{-}Stat3^+/BrdU^+$ double labelled cells. (B) 4C4 immunoflourescence (red) shows microglia, diffusely scattered throughout the uninjured retina, accumulate at the injury site, but do not proliferate (lack BrdU co-labeling, green). The $BrdU^+$ cells (green) are MG-derived progenitors confined to the INL. (C, D) Immunofluorescence shows ~50% of glutamine synthetase $(GS)^+$ MG (red) incorporate $BrdU^+$ 4 days after a 30 min exposure to UV light; n=3. Arrows point to $BrdU^+/GS^+$ double labelled cells. Arrowheads point to $BrdU^-/GS^+$ quiescent MG. (E) Immunofluorescence shows p-Stat3 is restricted to $BrdU^+$ MG-derived progenitors 4 days after a 30 min exposure to UV light. Asterisks in (A) point to the injury site (needle poke). Scale bar, 20 μm (C); 50 μm (A,E). INL, inner nuclear layer.

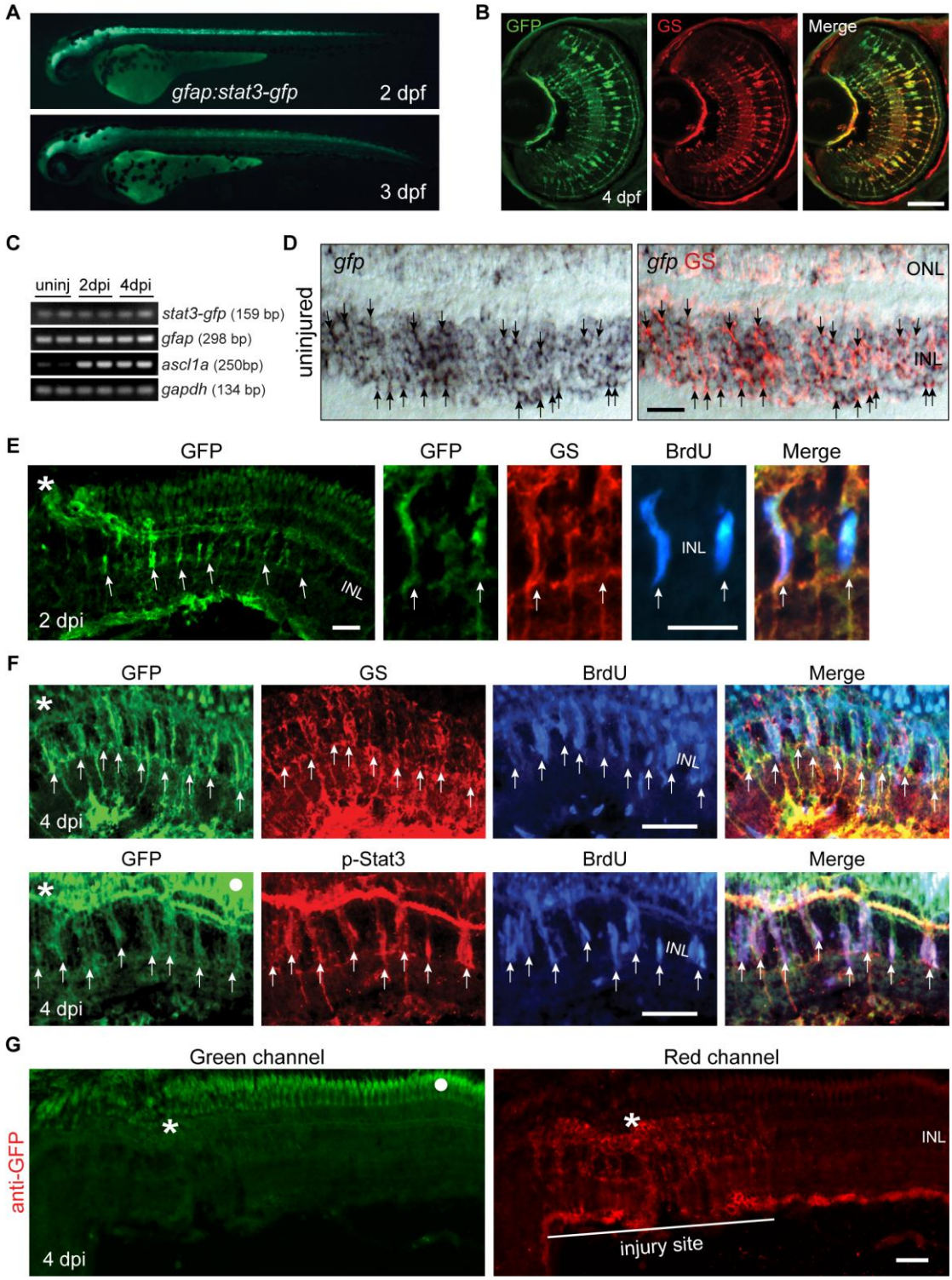

Figure S2. Stat3-GFP expression in developing and adult *gfap:stat3-gfp* fish. Related to Figure 1. (A) Stat3-GFP is expressed throughout brain and spinal cord in live gfap:stat3-gfp larva at 2 and 3 dpf. (B) Immunofluorescence on retinal sections shows co-localization of Stat3-GFP with glutamine synthetase $(GS)^+$ MG at 4 dpf. (C) Whole retina RT-PCR showing constitutive gfp mRNA expression in the uninjured and injured adult retina, while ascl1a mRNA was induced after retinal injury. (D) *In situ* hybridization and immunofluorescence shows expression of gfp RNA in GS^+ MG in adult uninjured retina

(arrows). (E) Immunofluorescence shows Stat3-GFP expression co-localizes with $GS^{+}/BrdU^{+}$ MG-derived progenitors localized to the injury site at 2 dpi in the adult retina. (F) Immunofluorescence shows co-localization of Stat3-GFP with $GS^{+}/p\text{-}Stat3^{+}/BrdU^{+}$ MG-derived progenitors at 4 dpi in the adult retina. White dot indicates autofluorescence unique to the green channel. Asterisks mark the injury site (needle poke) and arrows point to MG-derived progenitors. (G) Anti-GFP immunofluorescence on a retinal section prepared at 4 dpi from *gfap:stat3-gfp* fish using a secondary antibody coupled to a red fluor shows autofluorescence in the ONL when viewed in the green channel (left-hand panel, dot marks autofluorescence) that is not evident in the red channel (right-hand panel). Asterisk marks the injury site. Scale bar, 50 μm (B, D-G). INL, inner nuclear layer; dpf, days post fertilization.

