Optic nerve regeneration screen identifies multiple genes restricting adult neural repair

Graphical Abstract

Highlights

- Candidate genes are screened for optic nerve regeneration effects by AAV-shRNA
- Regeneration-limiting genes are confirmed by AAV CRISPR-Cas9 gene editing
- Validated regeneration-limiting genes do not show axotomy-regulated expression
- IL-22 loss activates both Stat3 and DLK, with upregulation of multiple pathways

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In brief

Lindborg et al. conduct an unbiased, in vivo, loss-of-function shRNA-AAV screen of nearly 400 candidate genes for optic nerve regeneration and confirm their role by AAV CRISPR-Cas9 gene editing. Together, their studies uncover 40 axon-regeneration-limiting genes and define a multifactorial role for IL-22 in limiting CNS regeneration.

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Optic nerve regeneration screen identifies multiple genes restricting adult neural repair

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SUMMARY

Adult mammalian central nervous system (CNS) trauma interrupts neural networks and, because axonal regeneration is minimal, neurological deficits persist. Repair via axonal growth is limited by extracellular inhibitors and cell-autonomous factors. Based on results from a screen in vitro, we evaluate nearly 400 genes through a large-scale in vivo regeneration screen. Suppression of 40 genes using viral-driven short hairpin RNAs (shRNAs) promotes retinal ganglion cell (RGC) axon regeneration after optic nerve crush (ONC), and most are validated by separate CRISPR-Cas9 editing experiments. Expression of these axon-regeneration-suppressing genes is not significantly altered by axotomy. Among regeneration-limiting genes, loss of the interleukin 22 (IL-22) cytokine allows an early, yet transient, inflammatory response in the retina after injury. Reduced IL-22 drives concurrent activation of signal transducer and activator of transcription 3 (Stat3) and dual leucine zipper kinase (DLK) pathways and upregulation of multiple neuron-intrinsic regeneration-associated genes (RAGs). Including IL-22, our screen identifies dozens of genes that limit CNS regeneration. Suppression of these genes in the context of axonal damage could support improved neural repair.

INTRODUCTION

Neuronal injuries cause debilitation and pain with few palliative options and no curative treatments. Achieving neuroprotection and neuroregeneration after injury remains a challenging and elusive goal. Glaucoma is characterized by retinal ganglion cell (RGC) axon degeneration and cell death, with vision loss (Weinreb et al., 2014), and overlaps with experimental optic nerve crush (ONC) (Templeton and Geisert, 2012). After ONC injury, less than 1% of spared axons regenerate and more than 80% of RGCs die (Nadal-Nicolás et al., 2015; Tran et al., 2019).

Although no intervention achieves full recovery, much has been learned about why axons fail to recover. Gial-based mechanisms extrinsic to neurons, including scar-derived chondroitin sulfate proteoglycans and myelin-associated inhibitors, have a substantial role (GrandPré et al., 2000; McKeon et al., 1991; McKerracher et al., 1994; Wang et al., 2002). Chondroitinase ABC augments axon sprouting and functional recovery after spinal cord injury (Bradbury et al., 2002; Cafferty et al., 2007; Massey et al., 2006). Targeting of individual myelin proteins or axonal Nogo receptor NgR1 enhances axon growth and functional recovery after CNS injury (Bregman et al., 1995; Fischer et al., 2004; Fournier et al., 2001; GrandPré et al., 2002; Wang et al., 2011, 2020a; Zai et al., 2011). Limited glial production of growth-promoting extracellular molecules also diminishes axon growth. For example, activators of the Jak-Stat pathway significantly enhance cultured RGC neurite outgrowth and neuronal survival (Leibinger et al., 2009).

Separate from the axon’s extracellular environment, adult central nervous system (CNS) neurons have limited intrinsic ability to regenerate axons. In contrast, peripheral nervous system (PNS) neurons respond adeptly to axotomy with upregulation of regeneration-associated genes (RAGs) and substantial regeneration. Strong induction of Atf3 (activating transcription factor 3) and Sprr1a (small proline-rich repeat protein 1a) demarcate the enhanced regeneration of axotomized PNS neurons (Bonilla et al., 2002; Hyatt Sachs et al., 2007; Starkey et al., 2009; Tsujino et al., 2000). Enhancing the intrinsic growth potential of RGCs leads to greater axon regeneration. Suppression of neuronal Pten (phosphatase and tensin homolog) or Socs3 (suppressor of cytokine signaling 3) expression disinhibits the Akt/mTOR and Jak-Stat signaling pathways, respectively, to support axon regeneration (Park et al., 2008; Smith et al., 2009). However, incomplete axonal restoration and the challenges of functional recovery compel more complete discovery of axon regenerative mechanisms.

Previously, we surveyed the mouse genome in an unbiased approach for loci that affect axon regeneration (Sekine et al., 2018). Using lentiviral short hairpin RNAs (shRNAs) to survey 17,000 genes after cortical neuron axotomy, we identified more than 400 genes whose suppression supported axon...
regeneration. Here, we leverage those in vitro results to characterize pathways that can be targeted to improve CNS neural repair in vivo. Using shRNA-AAV (adeno-associated virus) and CRISPR-AAV-mediated inhibition, we assayed 392 genes and identified 40 targets whose loss-of-function after ONC produced enhanced RGC regeneration. We studied one gene, interleukin-22 (IL22), in greater detail because of its bioinformatic linkage to the Jak-Stat signaling pathway and robust effects in multiple tests. We find that IL-22 inhibition leads to increased transcription factors, cytokines, and proteins critical for CNS regeneration. Conversely, exogenous IL-22 application abrogated expression of many pro-inflammatory and pro-regenerative factors. Together, our studies uncover more than three dozen genes limiting axon regeneration in vivo and define a multifactorial role for IL-22 in limiting axon regeneration.

**RESULTS**

**In vivo screen of candidate regeneration limiting genes by shRNA-mediated gene silencing**

Previously, we screened about 17,000 mouse genes for their functional role in CNS axonal regeneration, identifying more than 400 genes whose suppression via lentiviral shRNA knockdown stimulated regeneration in cortical neuron cultures after injury (Sekine et al., 2018). Here, we tested 392 of those genes individually by in vivo loss-of-function in optic nerve regeneration with delivery of recombinant AAVs expressing specific shRNA and GFP (Figure 1A; Table S1). High infection efficiency and minimal provocation of an immune response make AAVs an attractive option for knockdown and editing in animal studies (Daya and Berns, 2008). The shRNA sequence for a particular gene was selected based on the highest regeneration score from the in vitro regeneration screen. ONC surgeries were preceded, 14 d earlier, by intravitreal injections of shRNA-AAV and followed 14 d later by injection of cholera toxin β (CTB) subunit conjugated to a fluorescent dye to anterogradely trace axons (Figure 1B). Efficient transduction of retinal cells, including RGCs, was achieved with recombinant AAV serotype 2/2 (Hellstrom and Harvey, 2011; Leaver et al., 2006; Petr-Peterson et al., 2009), and AAV infection was confirmed with a GFP reporter in retina whole mounts (Figure S1A). Regeneration of CTB-labeled axons in the optic nerve extending 500 µm distal to the crush site for 392 genes was counted for an average seven nerves per gene, from 2,851 separate eyes (Figure 1C). The scrambled-sequence control background averaged eight axons (5.8–10.6, 95% confidence interval) compared with a positive control from Pten knockdown of 148 axons (111–186, 95% confidence interval) (e.g., Park et al., 2008; Yunghe et al., 2015; Zukor et al., 2013).

