# Supplemental Information

## Retinal injury, growth factors and cytokines converge on β-catenin and pStat3 signaling to stimulate retina regeneration

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Supplemental Figures: S1-S6

Supplemental Tables: S1-S2

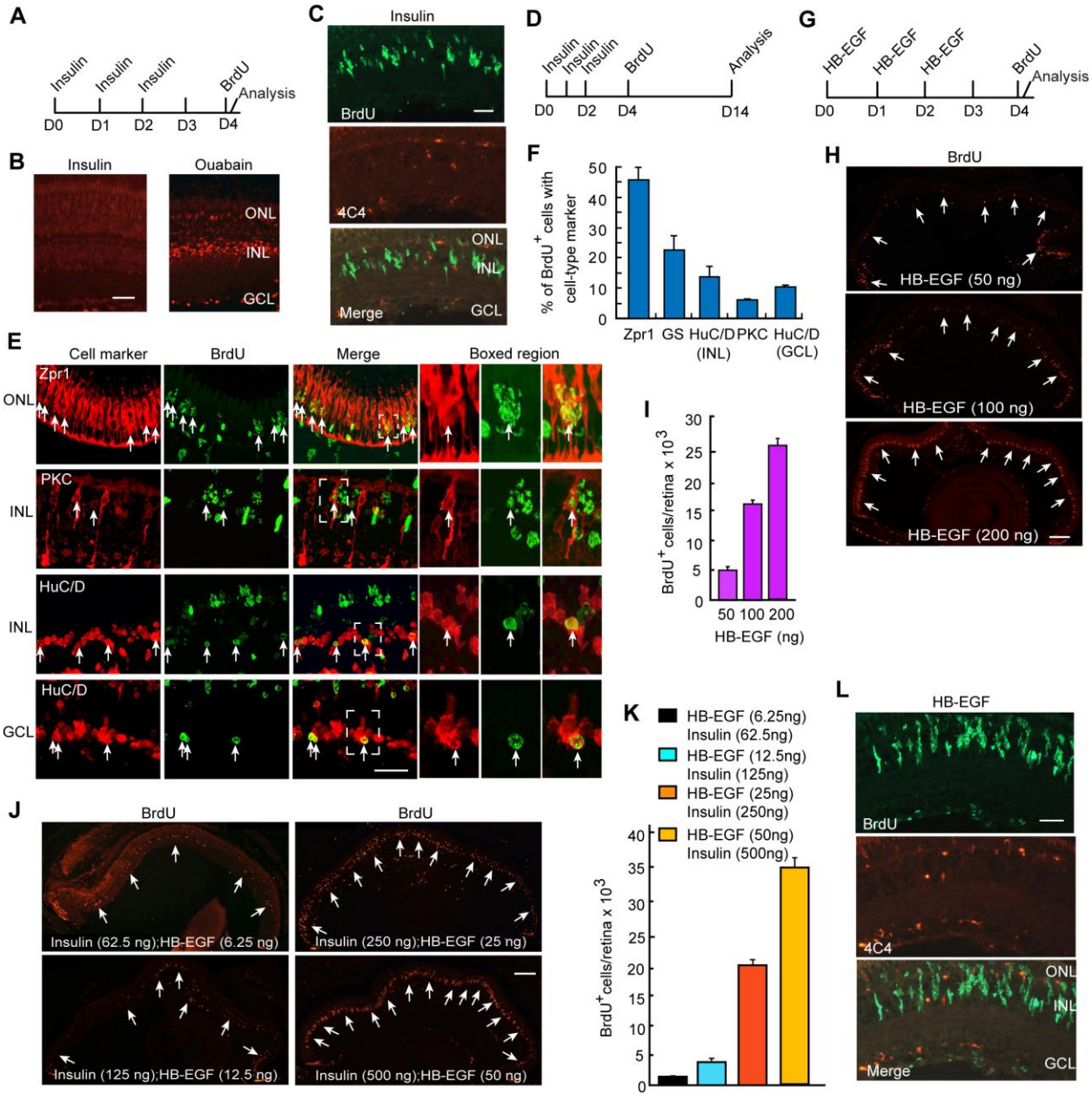


**Figure S1, related to Figures 1 and 2. Insulin and HB-EGF act in a synergistic fashion to stimulate the production multipotent progenitors in the uninjured retina.** (A) Diagram of the experimental protocol used for intravitreal Insulin injection used in (B & C). (B) TUNEL assay shows no detectable apoptosis in the Insulin-treated retina. Scale bar is 50 μm. (C) Immunoflourescence using anti-BrdU and anti-4C4 antibodies to detect proliferating cells and microglia, respectively, shows microglia do not proliferate in the uninjured retina treated with 2