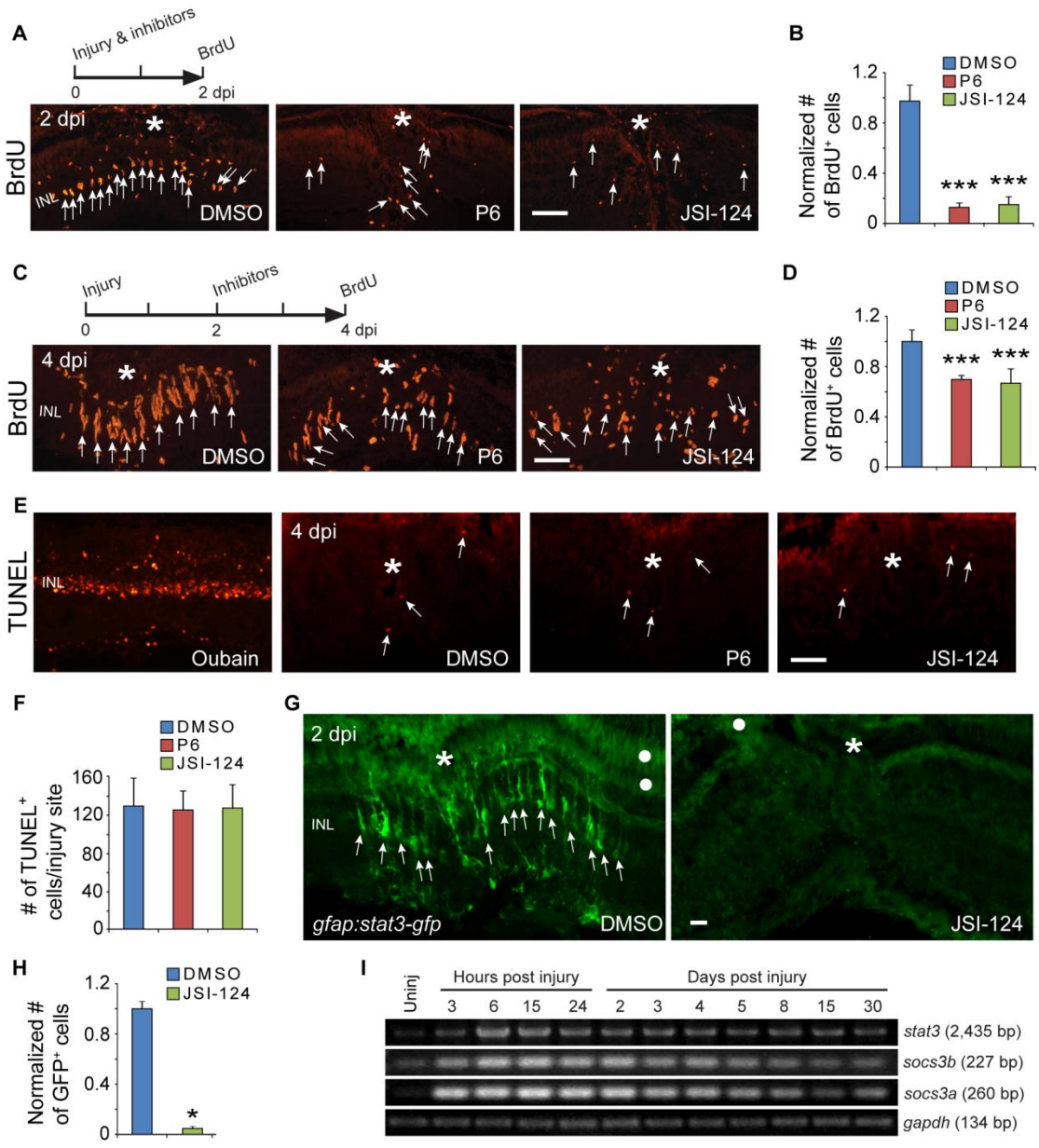

Figure S3. Jak/Stat signaling regulates proliferation of MG-derived progenitors and Stat3-GFP expression. Related to Figure 2. (A, B) BrdU immunofluorescence on retinal sections shows that exposing fish to Jak inhibitors P6 or JSI-124 from 0-2 dpi, inhibits progenitor proliferation at 2 dpi; ***P<0.001, n=4. (C, D) BrdU immunofluorescence shows that exposing fish to Jak inhibitors P6 or JSI-124 from 2-4 dpi, inhibits progenitor proliferation at 4 dpi; ***P<0.001, n=4. (E, F) TUNEL staining for apoptotic cells at 4 dpi in retinas treated with DMSO, P6 or JSI-124. Oubain was used as a positive control. In panel F, n=3. (G, H) GFP