This screen identified 40 genes with increased regeneration phenotypes relative to the control, as defined either by significant increases of the average number of axons compared with the scrambled sequence ($p < 0.05$) or by an average more than one control standard deviation above the scrambled value (Figures 1C–1E; Table S2). Of interest, Rassf3 and Tbc1d22b are two of several genes whose inhibition had the most significant effect on axon regeneration with counts nearly six times greater than that of the control. Rassf3 is a protein associated with the Ras family of small GTPases having a central role in RAFT/MEK/ERK signaling-mediated mechanisms of neuronal survival and repair (Zhong, 2016), whereas Tbc1d22b is part of the Rab-GTPase-activating protein domain with an as-yet-unknown Rab protein association (Ishibashi et al., 2009). Rab proteins have been implicated in axonal growth (Hernandez-Deviez et al., 2004), and our unbiased genome-wide cortical neuron regeneration
Figure 2. CRISPR-Cas9 gene editing in cortical neurons leads to targeted DNA, RNA, and protein reduction

(A) Schematic illustration of sgRNA-AAV and Cas9-AAV expression vectors. The sgRNA-AAV vector encodes an EGFP sequence for identification of infected retinal cells.

(B) Representative image and quantification of P1 cortical neurons co-transduced with sgRNA-AAV (green) and Cas9-AAV and stained with NeuN (red) to determine neuron-specific infection. Scale bar, 100 μm.

(C) Cellular makeup of untransduced cortical neuron cultures showing quantification and representative images of cultures stained with NeuN (green) and either GFAP (astrocytes), Iba1 (microglia), PDGFRα (oligodendrocyte precursors), or O4 (oligodendrocytes) (red). DAPI (blue) labels cell nuclei. Counts for each cell marker were made in ImageJ and were averaged across 10,350-μm x 350-μm images from different wells. Scale bar, 100 μm.

(D) Evaluation of Pten locus targeting using cortical neurons co-transduced with Cas9-AAV and one of two different sgRNA-AAVs targeting Pten (labeled Pten-1 and Pten-3). sgRNA-AAV targeting LacZ was used as the control and PCR was amplified using Pten-1 primers. Locus modification efficiencies were analyzed 14 d after transduction with a T7EI endonuclease assay in the presence (+) or absence (−) of the enzyme. Detected indel formation (%) within the Pten locus is indicated.

(E) Relative expression of Pten in cortical neurons 14 d after sgRNA-AAV and Cas9-AAV co-transduction was quantified as comparative Ct method (ΔΔCt) normalized to Gapdh. n = 3 samples of ~300,000 neurons each. **p < 0.01, ****p < 0.0001, one-way ANOVA with Tukey’s test.

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assessments revealed a functional role for 10 different Rab proteins in axon regeneration (Sekine et al., 2018). These findings affirm the translatability of our in vitro to in vivo regeneration screens and highlight 40 promising regeneration-limiting genes to target for further study.

**Axon regeneration after editing of regeneration-limiting genes by CRISPR-Cas9**

Having identified multiple in vivo axon regeneration phenotypes by shRNA knockdown, we further assessed those genes by CRISPR-Cas9 gene editing as validation of the shRNA knockdown phenotypes. To that end, we employed the dual-vector CRISPR system that packages single-guide RNA (sgRNA) and Cas9 (from *Streptococcus pyogenes*) cassettes in different viral vectors (Figure 2A; Table S3; Swiech et al., 2015). Cas9 expression is driven by the Mecp2 promoter, which isolates gene editing to neuronal populations. An sgRNA species targeting the bacterial enzyme β-galactosidase (LacZ) was used as a non-targeting control. Mouse cortical neuron cultures from P1 pups were used to quantify editing at the DNA, RNA, and protein levels. Neurons were co-transduced with sgRNA-AAV and Cas9-AAV at 3 d in vitro (DIV) and harvested for downstream applications at DIV17. The culture composition was 80% NeuN+ neurons, with the remaining cells being 8% GFAP+ (gliarial fibrillary acidic protein positive) astrocytes and 12% PDGFRα+ or O4+ oligodendrocyte lineage cells (Figure 2C). An 81% cortical neuron transduction efficiency was achieved with AAV serotype 2/1 (Figure 2B, Hammond et al., 2017). Two to five sgRNA species were generated for each gene because of variability in editing efficiency. For Pten, two sgRNAs, Pten-1 and Pten-3, showed remarkably different editing efficiencies at the DNA (Figure 2D), mRNA (Figure 2E; Table S4), and protein levels (Figure 2F). The reduction of DNA, mRNA, and protein for the Pten-1 versus Pten-3 species was 15% versus 82%, 32% versus 78%, and 80% versus 98%, respectively. Pten-3 (referred to hereafter as “Pten”) produced greater editing overall and was, therefore, selected for use in subsequent assays.

It is worth noting that although DNA editing efficiency was low for the Pten-1 species, its minimal disruption in the Pten locus was sufficient to cause a significant decrease in protein expression. Likewise, for the positive regeneration gene Rassf3, minimal disruption at the sgRNA-targeted DNA locus supported substantial protein reduction (Figure 2G). These findings support the view that downstream changes in protein expression, rather than DNA, may be more of an appropriate readout of editing efficiency, consistent with previous studies (Nelson et al., 2016; Swiech et al., 2015).

Subsequently, all 40 genes were tested in the cortical neuron regeneration assay using the CRISPR system (Figure 2H; Table S2). Axons were injured at DIV17 using a pin tool and analyzed for regeneration using anti–βIII-tubulin staining at DIV25. sgRNA-AAV targeting enhanced regeneration to a statistically significant level for 28 of the 40 genes, compared with the non-targeting LacZ control.

Next, we analyzed in vivo regeneration after sgRNA-AAV delivery (Figure 3A) using the viral injection and ONC injury model outlined for shRNA studies in Figure 1B. Because dual AAV injections may reduce infection efficiency in vivo (Carvalho et al., 2017), we used Cas9 transgenic mice in conjunction with single AAV delivery of sgRNAs at 10^{12}–10^{13} genome copies/mL. Transgenic mice express Cas9 endonuclease driven by a CAG promoter (Figure 3B), which expands the probability of editing in non-neuronal populations. Intravitreal injection of AAV serotype 2/2 is selective for the retinal ganglion layer (Leibinger et al., 2013a), but it can also infect Müller glia in the inner nuclear layer with a lower efficiency (Liang et al., 2003; Pang et al., 2008). Approximately 50% of RGCs were infected with any given sgRNA-AAV, as judged by GFP overlap with Rbpms antigenicity (Figures S1B and S1D), and, consistent with previous reports (Leibinger et al., 2013a), about 90% of AAV2-infected cells were RGCs (Figures S1C and S1D). Thus, direct effects on RGCs are likely to predominate, but we cannot completely rule out cell non-autonomous effects.