μg of Insulin. Scale bar is 50 μm. (D) Diagram of experimenetal protocol used for lineage tracing shown in (E). (E) Immunoflourescence using anti-BrdU and anti-retinal cell type-specific antibodies identifies MG-derived progenitors that differentiated into retinal neurons in Insulin-treated eyes. Arrows point to double-labelled cells. Boxed areas are enlarged in the 3 right-hand panels. Scale bar is 50 μm. (F) Quantification of newly generated lineage-traced cells shown in (E). Error bars are SD; n=3. (G) Diagram of experimental protocol used for intravitreal injection of HB-EGF into the uninjured retina and shown in (H, L). (H, I) Intravitreal injection of HB-EGF stimulates MG proliferation in a concentration-dependent manner in the uninjured retina. Arrows (H) point to $BrdU^+$ MG-derived progenitors. Scale bar is 150 μm. Error bars (I) are SD; n=3. (J, K) Intravitreal injection of Insulin and HB-EGF act in a synergistic fashion to stimulate MG proliferation in a concentration-dependent manner. Arrows (J) point to $BrdU^+$ MG-derived progenitors. Scale bar is 150 μm. Error bars (K) are SD; n=3. (L) Immunoflourescence using anti-BrdU and anti-4C4 antibodies to detect proliferating cells and microglia, respectively, show microglia do not proliferate in the uninjured retina treated with 200 ng HB-EGF. Scale bar is 50 μm.