immunofluorescence shows that exposing *gfap:stat3-gfp* fish to Jak inhibitors JSI-124 from 0-2 dpi, inhibits Stat3-GFP expression; *P<0.05, n=3. (I) RT-PCR analysis of retinal *stat3* and *socs3* gene expression at various times after injury. Asterisks mark the injury sites (needle poke) and arrows point to MG-derived progenitors. White dot marks regions with autofluorescence. Error bars, s.d. Scale bar, 20 μm (A, C, E, G). INL, inner nuclear layer.

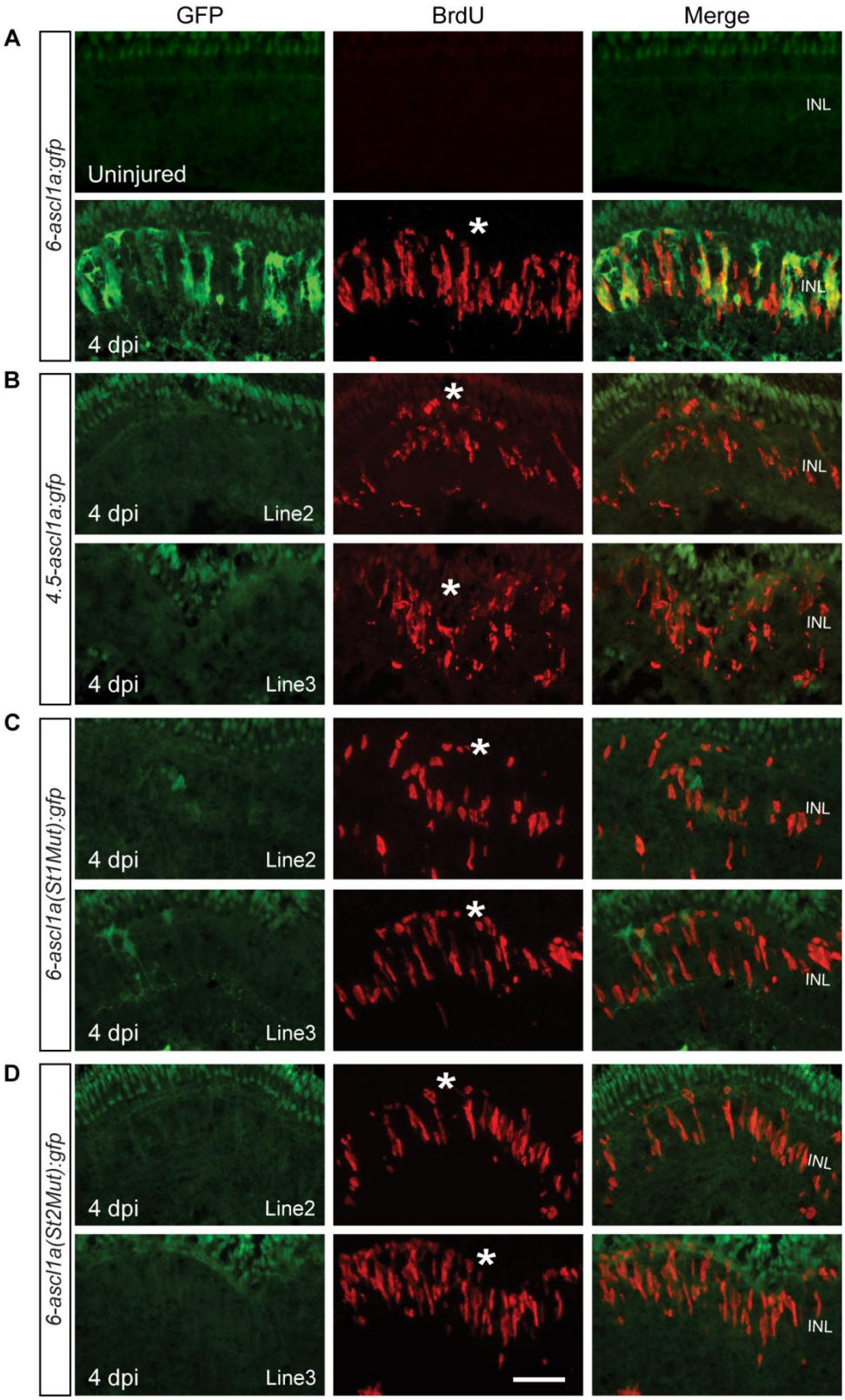
GFP
BrdU
Merge
A
6-ascl1a:gfp
Uninjured
4 dpi
INL
B
4.5-ascl1a:gfp
4 dpi
Line2
4 dpi
Line3
C
6-ascl1a(St1Mut):gfp
4 dpi
Line2
4 dpi
Line3
D
6-ascl1a(St2Mut):gfp
4 dpi
Line2
4 dpi
Line3