Optic nerve analysis for the 40 genes using CRISPR editing highlighted nine genes with significantly more regeneration phenotypes compared with LacZ (Figures 3A and 3C; Table S2). Only two sgRNA species were generated for most of the 40 genes, and likely, the reduction in the number of positive regeneration hits compared with shRNA can be attributed to the selection of suboptimal targeting sequences. Of the positive regeneration genes using sgRNA, the four most potent were Myl10 (myosin light chain 10), Airn (antisense of insulin growth factor 2 receptor [Igf2r] non-protein coding RNA), Prg2 (proteoglycan 2, proMMP), and IL-22. All showed comparable axonal regeneration to Pten, in addition to axon extension well beyond the 500-μm distance of measurement (Figure 2E). IL-22, a cytokine that participates in Jak-Stat signaling, (Leibinger et al., 2013a), about 90% of AAV2-infected cells were RGCs (Figures S1C and S1D). Thus, direct effects on RGCs are likely to predominate, but we cannot completely rule out cell non-autonomous effects.

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PNS and CNS regeneration and repair are well documented (Dupraz et al., 2013; Rabinovsky, 2004; Toy and Namgung, 2013; Wang et al., 2020b). Critically, this in vivo screen has identified many genes not previously linked to axon regeneration; all of which were identified by genome-wide shRNA screening in vitro, confirmed by in vivo knockdown studies in a different CNS population, and validated by gene editing both in vitro and in vivo.

**Effect of axon regeneration gene editing on RGC survival**

Some axon regeneration-promoting genes, such as DLK (dual leucine zipper kinase), and regeneration-inhibiting genes, such as Pten, participate in axotomy-induced RGC death (Park et al., 2008; Watkins et al., 2013). Therefore, we considered whether the CRISPR gene editing leading to enhanced regeneration after injury might be linked to changes in RGC survival. We assayed the number of RGCs 14 d after ONC, when approximately 80% of these neurons have undergone apoptosis (Berkelaar et al., 1994; Nadal-Nicolás et al., 2015; Tran et al., 2019). Survival was measured after sgRNA-AAV-targeting of the top six regeneration genes identified in Figure 3A. Retinal Pten^{fl/fl} mice and AAV-Cre delivery (45% in Park et al., 2008), this gap can be attributed to the different models used and the achieved degree of editing. In addition to Pten, editing of Xylt1 (xylosyltransferase1) rescued a small but significant percentage of RGCs from death. Editing of Myl10 showed a trend toward increased RGC numbers 14 d after injury, whereas IL-22, Prg2, and Airn did not alter survival. Reduction of Rassf3 expression significantly lowered survival, suggesting it has opposing effects on survival and regeneration.

To compare the strength of gene effects on regeneration and survival, the in vivo axon regeneration was normalized to RGC survival as a ratio (Figure S2D). Five genes had a regeneration index, per surviving RGC, equal to or greater than Pten, demonstrating improved axon regeneration irrespective of RGC survival, even for the presumptive survival-promoting Myl10 editing. For Xylt1, we observed a survival effect comparable to Pten, but a weaker effect on regeneration. In our recent single-cell RNA sequencing (scRNA-seq) dataset of adult mouse RGCs (Tran et al., 2019), Xylt1 was the mostly highly expressed in intrinsically photosensitive RGCs. However, it is unclear whether the survival and regeneration effects were specific to that population.
Axotomy regulation of regeneration-limiting gene expression

Insight into injury-induced gene expression changes and pathways shared with known axon regeneration genes may inform us about general mechanisms of repair. Our recent RGC scRNA-seq dataset analyzed genome-wide expression patterns across 46 adult mouse RGC types before and after ONC (Tran et al., 2019). We first used this dataset to assess expression in control RGCs. Transcripts for 248 of the 392 (63%) targeted genes assayed in Figure 1C had detectable expression profiles in RGCs (Figure 4A; Table S5). Of the 40 positive regeneration hits in Figure 1D; 24 (60%) are expressed in at least 1% of the RGCs that were designated as having a lesser regeneration phenotype after ONC.

We next assessed whether any of the 40 genes exhibited altered expression after injury. All genes detected in the control cells were also observed after injury, and none of the 40 positive regeneration hits overlapped with the global differentially expressed genes (771 genes) identified in our scRNA-seq dataset (Tran et al., 2019; Figure 4B; Table S6). Thus, the current screen identified targets whose expression is not globally altered by axonal injury, although some genes (e.g., Xylt1) were upregulated in a small subset of cells (Figure 4C). This finding was, perhaps, not unexpected, as previously described genes with inhibitory roles in RGC axon regeneration (e.g., Pten), also sustain relatively constant baseline expression levels with injury.

Figure 4. Regeneration genes are largely not regulated by axotomy and participate in multiple pathways

(A) Scatterplot showing expression of regeneration gene targets (average number of transcripts in expressing cells) and total fraction of RGCs with detected transcripts in control RGCs. The dotted gray line is drawn at 0.01 and demarcates a threshold for genes expressed in 1% of cells (below threshold targets are colored gray dots). Twenty-four of the 40 positive regeneration genes (red dots) are expressed in >1% of RGCs. Black dots indicate genes expressed in >1% of RGCs that were designated as having a lesser regeneration phenotype after ONC.

(B) Scatterplot showing average expression of regeneration gene targets and fraction expressing cells in injured RGCs (merged data from 0.5, 1, 2, 4, 7, and 14 d after ONC).

(C) Expression levels before and 14 d after ONC of the 24 positive regeneration genes expressed in >1% of RGCs. The overall average expression level is calculated as (average number of transcripts in expressing cells) x (total fraction of RGCs with detected transcripts). “Atlas” data are the average expression level from 35,699 P56 mouse RGCs pooled across types.

(D) The interaction network for the list of regeneration genes constructed using STRING database. Known and predicted interactions, as well as co-expression and text-mining, are shown by connecting lines.

(E) Expansion of the interaction network for genes with cytokine signaling function (IL-22, IL-17c, IL-17rb, and Soc5) identifies Stat3 as a binding partner for IL-22, one of the top regeneration genes identified in Figures 1, 2, and 3. See also Figures S3 and S4 and Tables S5, S6, and S7.
AAV-infected GFP+/Rbpms+/Brn3a+ RGCs were collected by retina using ELISA (Figure S4D) and immunoblotting (Figure S4E), translated into a significant reduction of IL-22 protein in the total in vivo specifically in RGCs after analyzed for enrichment in protein-protein interaction (PPI) network of RGCs. Among the four genes with involvement in Jak-Stat signaling, among those genes using STRING database, is a recognized regulator of regeneration (Qi et al., 2005). Among the four genes with involvement in Jak-Stat signaling, IL-22 editing showed the most significant regeneration phenotype in vivo (Figures 3A and 3C). Therefore, we explored the mechanism by which IL-22 editing supports optic nerve regeneration in more detail.

Expression of IL-22 and related signaling components
To initiate analysis of IL-22, we assessed the RGC expression profile of IL-22 and its co-receptors, IL-10Rb and IL-22Ra1 (Kotenko et al., 2001; Xie et al., 2000). IL-22 and IL-10Rb were expressed at low levels in RGCs but were present across many RGC types (Figure S3A). Uninjured whole-retina RNA-seq analysis of IL-22 levels reports expression in multiple neuronal populations, including RGCs, amacrine cells, rods, cones, and bipolar cells (Figure S3B; Macosko et al., 2015; Shekhar et al., 2016; Tran et al., 2019; Yan et al., 2020). Low or absent levels of IL-22 are found in non-neuronal cell populations. Because AAV2/2 delivery of sgRNA targets predominantly RGCs (approximately 90%; Figures S1C and S1D), we asked whether the IL22 gene was edited specifically in RGCs after in vivo AAV delivery of IL-22 sgRNA. AAV-infected GFP+/Rbpms+/Bm3a+ RGCs were collected by fluorescence-activated cell sorting (FACS) 14 d after intravitreal injection. With targeted next-generation sequencing of a 302-bp PCR amplicon, we confirmed indel mutations in the IL-22 locus of RGCs; most of which displayed a single base-pair insertion or deletion (Figures S4A–S4C). Editing of RGCs at the DNA level translated into a significant reduction of IL-22 protein in the total retina using ELISA (Figure S4D) and immunoblotting (Figure S4E), resulting in a large increase in the number of regenerating RGC axons under those conditions (Figure 3A).