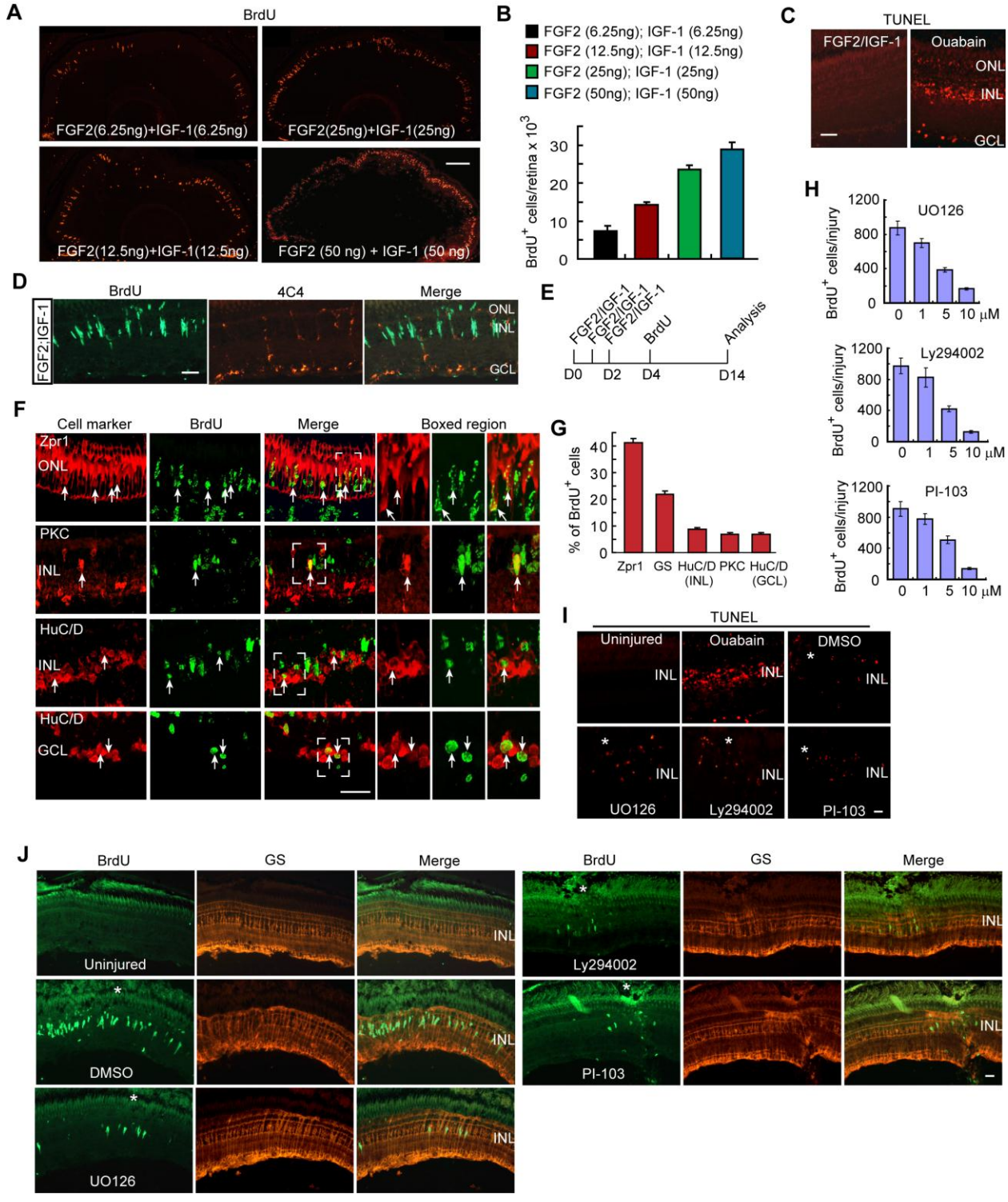


**Figure S2, related to Figures 2 and 3. FGF2 and IGF-1 act in a synergistic fashion to stimulate production of multipotent progenitors in the uninjured retina.** (A, B) Intravitreal injection of FGF2 and IGF-1 synergistically stimulate MG proliferation in a concentration-

dependent manner. Scale bar (A) is 150 μm. Error bars (B) are SD; n=3. (C) TUNEL assay shows no detectable apoptosis in the FGF2/IGF-1-treated retina. Scale bar is 50 μm. (D) Immunoflourescence using anti-BrdU and anti-4C4 antibodies to detect proliferating cells and microglia, respectively, show microglia do not proliferate in the uninjured retina treated with 400 ng FGF2 + 400 ng IGF-1. Scale bar is 50 μm. (E) Diagram of the experimental protocol used for lineage tracing shown in (F). (F, G) Immunoflourescence using anti-BrdU and anti-retinal cell type-specific antibodies identifies MG-derived progenitors that differentiated into retinal neurons and glia in FGF2;IGF-1-treated eyes. Scale bar (F) is 50 μm. Error bars (G) are SD; n=3. (H) Concentration-dependent inhibition of MG proliferation in injured retinas treated with Mapk inhibitor (UO126) or PI3K inhibitors (Ly294002 and PI-103). (I) TUNEL stain shows Mapk and PI3K inhibitors have little effect on injury-induced cell death. Scale bar is 50 μm. (J) Immunoflourescence using anti-BrdU and anti-GS antibodies shows normal MG numbers and morphology, but reduced MG proliferation after treating injured retinas with Mapk (UO126) and PI3K (Ly294002 and PI-103) inhibitors. Scale bar is 50 μm. Asterisks mark injury sites.