Figure S4. Jak/Stat3 signaling mediates injury-dependent activation of the *ascl1a* promoter. Related to Figure 3. (A) GFP and BrdU immunofluorescence co-localize beneath the injury site in *6-ascl1a:gfp* fish at 4 dpi. (B) A distal 1.5 kb *ascl1a* promoter fragment is necessary for injury-dependent transgene induction. (C, D) Consensus Stat3 sites located within this distal 1.5 kb region are necessary for injury-dependent transgene expression. $BrdU^{+}$ immunofluorescence identifies the injury site and a normal regenerative response. The asterisks mark the injury sites. See Figure 3 for promoter schematic. Scale bar, 50 μm. INL, inner nuclear layer.

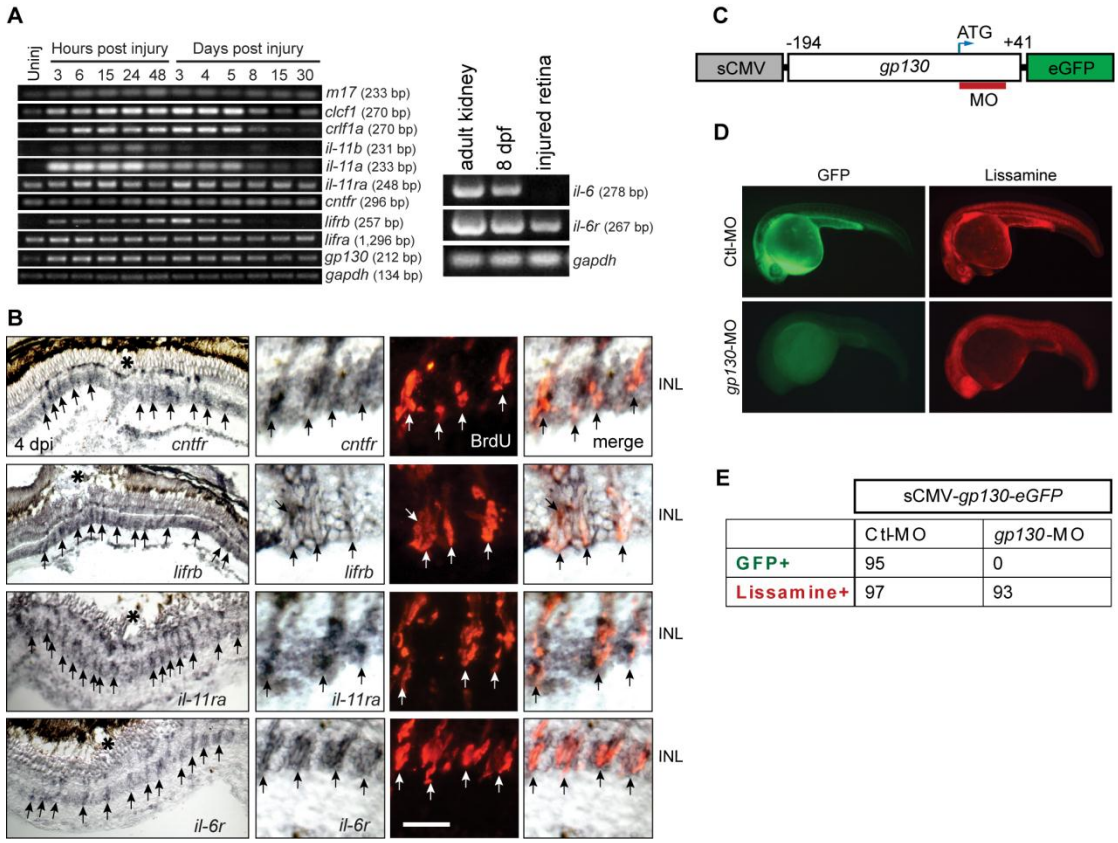

Figure S5. Genes encoding IL-6 family cytokines are expressed in MG-derived progenitors upon retinal injury. Related to Figure 4. (A) RT-PCR analysis of retinal gene expression at various times after injury. *il-6* is expressed in adult kidney and 8 dpf larva, but undetectable in the injured retina. (B) *In situ* hybridization shows induction of *il-6* family member genes in $BrdU^{+}$ MG-derived progenitors at the injury site. The asterisks mark the injury sites and the arrows point to MG-derived progenitors. Scale bars, 20 μm. (C) Diagram of sCMV:*gp130-egfp* reporter in which a fragment of *gp130* DNA, containing the MO target sequence, is in-frame with the *egfp* coding sequence and under control of the sCMV promoter. (D) Injection of the sCMV:*gp130-egfp* reporter together with either lissamine-tagged control (Ctl) MO or *gp130*-targeting MO into one cell stage of zebrafish embryos and examined by fluorescence 24 hours later. Red fluorescence shows embryos received lissamine-tagged MOs. (E) Quantification of GFP-expressing embryos at 24 hpf. No GFP was detected in the *gp130*-targeting MO injected group, while GFP was readily detected in the control group. Similar results were obtained in 3 independent experiments. dpf, days post fertilization ; INL, inner nuclear layer.