In contrast to expression of IL-22 and IL-10Rb, IL-22Ra1 expression was not detected in RGCs (data not shown). Immunostaining with an anti-IL-22Ra1 antibody indicated that IL-22Ra1 expression is chiefly restricted to the ganglion cell layer (GCL) but did not colocalize with Rbpms+ RGC somas or IL-22-sgRNA-infected GFP+ cells (Figures S3C–S3E). Visualization of the GFAP+ glial population, which does express IL-10Rb (Burmeister and Marriott, 2018; Shekhar et al., 2016), showed some astrocytes with GFAP+ filaments surrounded by IL-22Ra1, although colocalization was not observed (Figure S3E, left inset). Indeed, there were some IL-22Ra1+ cells spatially distinct from GFAP+ astrocytes (Figure S3E, right inset). These data suggest that IL-22 released from neurons (RGCs or other neuronal cells) may limit axon growth by autocrine signaling via RGC-expressed IL-10Rb, by paracrine signaling via immune or glial-expressed IL-22Ra1, or by a combination of both modalities. Regardless, the apposition and confinement of IL-22, IL-10Rb and IL-22Ra1 expression to the GCL supports the hypothesis that axon regeneration triggered by IL-22 inhibition is initiated from RGC-intrinsic changes of the secreted cytokine and local action within the GCL.

IL-22 editing initiates a pro-regenerative transcriptional program including Stat3, DLK, and RAGs
A hallmark of successful nerve regeneration after injury is the stimulation of a neuron’s intrinsic growth capacity (Ma and Willis, 2015). Because RAG upregulation is often a clear indication of the regenerative state of neurons, we evaluated how effectively IL-22 inhibition primed RGC neurons to regenerate. Transcriptional activation of RAGs was measured 3 d after injury. We observed a 2-fold increase in Atf3 mRNA in edited retina compared with unedited retina, in addition to marked 4-fold and 3-fold increases in Spp7a and Galanin transcripts, respectively (Figure 5A). Furthermore, a substantial increase in Atf3 immunoreactivity was observed in the GCL of IL-22-edited retinas 3 d after injury compared with non-targeting LacZ (Figure 5B).

We next sought to identify signaling changes that link IL-22 suppression to RAG activation. Stat3 is a key factor in promoting axon regeneration, in large part via its upregulation of RAG expression (Mehta et al., 2016; Smith et al., 2011). Stat3 transcript levels were quantified 3 d after ONC (Figure 5C). Both LacZ- and IL-22-edited retinas displayed a marked increase over their respective uninjured controls, but a significantly larger increase in Stat3 was detected in injured and IL-22-edited retina compared with the non-targeting injured control. The retinal distribution of phosphorylated Stat3 (p-STAT3) 14 d after injury was localized predominantly in RGCs in the GCL of IL-22-edited retinas (Figure S5A). Astrocytes also expressed p-STAT3 after injury (Figure S5B). Similar to RGC-specific p-STAT3 expression, induction of p-STAT3 in astrocytes has historically been shown to support neuronal regeneration in acute models of injury (Anderson et al., 2016). Assessment of the total protein levels in the retina confirmed that IL-22 inhibited significantly enhanced p-STAT3 expression 14 d after ONC compared with injured control retina (Figure 5D). Remarkably, injection of sgRNA-AAV targeting IL-22 was sufficient to cause significant enrichment of activated p-STAT3 protein, with levels exceeding that observed in injured and unedited retina. Thus, IL-22 editing partially bypasses the requirement for injury to drive Stat3 activation.

We considered whether endogenous IL-22 levels were at saturating concentrations with respect to that of post-axotomy RAG
Figure 5. Reduction of IL-22 drives a pro-regenerative RAG and Stat3 expression program in the retina after injury

(A) Relative mRNA expression of RAGs in the retina after LacZ or IL-22-targeted sgRNA-AAV infection and 3 d after ONC was quantified using the ΔΔCt method normalized to the housekeeping gene Gapdh. n = 4 retinas/group/time. Data are means ± SEM of comparative quantification (ΔΔCq), with one-way ANOVA of Ln-transformed values and Tukey’s test. **p < 0.01, ***p < 0.001, ****p < 0.0001.

(B) Quantification and representative images of Atf3 immunostaining in LacZ- or IL-22-sgRNA-targeted retinas 3 d after injury. n = 5 retinas/group. Data are means ± SEM. **p < 0.01, Student’s t test. Scale bar, 50 μm. GCL, ganglion cell layer. A threshold fluorescence was used to count Atf3+ cells. Counts are from four separate retina cross-sections from each animal. The GCL was identified by DAPI, and counts were normalized to the layer area.

(C) Relative Stat3 mRNA expression in retina after LacZ- or IL-22-targeted sgRNA-AAV infection and 3 d after ONC was quantified using ΔΔCt normalized to Gapdh. n = 4 retinas/group/time. Data are means ± SEM of ΔΔCq, with one-way ANOVA of Ln-transformed values and Tukey’s test. *p < 0.05, ***p < 0.001, ****p < 0.0001.

(D) Retinal p-Stat3 protein expression was quantified 14 d after ONC. IL-22 editing with injury significantly increases p-Stat3 protein levels compared with the LacZ control. n = 3 retinas/group/time. Data are means ± SEM. *p < 0.05, Student’s t test within uninjured or injured groups because experiments were performed separately.

(E) Intravitreal delivery of r-IL-22 increased IL-22 protein in the retina 1 d after injection.

(F) PBS or r-IL-22 was injected into the vitreous cavity of WT mice 1 d before ONC. Retinas were isolated 3 d after injury for qPCR. Relative mRNA expression in the retina 3 d after ONC was quantified as ΔΔCt normalized to Gapdh and compared with controls, the average values of injured LacZ-AAV and PBS retinas. n = 4 retinas/group/time. Data are means ± SEM of ΔΔCq, with one-way ANOVA of Ln-transformed values and Tukey’s test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. sgRNA LacZ and IL-22 qPCR raw data from (A) were used for analysis.

See also Figure S5 and Table S4.
expression by injecting excess recombinant IL-22 (r-IL-22) into the vitreous. r-IL-22 was injected 1 d before injury because marked increases in IL-22 protein were detected in the retina at that post-injection time point compared with the PBS-injected control retina (Figure 5E), which made it suitable for assessing the effects of increased IL-22 on RAG expression. Four days after r-IL-22 injection and 3 d after injury, we measured retinal Stat3 and RAG transcript expression in comparison to that of control and IL-22 loss-of-function gene-edited retina (Figure 5F). Increased levels of retinal IL-22 significantly diminished Atf3 expression relative to both the control and the IL-22-edited eyes. For other RAGs and Stat3, excess IL-22 retina yielded expression levels significantly less than it did for sgRNA-IL-22 retina, and non-significantly less than it did for control eyes. These data define a monophasic function for suppression of Stat3 and RAG expression by IL-22 after axotomy, which bears saturation at physiological levels of IL-22.