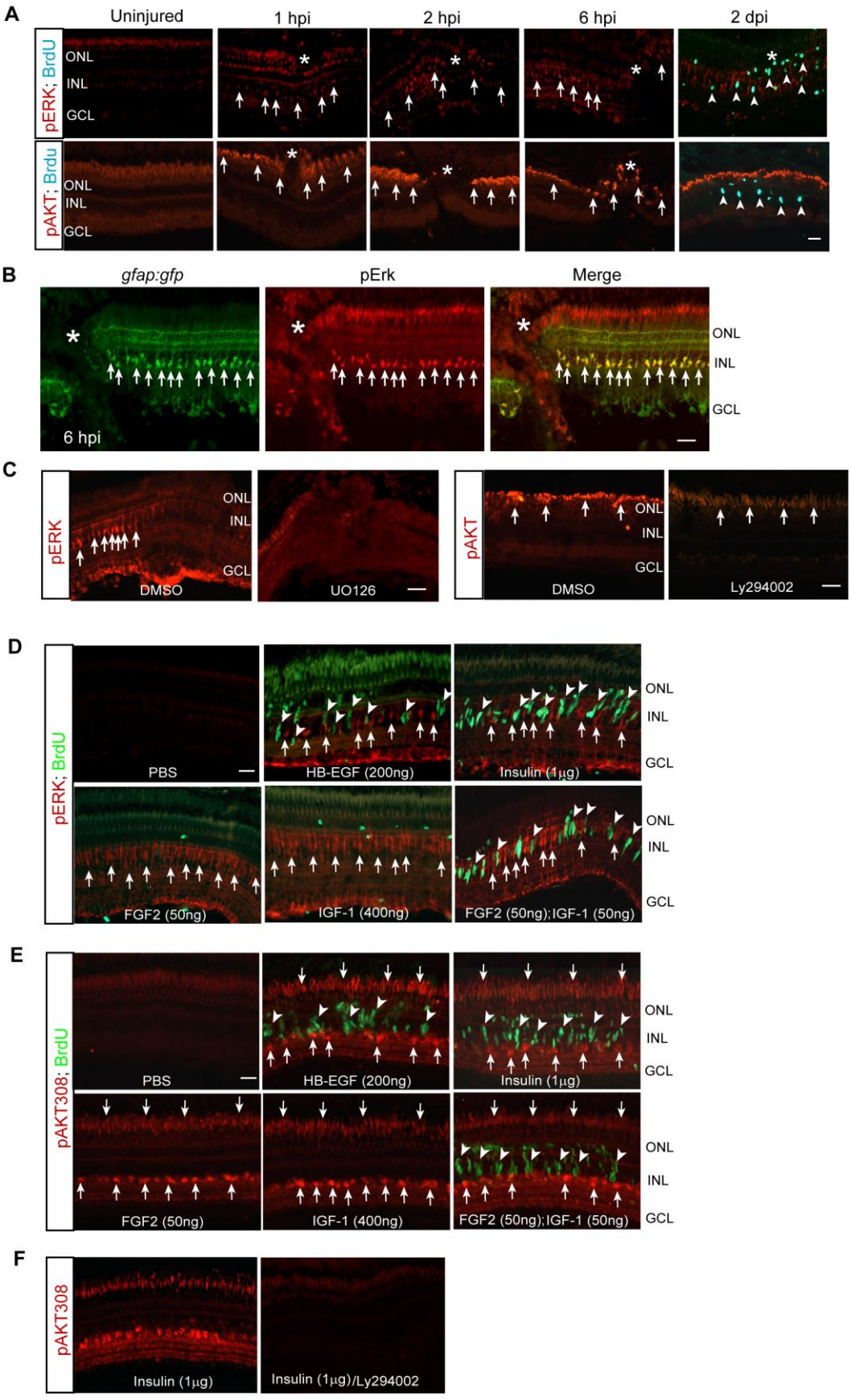
A
Uninjured
1 hpi
2 hpi
6 hpi
2 dpi
pERK; BrdU
pAKT; Brdu
ONL
INL
GCL
B
gfap:gfp
pErk
Merge
6 hpi
C
pERK
pAKT
DMSO
UO126
Ly294002
D
pERK; BrdU
PBS
HB-EGF (200ng)
Insulin (1μg)
FGF2 (50ng)
IGF-1 (400ng)
FGF2 (50ng);IGF-1 (50ng)
E
pAKT308; BrdU
F
pAKT308

**Figure S3, related to Figure 3. pErk and pAkt expression in the injured and growth factor-treated uninjured retina.** (A) pErk, pAkt and BrdU immunofluorescence at various times post retinal injury. Asterisk identifies the injury site. Arrows point to $pErk^+$ and $pAkt^+$ cells; arrowheads point to $BrdU^+$ cells. Scale bar is 50 μm. (B) Retinal injury in *gfap:gfp* transgenic fish shows essentially all $GFP^+$ MG express pErk at 6 hpi. Arrows point to double-labeled cells. Asterisk identifies the injury site. Scale bar is 50 μm. (C) Injury-dependent pERK expression is suppressed by treating retinas with the Mapk inhibitor UO126 and injury-dependent pAKT expression is suppressed by treating retinas with the PI3K inhibitor LY294002. Scale bar is 50 μm. (D) pErk and BrdU immunofluorescence shows that $BrdU^+$ progenitors do not co-label with pErk in the HB-EGF, Insulin or FGF2/IGF-1 treated uninjured retina. Arrows point to $pErk^+$ cells; arrowheads point to $BrdU^+$ cells. Scale bar is 50 μm. (D) pAkt and BrdU immunofluorescence shows that $BrdU^+$ progenitors do not co-label with pAkt in the HB-EGF, Insulin, FGF2/IGF-1 treated uninjured retina. Arrows point to $pAkt^+$ cells; arrowheads point to $BrdU^+$ cells. Scale bar is 50 μm.