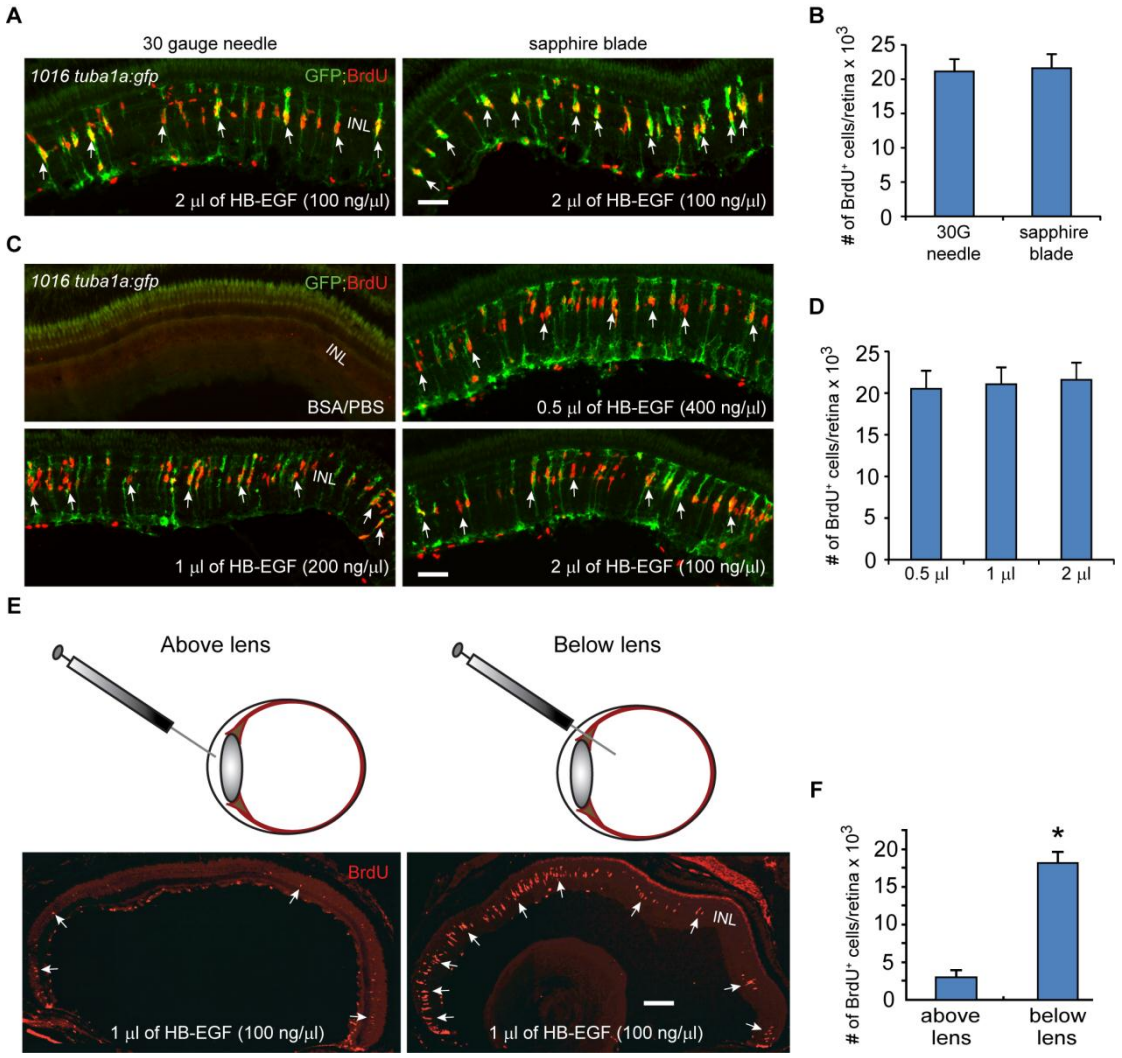

Figure S6. HB-EGF stimulates MG reprogramming and proliferation in the uninjured retina. Related to Figure 4. (A) A corneal puncture with a 30 gauge needle or a corneal incision made with a sapphire blade was followed by intravitreal injection of recombinant HB-EGF into the uninjured eye of *1016tuba1a:gfp* fish. HB-EGF stimulated GFP expression and BrdU incorporation in MG throughout the retina's inner nuclear layer. No GFP expression or BrdU incorporation is observed in PBS/BSA-injected eyes. (B) Quantification of $BrdU^+$ cells in (A) shows similar effect of intravitreally delivered HB-EGF on MG proliferation when the cornea was either punctured with a needle or cut with a sapphire blade; n=3 per group. (C) A sapphire blade was used to make a small incision in the cornea and 200 ng of HB-EGF in 0.5 to 2 µl volumes was intravitreally injected into uninjured eyes of *1016tuba1a:gfp* fish. (D) Quantification of $BrdU^+$ cells following intravitreal delivery of different HB-EGF volumes reveals a similar effect on MG proliferation regardless of injection volume; n=3 per group. (E, F) HB-EGF must be delivered below the lens to stimulate MG proliferation; *P<0.05, n=3. Arrows point to MG-derived progenitor. Error bars, s.d. Scale bar, 50 µm (A, C); 150 µm (E). INL, inner nuclear layer.