In addition to Stat3, induction of RAG expression is aided by the retrograde transport of injury-induced axonal signals (Ben-Yaakov et al., 2012; Cox et al., 2008). DLK is one such critical signal that not only engages RAG cascade activation but also ferries axonal p-STAT3 to the cell body to augment a neuron’s pro-regenerative program (Shin et al., 2012). Because DLK has also been shown to initiate regenerative responses in RGCs (Watkins et al., 2013), we evaluated its expression in the retina 3 d after injury. scRNA-seq analysis of Dlk expression in RGCs revealed a non-significant trend to increase early after injury with a decrease by 4 days after ONC (Figure 6A; Tran et al., 2019), most likely because of the initiation of RGC death. In line with our scRNA-seq dataset, unchanged levels of Dlk transcripts were measured between uninjured and injured control retinas 3 d after injury, and a slight yet significant increase in transcript levels between uninjured and injured IL-22-edited samples (Figure 6B). Injection of r-IL-22 did not significantly alter expression compared with control baseline (Figure 6C). Notably, significant increases in Dlk protein were observed with IL-22 editing 14 d after injury by immunoblotting (Figure 6D). Few Dlk+ cells were observed in the GCL of control retinas compared with that of IL-22-edited retinas 14 d after injury (Figure 6E). After injury and IL-22 inhibition, Dlk expression increases in both jilI1-tubulin+ neuronal and GFAP+ non-neuronal populations in the GCL. Those cellular expression patterns of Dlk are similar to the findings in the uninjured whole-retina RNA-seq datasets, in which Dlk is expressed in all retinal cell types (Figure SSC; Macosko et al., 2015; Shekhar et al., 2016; Tran et al., 2019; Yan et al., 2020). Here, we find that a population of neurons in the GCL upregulate Dlk after IL-22 editing, and these molecular changes are concurrent with enhanced regeneration of RGC axons. Taken together, these results suggest that amplified expression of RAGs after IL-22 inhibition is driven by both increased Dlk and activated Stat3. This triad of neuronal changes that occur because of IL-22 editing demonstrate a shift of RGCs to a primed regenerative state.

Potential mediators of Stat3 activation after IL-22 inhibition
To establish a connection between IL-22 suppression and Stat3 activation, we surveyed retinal expression of multiple Stat3-activating cytokines in response to IL-22 loss. Early transcript changes were analyzed 3 d after ONC in IL-22-edited and unedited retinas (Figure 7A). Lif mRNA was significantly increased by 3-fold in injured IL-22-edited retinas compared with the control. Importantly, Lif is a member of the glycoprotein-130 (gp130)-activating cytokine family, which is neuroprotective and activates Stat3 (Leibinger et al., 2009, 2013a). IL-22 silencing also stimulated a 6-fold and a significant 13-fold increase in Il1β and Il6 mRNA transcripts, respectively, over injured LacZ control. IL-6, similar to Lif, is a member of the gp130 cytokine family that activates Stat3 and promotes axon regeneration in both CNS and PNS neurons (Cao et al., 2006; Leibinger et al., 2013b; Yang et al., 2012). IL-22 belongs to a family of cytokines that includes IL-10, IL-19, IL-20, and IL-24 (Sabat, 2010), all of which signal via the Jak-Stat3 pathway (Kiu and Nicholson, 2012; Kragstrup et al., 2018). Of those, we observed large transcript increases across all species after IL-22 editing and injury compared with the injured control. Most notably, Il10 and Il24 boasted transcript increases six times and 28 times, respectively, higher than that of LacZ. Clearly, loss of IL-22 stimulates a shift in the expression of cytokines that are well known in their role of potentiating Stat3 activation leading to axon regeneration.

The increased expression of pro-inflammatory cytokines suggests an enhanced immune reaction in response to the loss of IL-22. Therefore, we evaluated markers of immune and glial cell activation after IL-22 editing in the injured retina. Substantial increases in transcript expression of the microglia/macrophage chemokine Ccl2 was observed 3 d after ONC in IL-22-edited retinas compared with that of the control (Figure 7B). These microglia/macrophage injury-induced changes were concurrent with significant increases in Cds8 transcript levels in both edited and unedited retinas, but a larger increase was observed after IL-22 inhibition. Notably, IL-22 editing was sufficient to cause a significant increase in Ccl2 transcript over that of LacZ in uninjured tissue, suggesting possible changes in cell chemotaxis or activity before injury. The observed increases in Ccl2 mRNA with IL-22 loss are supported by a prior study that reported reduced Ccl2 transcript in the retina after intravitreal injection of recombinant IL-22 (Mattapallil et al., 2019). Significant increases in mRNA for injured, unedited and IL-22-edited retinas, compared with their respective uninjured controls, were also observed for the reactive astrocytic indicator Gfap, although no differences between injection groups were noted. Evaluation of GFAP in the retina 3 and 14 d after ONC did not reveal any differences in astrocyte reactivity between IL-22-edited and unedited retinas (Figures S6A and S6B).

Greater immune cell activity after IL-22 inhibition
To characterize the immune cell response to IL-22 suppression, we analyzed the localization of microglia/macrophages among retinal layers after injury. Their distribution showed a specific cellular migration pattern for both LacZ and IL-22 groups, with accumulation in the outer plexiform layer at 3 d, extending ventrally to the GCL at 14 d after injury (Figures 7E, 7F, S6C, and S6D). This effect, however, was more pronounced after IL-22 editing in which almost all microglia/macrophages were confined to the GCL.
to the inner and outer plexiform layers early after injury has been described as a process that is related to microglial function in regulating retina synapse formation and refinement during development (Li et al., 2019; Santos et al., 2008). Retinal injury and degeneration similarly result in reorganization of synapses requiring regulation by microglia (Silverman and Wong, 2018).

In addition to the divergent patterns of microglia/macrophage localization among injection groups, clear distinctions in their accumulation and activation were apparent among injured groups. Iba1+ (Figures 7C and 7E) and CD68+ (Figures 7D and 7F) populations were evaluated at both 3 and 14 d after crush injury. Significantly more of both Iba1+ and CD68+ cells were identified after IL-22 editing at 3 d after ONC compared with LacZ, although numbers were comparable between groups at the later time point. These data suggest that, compared with the control, IL-22 inhibition drives earlier microglia/macrophage accumulation and activation. The normalization of the cellular inflammatory response to control levels mirrors the reduction 14 d after ONC in transcript levels of the proinflammatory factors evaluated in Figure 7 (data not shown).
Figure 7. IL-22 editing stimulates the transcriptional expression of numerous genes implicated in axon regeneration and increases microglia/macrophage transcripts and cell accumulation in the retina

(A and B) Relative mRNA expression of inflammatory cytokine (A) and glial cell (B) activators in the retina after LacZ- or IL-22-targeted sgRNA-AAV infection and 3 d after ONC was quantified as ∆∆Ct normalized to Gapdh. n = 4 retinas/group/time. Data are means ± SEM of ∆ΔCq, with one-way ANOVA of Ln-transformed values and Tukey’s test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(C and E) Quantification (C) and representative images (E) of Iba1+ microglia/macrophages in the unedited or edited retina 3 and 14 d after ONC are represented as the total number of Iba1+ cells counted in four separate images of retinal cross-sections from the same animal. Retinal layers were identified by DAPI, and counts were normalized to the layer area. n = 5 retinas/group/time. Data are means ± SEM. *p < 0.05, **p < 0.01, one-way ANOVA and Tukey’s test. Scale bar, 50 μm.