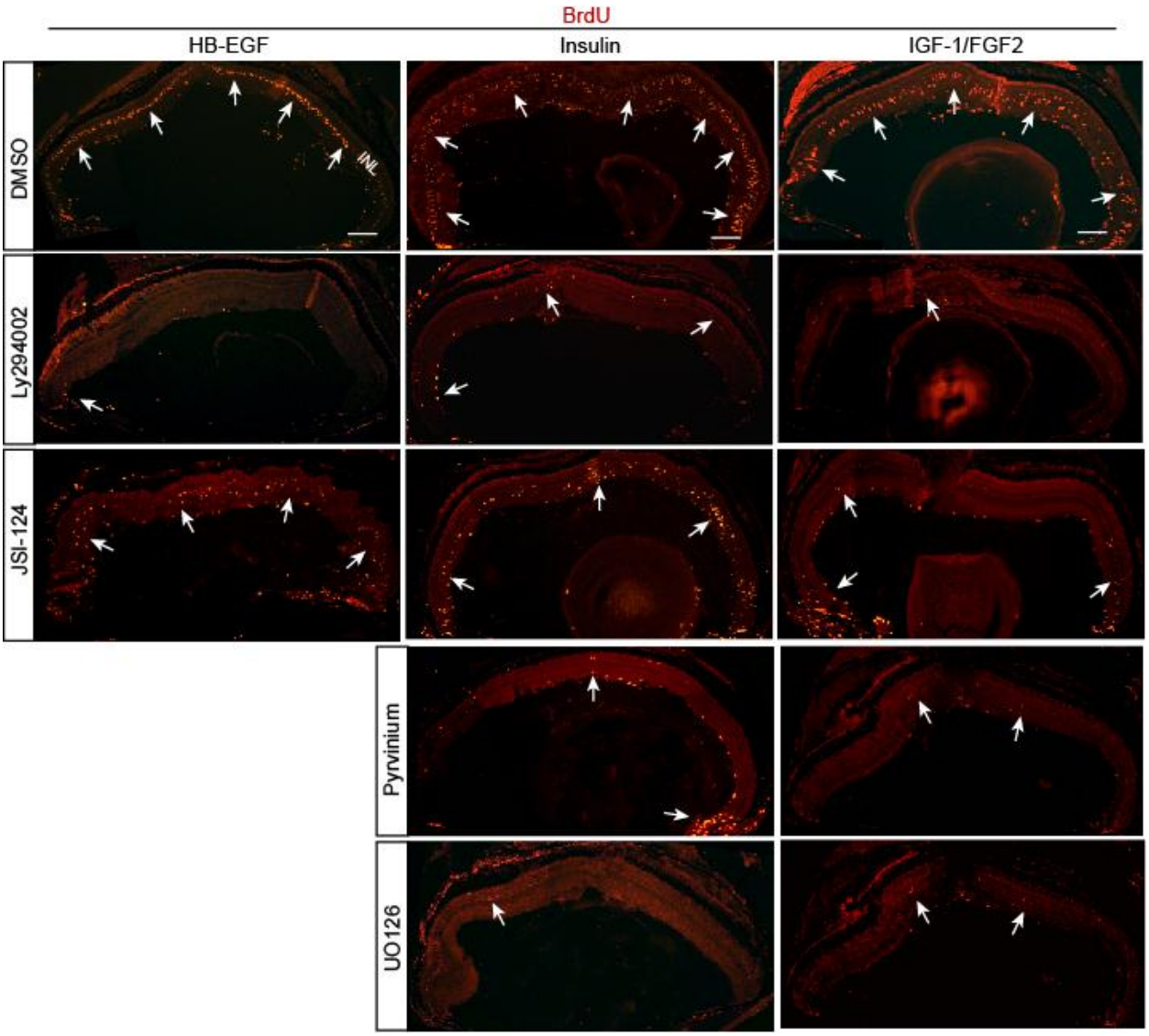


**Figure S4, related to Figure 3. HB-EGF, Insulin and IGF-1/FGF2 stimulate Müller glia proliferation via Mapk/Erk, PI3K, β-catenin and Jak/Stat signaling pathways.** BrdU immunofluorescence shows that Insulin or IGF-1/FGF2-dependent MG proliferation is suppressed by the Mapk inhibitor, UO126; the PI3K inhibitor, Ly294002; the Jak/Stat inhibitor, JSI-124 and the β-catenin inhibitor, pyrvinium. Also shown is inhibition of HB-EGF-dependent MG proliferation by the PI3K inhibitor, Ly294002; the Jak/Stat inhibitor, JSI-124 (See Figure 3E and Wan et al., 2012 for effects of inhibiting the Mapk and β-catenin signaling pathways on HB-EGF-induced MG proliferation). Arrows point to $BrdU^+$ cells. Scale bar is 150 μm.

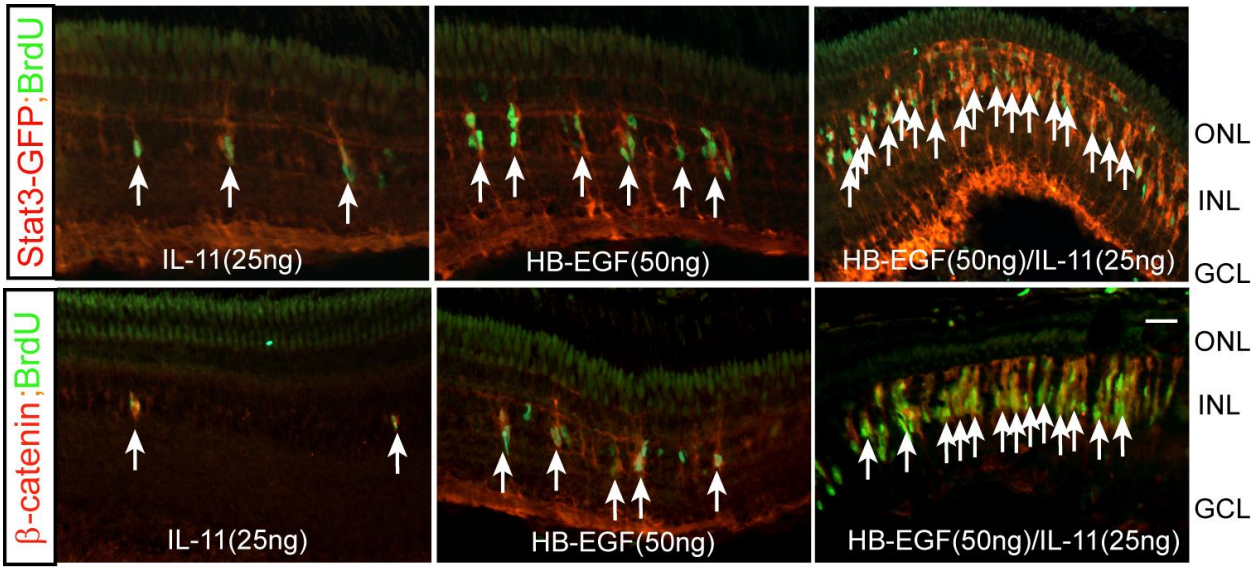


**Figure S5, related to Figure 5. Activated Stat3-GFP and β-catenin are localized to proliferating Müller glia-derived progenitors in the IL-11 and HB-EGF treated retina.** HB-EGF and IL-11 synergize with each other to stimulate the generation of $BrdU^+$ progenitors and the accumulation of Stat3-GFP and β-catenin in the uninjured retina of *gfap:stat3-gfp* transgenic fish. In this figure we used a secondary antibody coupled to a red fluor to detect Stat3-GFP and β-catenin expression. Note that the immunofluorescence observed in the photoreceptor layer of Figures 4 and 5 does not appear, suggesting it is a non-specific signal. Importantly, the specific Stat3-GFP and β-catenin immunofluorescence observed in $BrdU^+$ progenitors is retained. Scale bar is 50 μm.