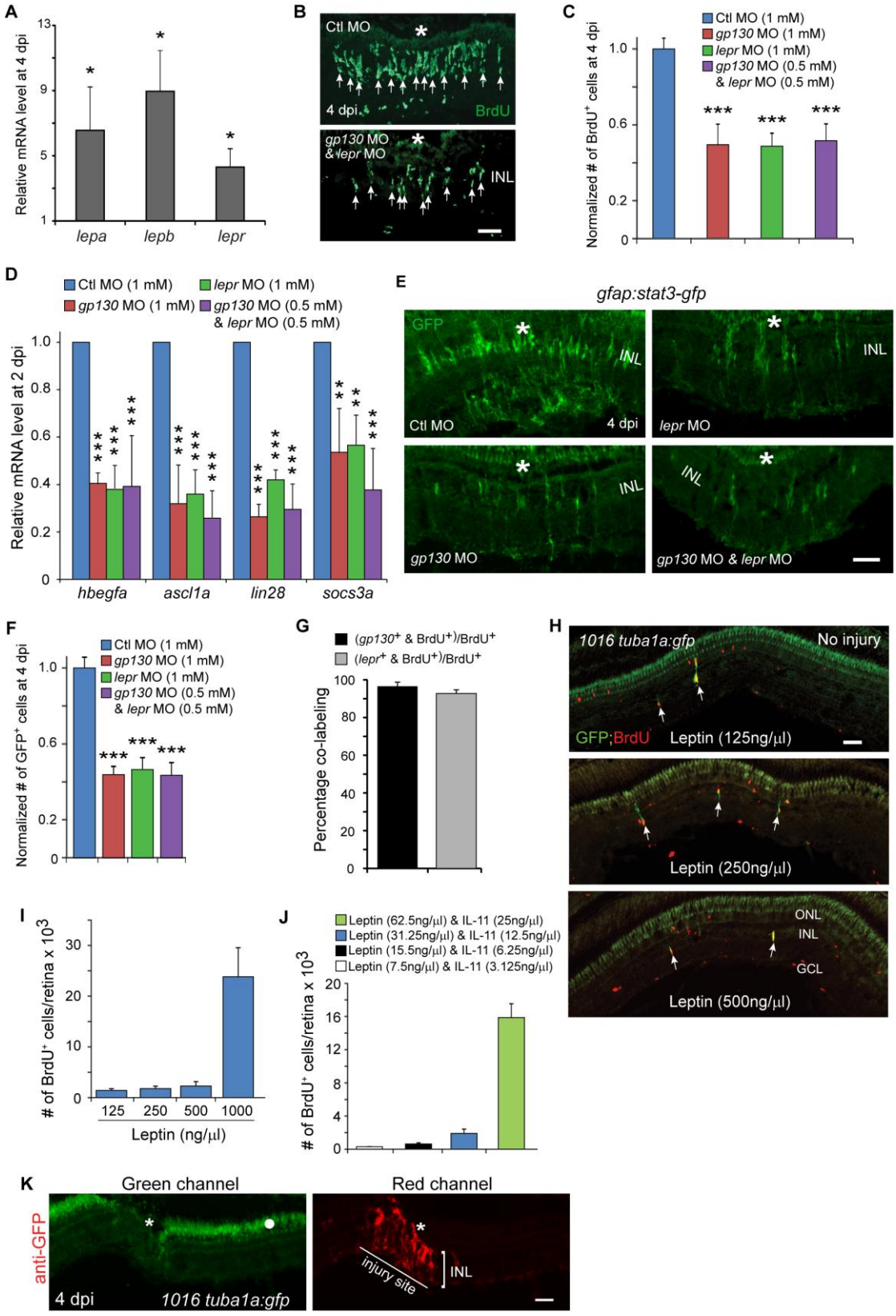

Figure S7. Leptin signaling regulates the generation of MG-derived progenitors. Related to Figure 5. (A) qPCR quantifying induction of gene expression in FACS purified GFP^{+} MG-derived progenitors from the retina of *1016tuba1a:gfp* fish at 4 dpi in comparison

to that in FACS purified GFP^+ MG from the uninjured retina of *gfap:gfp* fish; *P<0.05, n=3. (B) BrdU immunofluorescence shows that Leptin receptor and Gp130 knockdown inhibits the generation of $BrdU^+$ MG-derived progenitors at 4 dpi. Asterisks mark the injury sites and the arrows point to MG-derived progenitors. (C) Quantification of the effects Gp130 and Leptin receptor knockdown have on progenitor formation (Figure 4C; Figure 5C); ***P<0.001, n=4. (D) qPCR showing Gp130 and Leptin receptor knockdown, individually or in combination, inhibits injury-dependent induction of reprogramming genes at 2 dpi; **P<0.01, ***P<0.001, n=4. (E) Knockdown of Gp130 inhibits the expression of Stat3-GFP at 4 dpi. (F) Quantification of the number of GFP^+ cells in the retina at 4 dpi following Gp130 or Leptin knockdown; ***P<0.001, n=4. Error bars, s.d. (G) Quantification of the number of $BrdU^+$ cells expressing *gp130* or *lepr* RNA in the retina at 4 dpi; n=3. Error bars, s.d. (H) Effects of different Leptin concentrations on the generation of $BrdU^+$ MG-derived progenitors and transgene expression in the uninjured retina of *1016tuba1a:gfp* fish. The arrows point to MG-derived progenitors. (I, J) Quantification of the effect different Leptin (I) or Leptin/IL-11 (J) concentrations have on progenitor formation in the uninjured retina; n=3. Error bars, s.d. (K) GFP immunofluorescence using a secondary antibody coupled to a red fluor shows autofluorescence in the green channel (left-hand panel, dot) that is localized to the ONL and pigment epithelium, while the red channel shows GFP expression restricted to the injury site (right-hand panel, asterisk). Scale bars, 20 μm (B); 50 μm (E, H, K). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

Table S1. Primer sequences for PCR. Related to Experimental Procedures.