(legend continued on next page)
We also examined the effect of excess IL-22 on inflammation in the retina. r-IL-22 was injected intravitreally into wild-type (WT) mice 1 d before ONC. Supplementation with r-IL-22 reversed the inflammatory transcriptomic profiles observed in the retina with both control injections and with IL-22 inhibition (Figure 7G). Specifically, significant reductions in Cd68, Tnfa, and Il1β transcripts compared with the control underscored an overall trend in the expression of pro-inflammatory factors because of IL-22 expression. Moreover, neither microglia/macrophage nor astrocyte accumulation or activation were significantly altered 3 d after ONC with enhanced IL-22 levels compared with that of the PBS control (Figures S7A–S7E). Although glial composition was unaffected by injection of r-IL-22 early after injury, changes in the production of pro-inflammatory and pro-regenerative indicators define the contribution of IL-22 to the injury response. Overall, these studies indicate that titration of IL-22 levels dampsens the inflammatory response after injury and suppresses the transcriptional expression of important Stat3 activators contributing to enhanced RAG expression and ultimately, axon regeneration.

**DISCUSSION**

We applied genome-wide in vitro and in vivo loss-of-function screening methods to define mechanisms of adult mammalian CNS axonal regeneration. We began with a set of about 400 genes identified previously by our unbiased and comprehensive loss-of-function screening of the mouse genome (Sekine et al., 2018). Using both shRNA and CRISPR virally-mediated gene targeting, we distilled that list to 40 genes whose loss of function enhanced regeneration in vivo. Although the in vivo shRNA screen provided 40 axon regeneration-limiting genes, the results of the sgRNA screen of those 40 genes yielded 28 in vitro confirmations and 9 in vivo confirmations. The lack of exact matching between the two methods is likely attributable primarily to the selection of shRNA or sgRNA sequences. Notably, five or more shRNAs were evaluated for each gene for the in vitro screen of Sekine et al. (2018). The most effective shRNA sequence was used here for optic nerve regeneration. Conversely, an average of only two sgRNAs were generated for most of the 40 genes, and likely, the reduction in the number of positive regeneration hits compared with that of shRNA can be attributed to suboptimal targeting sequences. As an example, efficiency for PTEN suppression varied widely with different sequences.

Here, single regeneration targets that emerged from the 40-gene list included Myl10, Airn, Prg2, Rassf3, Tbc1d22b, and Xylt1. Literature regarding the function of Myl10 is scarce, but myosin light chain kinase has been shown to regulate actin-myosin interactions in neuronal growth cones (Jian et al., 1996). Given that myosin light chains are substrates for myosin light chain kinase (Tan and Leung, 2009), we hypothesize that Myl10 controls extension and retraction of regenerating axons. Aim is an RNA that paternally silences the Igf2R gene (Santoro et al., 2013), whose unimpeded function is to bind and traffic Igf2 for lysosomal degradation (Oka et al., 1985; Wang et al., 1994). Although Igf1 enhances RGC survival after ONC (Bray et al., 2019), the mechanism of IgfR2/Igf2-regulated axon regeneration requires further study. Prg2 is a secreted glycoprotein known to bind heparan sulfate proteoglycans (Glærup et al., 2006), which regulate axonal growth. Prg2 also forms a complex with the protease PAPP-A (Overgaard et al., 2000), which releases active IGF-I from its antagonistic binding protein IGFBP-4.5 (Weyer and Glærup, 2011). Thus, the role or Prg2 in axon-limiting axonal regeneration may relate to IGF. Rassf3 is consistent with Ras/RAF function in neuronal survival and repair (Zhong, 2016) and with Tbc1d22b in Rab-GTPase regulation of axonal regeneration (Hernández-Deviez et al., 2004). Xylt1 encodes a key enzyme in the synthesis of chondroitin sulfate proteoglycans (Esko et al., 1985; Müller et al., 2006; Prante et al., 2006), so its suppression should reduce those extracellular inhibitors.

Here, we focused on IL-22 for two reasons. First, it was one of the strongest hits and was consistent across knockdown methods. Second, IL-22 is a cytokine regulating Jak-Stat signaling, which is implicated in nerve regeneration. Although the role of IL-22 had not been explored until now, we explored the Jak-Stat pathway to delineate that cytokine’s mechanism of action. Our results place IL-22—alongside Stat3, DLK, and RAG expression (Bollaerts et al., 2017)—as a critical node in the inflammatory response to nerve injury. Analysis of IL-22 inhibition revealed that endogenous IL-22 suppresses the inflammation after ONC and prevents upregulation of Stat3 activators DLK and RAGs. In the retina, enhanced inflammation induced by ONC and lens injury or injection of the Toll-like receptor 2 (TLR2) agonist zymosan elicits a regenerative RGC response (Leon et al., 2000; Yin et al., 2003), which is due, in large part, to glial activation and release of growth-promoting factors, such as LIF and IL-6, which stimulate Stat3 and RAG expression in RGCs (Leibinger et al., 2009, 2013a, 2013b). Mechanistically, we propose that IL-22 limits cytokine signaling and inflammation at the baseline and after axotomy. Before injury, IL-22 has little effect on glial or neuronal cells in the retina, but it primes an injury response, including Stat3. In mice with endogenous IL-22, ONC induces limited RAG expression for Aft3, Sprr1a, and Galanin, with a limited inflammatory glial reaction in the retina and minimal regeneration of axons in the optic nerve. After suppression of IL-22, the axotomy signal mediated by DLK and p-STAT3 initiates greater and more-rapid cytokine and growth factor induction.
as well as glial migration and activation. In that setting, cell-autonomous and/or cell-non-autonomous upregulation of RAGs in RGCs are markedly enhanced and axon regeneration is increased. Further, injection of recombinant IL-22 suppresses cytokine and RAG expression, placing IL-22 as a key regulator of regeneration.

Stat3 activation is essential to bridge inflammation with RAG induction and axon regeneration in both PNS and CNS (Bareyre et al., 2011; Leibinger et al., 2013a; O’Brien and Nathanson, 2007; Pellegrino and Habeker, 2013; Qi et al., 2005). Although IL-22 can activate Stat3, it has complicated signaling functions (Dudakov et al., 2015). The injury-induced increases in retinal Stat3 transcript and protein after ONC are significantly larger when IL-22 has been silenced. Moreover, IL-22 editing without injury is sufficient to increase expression of Ccl2, Il1b, Il6, and Il10 transcripts and expression of p-STAT3 protein. Although IL-22 may promote Stat3 activation in other contexts, its loss in RGCs allows compensatory increases of alternate potent Stat3 activators, such as Lif, Il-6, Il-10, Il-19, Il-20, and Il-24 (Leibinger et al., 2009; Martin et al., 2003). Our results indicate that IL-22 actively quells an immune response, consistent with studies of experimental autoimmune encephalomyelitis (Laaksonen et al., 2014) and of experimental autoimmune uveitis (Mattapallil et al., 2019). After ONC, the enhanced cytokine-driven immune response is pro-regenerative. In addition to Stat3, IL-22 inhibition produced marked increases in retinal expression of the injury-induced signal DLK (Watkins et al., 2013).