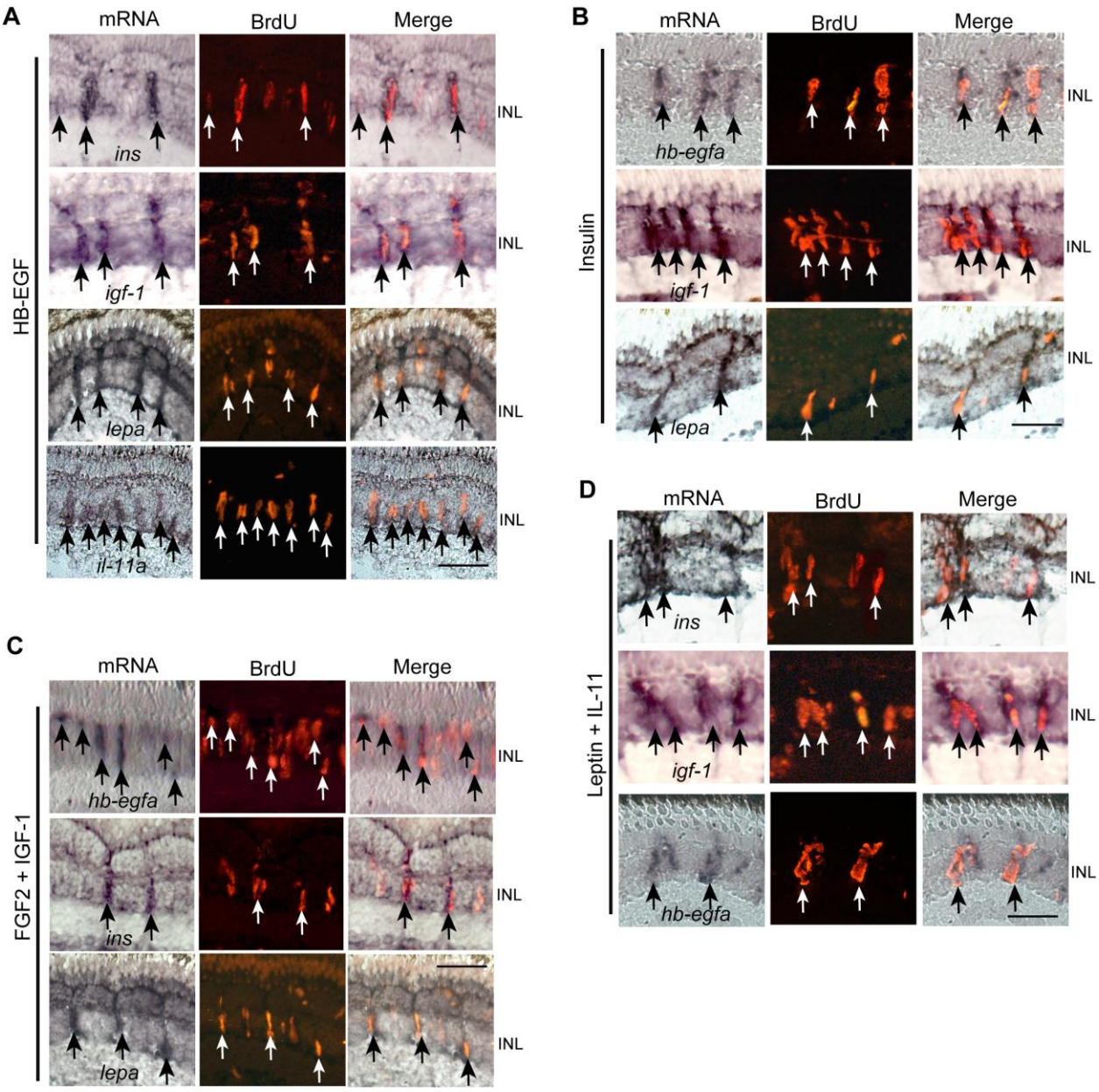


**Figure S6, related to Figure 6. Growth factors and cytokines stimulate growth factor and cytokine gene expression in Müller glia-derived progenitors.** In the uninjured retina treated with HB-EGF (A), Insulin (B), FGF2/IGF-1 (C) or Leptin/IL-11, *in situ* hybridization assays show that the indicated mRNAs were induced in proliferating MG-derived progenitors. Arrows point to $BrdU^{+}$ cells expressing the indicated mRNA. Scale bar is 50 μm.