Gene	Sequence 5'- 3'
lepa	F, TTTCCAGCTCTCCGCTCAACC R, CGGCGTATCTGGTCAACATGC
lepb	F, CATTGCTCGAACCACCATCAGC R, TCTTTATGCACCGGGGTCTCG
lepr	F, CAGTACGAGCTGCAATTCAAGG R, TAAAATGCGCCAGAAGTCTGG
m17	F, CTTGATTGCCGTTCAGTTAGTGC R, TTAAGATGCGCTCCGATTCAGT
clcf1	F, GAAAGTTGGTCAGGTTGCTGTGC R, CATAAGTCCACACGTGTTGCTGC
crlf1a	F, GGGATTCTGGGATCTAGGAAAGC R, TCCTTGAAGAACCTGGTTGCG
il-11a	F, CTCCTCATCGCTGCTTCTCTCG R, TTGCGAAGTCACTGGCTCTGC
il-11b	F, GCTAACAGTGTCGCCTGACTCC R, CTGTAGTTCAGTGAGGGCAGGG
il-11ra	F, GTTGGACTGTTGGTTTTGTTGG R, TGGATTGTGGGTAATGAAGGC
cntfr	F, ACACCATCACCGACGCCTATGC R, GAAGCTCACACATCACATGATGG
lifra	F, AAGCCGTCTCCACACAGTCTGG R, TTCCCCCCATTCTGCTTTCC
lifrb	F, TCACAGTTGACCAGATGCTTGC R, ATGTGGGTTTTCTGAGGTGGG
gp130	F, AATGAAGTTCGCCGATGGAGAGG R, CGTCTTCCTTGGGCATTTCGG
il-6	F, GCTATTCCTGTCTGCTACACTGG R, TGAGGAGAGGAGTGCTGATCC
il-6r	F, TCAGCTCCTGAGACAACTACTGC R, AAACGGCATAGTCTGTTTCCC
stat3	F, ACACATTAAGGGGTCAACATGGCCCAG R, GCATTTCGGCAGGTGTCCATATCCGAG
socs3a	F, CACTAACTTCTCTAAAGCAGGG R, GGTCTTGAAGTGGTAAAACG
socs3b	F, GAAAACTCCCAAGATTGAGTCG R, TACTATGCGTTACCATGGCG
hbegfa	F, ATGTCTGACCATCATTGGCCTCC R, ACCATTCAGCTTGCTGTGCCC
ascl1a	F, ATCCGCGCGCTGCAGCAGCTTCTGGACG

	R, CGAGTGCTGATATTTTAAGTTTCCTTTTAC
lin28	F, TAACGTGCGGATGGGCTTCGGATTTCTGTC R,ATTGGGTCCTCCACAGTTGAAGCATCGATC
gfp	F, CAGCAGAACACCCCCATC R, CTCGTCCATGCCGAGAGT
gfap	F, GGATGAGATCCAGATGCTGAAGG R, CAGATCCTTCCTCTCCGTAGTGG
gapdh	F, ATGACCCCTCCAGCATGA R, GGCGGTGTAGGCATGAAC

Table S2. Kd, ED_{50} and intravitreal concentration of recombinant mammalian cytokines. Related to Experimental Procedures.

Cytokine	Kd and ED_{50}	Estimated concentration after intravitreal injection*
Recombinant human Leptin	Kd ~0.06-3nM (Verkerke et al., PLoS One 9:e94843, 2014; Uotani et al., Diabetes 48:279, 1999). ED_{50} ~0.4-2 ng/ml in a cell proliferation assay (http://www.rndsystems.com/Products/398-LP).	62.5 ng = 0.195 μM 1 μg = 3.125 μM
Recombinant rat CNTF	Two classes of receptor binding sites: high affinity site, Kd ~1pM and low affinity site Kd ~1nM (Wong et al., J Biol Chem 270:313, 1995). ED_{50} ~1 ng/ml in a cell proliferation assay (Gearing et al, Proc Natl Acad Sci USA 91:1119, 1994).	25 ng = 54.8 nM 100 ng = 219 nM
Recombinant human IL-6	Two classes of receptor binding sites: high affinity site, Kd ~10pM and low affinity site, Kd ~1nM (Yamsaki et al., Science 241:825, 1988). ED50 ~10 ng/ml in a cell proliferation assay (Hibi et al., 63:1149, 1990).	100 ng = 211 nM
Recombinant mouse IL-11	Two classes of receptor binding sites: high affinity site, Kd ~0.3-0.8nM and low affinity site, Kd ~10nM; ED_{50} ~5 ng/ml in a cell proliferation assay (Hilton et al., EMBO J. 13:4765, 1994).	25 ng = 65.3 nM 100 ng = 2.6 μM

*Cytokine concentrations in the vitreous of injected eyes was estimated as previously described using a 20 μl vitreal volume (Fimbel et al., J Neurosci 27:1712, 2007; Ritchey et al., Exp Eye Res 99:1, 2012; Raymond et al., J Neurobiol 19:431, 1988).