Recognition of the role of IL-22 in CNS regeneration highlights the cooperation between neuronal intrinsic mechanisms and non-neuronal cells. IL-22 expression is induced by IL-6 and IL-23 (Liang et al., 2006; Zheng et al., 2007) and is found in RGCs and bipolar cells (Figure S3A; Shekhar et al., 2016; Tran et al., 2019). IL-22 signals via the heterodimer IL-22Ra1 and IL-10Rb (Zenevich and Flavell, 2008). Although expression of IL-10Rb is pervasive in the retina (Boyd et al., 2003), RGC-specific expression of IL-22Ra1 mRNA was below the detection limit here. IL-22Ra1 was more abundant on glial cells, as previously described (Perriard et al., 2015). Thus, RGC release of IL-22 after ONC may act on glia to initiate inflammation and cytokine expression. Paracrine factors may then stifle neuronal-specific increases in activated Stat3, DLK, and RAG expression to attenuate axon regeneration with endogenous IL-22.

In conclusion, IL-22 editing permits disinhibition of inflammation after ONC, leading to the upregulation of growth-promoting transcription factors that stimulate Stat3, DLK, and finally, downstream RAGs, culminating in augmented axonal regeneration. Here, we focused on IL-22, but our in vivo screen identified more than three dozen additional genes whose endogenous expression limits axon regeneration. Strikingly, none of those genes were strongly upregulated after axotomy. Therefore, expression surveys may fail to identify many negative regulators of axon regeneration. Future studies of the multiple pathways demonstrated here are expected to provide new molecular avenues to promote neural repair. Importantly, the identification of multiple pathways provides the opportunity for multiplexed gene editing or combined pharmacological interventions.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.108777.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Anderson, M.A., Burda, J.E., Ren, Y., Ao, Y., O’Shea, T.M., Kawaguchi, R., Coppola, G., Khakh, B.S., Deming, T.J., and Sofroniew, M.V. (2016). Astrocyte scar formation aids central nervous system axon regeneration. Nature 532, 195–200.

Bareyre, F.M., Garzon, N., Lang, C., Misgeld, T., Bünig, H., and Kerschensteiner, M. (2011). In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration. Proc. Natl. Acad. Sci. USA 108, 6282–6287.

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.

Berkelaar, M., Clarke, D.B., Wang, Y.C., Bray, G.M., and Aguayo, A.J. (1994). Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. J. Neurosci. 14, 4368–4374.

Bollaerts, I., Van Houcke, J., Andries, L., De Groef, L., and Moons, L. (2017). Neuroinflammation as fuel for axonal regeneration in the injured vertebrate central nervous system. Mediators Inflamm. 2017, 9478542.

Bonilla, I.E., Tanabe, K., and Strittmatter, S.M. (2002). Small proline-rich repeat protein 1A is expressed by axotomized neurons and promotes axonal outgrowth. J. Neurosci. 22, 1303–1315.

Boyd, Z.S., Kriatchko, A., Yang, J., Agarwal, N., Wax, M.B., and Patil, R.V. (2003). Interleukin-10 receptor signaling through STAT-3 regulates the apoptosis of retinal ganglion cells in response to stress. Invest. Ophthalmol. Vis. Sci. 44, 5206–5211.

Bradbury, E.J., Moon, L.D., Popat, R.J., King, V.R., Bennett, G.S., Patel, P.N., Fawcett, J.W., and McMahon, S.B. (2002). Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 416, 636–640.

Bray, E.R., Yunghe, B.J., Levay, K., Ribeiro, M., Dvoryanchikov, G., Ayupe, A.C., Thakor, K., Marks, V., Randolph, M., Danzi, M.C., et al. (2019). Thrombospondin-1 mediates axon regeneration in retinal ganglion cells. Neuron 103, 642–657.e7.

Bregman, B.S., Kunkel-Bagden, E., Schnell, L., Dai, H.N., Gao, D., and Schwab, M.E. (1995). Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 378, 498–501.

Beumer, M.R., and Marriott, I. (2018). The interleukin-10 family of cytokines and their role in the CNS. Front. Cell. Neurosci. 12, 458.

Cafferty, W.B., Yang, S.H., Duffy, P.J., Li, S., and Strittmatter, S.M. (2007). Functional axonal regeneration through astrocytic scar genetically modified to digest chondroitin sulfate proteoglycans. J. Neurosci. 27, 2176–2185.

Cao, Z., Gao, Y., Bryson, J.B., Hou, J., Chaudhry, N., Siddiq, M., Martinez, J., Spencer, T., Carmel, J., Hart, R.B., and Filbin, M.T. (2006). The cytokine interleukin-6 is sufficient but not necessary to mimic the peripheral conditioning lesion effect on axonal growth. J. Neurosci. 26, 5565–5573.

Carvalho, L.S., Turunen, H.T., Wassmer, S.J., Luna-Velez, M.V., Xiao, R., Bennett, J., and Vandenbergh, L.H. (2017). Evaluating efficiencies of dual AAV approaches for retinal targeting. Front. Neurosci. 11, 503.

Choi, S.H., Winters, I.P., Wang, J., Naranjo, S., Dudgeon, C., Tamburini, F.B., Brady, J.J., Yang, D., Grüner, B.M., Chuang, C.H., et al. (2015). Pancreatic cancer modeling using retroviral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing. Genes Dev. 29, 1576–1585.

Cox, L.J., Hengst, U., Gurskaya, N.G., Lukyanov, K.A., and Jeffrey, S.R. (2008). Intra-axonal translation and retrograde trafficking of CREB promotes neuronal survival. Nat. Cell Biol. 10, 149–159.

Daya, S., and Berns, K.I. (2008). Gene therapy using adeno-associated virus vectors. Clin. Microbiol. Rev. 21, 583–593.

Dudakov, J.A., Hanash, A.M., and van den Brink, M.R. (2015). Interleukin-22: immunobiology and pathology. Annu. Rev. Immunol. 33, 747–785.

Dupraz, S., Grassi, D., Karnas, D., Nieto Guíl, A.F., Hicks, D., and Quiroga, S. (2013). The insulin-like growth factor 1 receptor is essential for axonal regeneration in adult central nervous system neurons. PLoS ONE 8, e64462.
Kwong, J.M., Quan, A., Kyung, H., Piri, N., and Caprioli, J. (2011). Quantitative analysis of retinal ganglion cell survival with Rbpms immunolabeling in animal models of optic neuropathies. Invest. Ophthalmol. Vis. Sci. 52, 9694–9702.

Laaksonen, H., Guerreiro-Cacais, A.O., Adzemovic, M.Z., Parsa, R., Zeitelhofer, M., Jagodic, M., and Olsson, T. (2014). The multiple sclerosis risk gene IL22RA2 contributes to a more severe murine autoimmune neuroinflammation. Genes Immun. 15, 457–465.

Leaver, S.G., Cui, Q., Plant, G.W., Arulpragasam, A., Hisheh, S., Verhaagen, J., and Harvey, A.R. (2006). AAV-mediated expression ofCNTF promotes long-term survival and regeneration of adult rat retinal ganglion cells. Gene Ther. 13, 1328–1341.

Leibinger, M., Muller, A., Andreadaki, A., Hauk, T.G., Kirsch, M., and Fischer, D. (2009). Neuroprotective and axon growth-promoting effects following inflammatory stimulation on mature retinal ganglion cells in mice depend on cytokine neurotrophic factor and leukemia inhibitory factor. J. Neurosci. 29, 14334–14341.