**Table S1, related to Experimental Procedures. Primers used in this study.**

| **Gene** | **Primers for Real-time PCR** |
|---|---|
| *insra* | F 5' AAAGAGGCGGAAGAGACG<br>R 5' CCATTGGGGAGTGAGGAG |
| *insrb* | F 5' GTTTTTCAACTACGCCCTAG<br>R 5' CGATATAATTGTCCTCCACC |
| *ins* | F 5' TCTACAACCCCAAGAGAGACG<br>R 5' AAAACAAACGGAGAGCATTAA |
| *igf1ra* | F 5' AGCACTCAGGACAGGTAGCG<br>R 5'GACAAAGGGAGGAGGGAAAT |
| *igf1rb* | F 5' ACCTACTACGTGCTCCGCT<br>R 5' GGGTTTTGTCTCGTCCTCC |
| *Igfbp3* | F 5' AAGGGGGACGTGTGAACAT<br>R 5'GGCAGAAACAAGAATTGGG |
| *Igf-1* | F 5' CAATGGAACAAAGTCGGA<br>R 5' GCACAGCACCAGTGAGAG |
| **Gene** | **Primers for PCR** |
| *Igf-1* | F 5' TCCAAATCCGTCTCCTGTTC<br>R 5' ATTCCTCCCGCTGTCCTCTA |
| *ins* | F 5' ACCATTCCTCGCCTCTGCTT<br>R 5' GGGGCTCAACGTCTCTCTTG |
| *hb-egf*$_a$ | F 5'CTGTGATTGATTTATTGGAGG<br>R 5'GTCTGGTTTAGTGGTGTGGAG |
| *leptina* | F 5' TTTCCAGCTCTCCGCTCAACC<br>R 5' CGGCGTATCTGGTCAACATGC |

| | |
|---|---|
| *wnt4a* | F 5’ CAATGCGAGCAACTGGCTATAC<br>R 5’ AATGCAGCTTCCCTCGTACCTT |
| *wnt8b* | F 5’ GGCTCACATGAAGACTTGCTGC<br>R 5’ CAGCAGAACTGATGGCATGGA |
| *il-11a* | F 5’ CTCCTCATCGCTGCTTCTCTCG<br>R 5’ TTGCGAAGTCACTGGCTCTGC |
| | **Primers for amplifying *in situ* hybridization probes** |
| *igf-1* | T7F:5’ TGAATTGTAATACGACTCACTATAGGGCTCTCTACGAGCACAACGACACA<br>T3R:5’ AAGCTCGAAATTAACCCTCACTAAAGGGGGCTTTTGAATAATCCCCTTTG |

**Table S2. $ED_{50}$ and intravitreal concentration of recombinant mammalian growth factors and cytokines. Related to Experimental Procedures.**

| Growth factor cytokine | $ED_{50}$ | Estimated concentration after intravitreal injection* |
|---|---|---|
| Recombinant human HB-EGF | $ED_{50}$: 0.15-0.75 ng/ml. (Rubin, J.S. et al. Proc Natl Acad Sci USA 88:415, 1991; http://www.rndsystems.com/Products/259-HE). | 50 ng = 257 nM<br>200 ng = 1.03 μM |
| Recombinant human Insulin | $ED_{50}$: 0.03-0.15 μg/ml. (Levy et al., Endocrinology 119:1786, 1986; Backer et al., J Cell Biol 115:1535, 1991; http://www.rndsystems.com/Products/1544-IR). | 500 ng = 225 nM<br>2 μg = 900 nM |
| Recombinant human FGF-2 | $ED_{50}$: 0.1-0.6 ng/ml (Raines, E.W. et al. Methods Enzymol. 109:749, 1985; Zeytin and Delellis Endocrinology 121:352, 1987; http://www.rndsystems.com/Products/233-FB). | 50 ng = 140 nM<br>400 ng = 1.12 μM |
| Recombinant human IGF-1 | $ED_{50}$:0.3-1.5 ng/ml (Karey, K.P. et al. Cancer Research 48:4083, 1988; Tollefsen et al., J Clin Invest 87:1241, 1991; http://www.rndsystems.com/Products/291-G1). | 50 ng = 320 nM |
| Recombinant mouse IL-11 | $ED_{50}$ ~5 ng/ml in a cell proliferation assay (Hilton et al., EMBO J. 13:4765, 1994; http://www.rndsystems.com/Products/418-ML). | 25 ng = 65.3 nM<br>100 ng = 2.6 μM |
| Recombinant human Leptin | $ED_{50}$ ~0.4-2 ng/ml in a cell proliferation assay (http://www.rndsystems.com/Products/398-LP). | 62.5 ng = 0.195 μM<br>1 μg = 3.125 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).