Leibinger, M., Andreadaki, A., Diekmann, H., and Fischer, D. (2013a). Neuronal STAT3 activation is essential for CNTF- and inflammatory stimulation-induced CNS axon regeneration. Cell Death Dis. 4, e805.

Leibinger, M., Muller, A., Gobrecht, P., Diekmann, H., Andreadaki, A., and Fischer, D. (2013b). Interleukin-6 contributes to CNS axon regeneration upon inflammatory stimulation. Cell Death Dis. 4, e609.

Leon, S., Yin, Y., Nguyen, J., Irwin, N., and Benowitz, L.I. (2000). Lens injury stimulates axon regeneration in the mature rat optic nerve. J. Neurosci. 20, 4615–4626.

Li, F., Jiang, D., and Samuel, M.A. (2015). Microglia in the developing retina. Neural Dev. 12, 12.

Liang, F.Q., Surace, E., Dejneka, N.S., Maguire, A.M., and Bennett, J. (2003). Muller cell transduction by AAV2 in normal and degenerative retinas. Adv. Exp. Med. Biol. 533, 439–445.

Liang, S.C., Tan, X.Y., Luxenberg, D.P., Karim, R., Dunussi-Joannopoulos, K., Collins, M., and Fouser, L.A. (2006). Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J. Exp. Med. 203, 2271–2279.

Ma, T.C., and Willis, D.E. (2015). What makes a RAG-regeneration associated? Front. Mol. Neurosci. 8, 43.

Macosko, E.Z., Basu, A., Satija, R., Nemesh, J., Shekhar, K., Goldman, M., Tirosh, I., Bialas, A.R., Kamitaki, N., Martersteck, E.M., et al. (2015). Highly parallel genome-wide expression profiling of individual cells using nanoliter drop-lets. Cell 161, 1202–1214.

Martin, A., Hofmann, H.D., and Kirsch, M. (2003). Glia1 reactive in ciliary neuroepithelial factor-deficient mice after optic nerve lesion. J. Neurosci. 23, 5416–5424.

Massey, J.M., Hubscher, C.H., Wagoner, M.R., Decker, J.A., Aments, J., Silver, J., and Onifer, S.M. (2006). Chondroitinase ABC digestion of the perineuronal net promotes functional collateral sprouting in the cuneate nucleus after cerebellar spinal cord injury. J. Neurosci. 26, 4404–4414.

Mattapallil, M.J., Kielczewski, J.L., Zárate-Bladés, C.R., St Leger, A.J., Ray, J., and激活 transcription factor 3 activation. J. Neurosci. 25, 1645–1653.

McKeon, R.J., Schreiber, R.C., Rudge, J.S., and Silver, J. (1991). Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. J. Neurosci. 11, 3388–3411.

McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J., and Braun, P.E. (1994). Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13, 805–811.

Mehta, S.T., Luo, X., Park, K.K., Bixby, J.L., and Lemmon, V.P. (2016). Hyper-activated Stat3 boosts axon regeneration in the CNS. Exp. Neurol. 280, 115–120.

 Müller, S., Disse, J., Schüttler, M., Schön, S., Prante, C., Brinkmann, T., Kuhn, J., Kleesiek, K., and Götting, C. (2006). Human tyrosine phosphatase 1 and N-terminal truncated forms: functional characterization of the core enzyme. Biochem. J. 394, 163–171.

Nadal-Nicolás, F.M., Sobrado-Calvo, P., Jiménez-López, M., Vidal-Sanz, M., and Agudo-Barriloso, M. (2015). Long-term effect of optic nerve axotomy on the retinal ganglion cell layer. Invest. Ophthalmol. Vis. Sci. 56, 6095–6112.

Nelson, C.E., Hakim, C.H., Ousterout, D.G., Thakore, P.I., Moree, E.A., Castellanos Rivera, R.M., Madhavan, S., Pan, X., Ran, F.A., Yan, W.X., et al. (2016). In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. Science 351, 403–407.

O’Brien, J.J., and Nathanson, N.M. (2007). Retrograde activation of STAT3 by leukemia inhibitory factor in sympathetic neurons. J. Neurochem. 103, 288–302.

Oka, Y., Rozeik, L.M., and Czech, M.P. (1985). Direct demonstration of rapid insulin-like growth factor II Receptor internalization and recycling in rat adipocytes: insulin stimulates 125I-insulin-like growth factor II degradation by modulating the IGFI receptor recycling process. J. Biol. Chem. 260, 9435–9442.

Overgaard, M.T., Haaning, J., Boldt, H.B., Olsen, I.M., Laursen, L.S., Christiansen, M., Gleich, G.J., Slotrup-Jensen, L., Conover, C.A., and Ovig, C. (2000). Expression of recombinant human pregnancy-associated plasma protein-A and identification of the proform of eosinophil major basic protein as its physiological inhibitor. J. Biol. Chem. 275, 31128–31133.

Pang, J.J., Lauramore, A., Deng, W.T., Li, Q., Doyle, T.J., Chioudo, V., Li, J., and Hauswirth, W.W. (2008). Comparative analysis of in vivo and in vitro AAV vector transduction in the neonatal mouse retina: effects of serotype and site of administration. Vision Res. 48, 377–385.

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M., and He, Z. (2008). Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 323, 963–966.

Pellegrino, M.J., and Habeker, B.A. (2013). STAT3 integrates cytokine and neurotrophin signals to promote sympathetic axon regeneration. Mol. Cell. Neurosci. 56, 272–282.

Perriard, G., Mathias, A., Enz, L., Canales, M., Schuep, M., Gentner, M., Schena-Wiemers, N., and Du Pasquier, R.A. (2015). Interleukin-22 is increased in multiple sclerosis patients and targets astrocytes. J. Neuroinflammation 12, 119.

Petrus-Silva, H., Dinculescu, A., Li, Q., Min, S.H., Chioudo, V., Pang, J.J., Zhong, L., Zolotukhin, S., Srivastava, A., Lewin, A.S., et al. (2009). High-efficiency transduction of the mouse retina by tyrosine-mutant AAV serotype vectors. Mol Ther. 17, 463–471.

Prante, C., Bieback, K., Funke, C., Schön, S., Kern, S., Kuhn, J., Gastes, M., Kleesiek, K., and Götting, C. (2008). The formation of extracellular matrix during chondrogenic differentiation of mesenchymal stem cells correlates with increased levels of tyrosine phosphatase 1 and N-terminal truncated forms: functional characterization of the core enzyme. Biochem. J. 394, 163–171.

Santos, A.M., Calvente, R., Tassi, M., Carrasco, M.C., Martin-Oliva, D., Marin-Teva, J.L., Navascués, J., and Cuadros, M.A. (2008). Embryonic and postnatal development of microglial cells in the mouse retina. J. Comp. Neurol. 506, 225–239.

Sekine, Y., Lin-Moore, A., Chenette, D.M., Wang, X., Jiang, Z., Cafferty, W.B., Hammal, M., and Strittmatter, S.M. (2018). Functional genome-wide screen identifies pathways restricting central nervous system axonal regeneration. Cell Rep. 23, 415–428.
Shen, J.E., Cho, Y., Beiroukas, B., Milbrandt, J., Cavalli, V., and DiAntonio, A. (2012). Dual leucine zipper kinase is required for retrograde injury signaling and axonal regeneration. Neuron 74, 1015–1022.

Smith, P.D., Sun, F., Park, K.K., Cai, B., Wang, C., Kuvurako, K., Martinez-Carrasco, I., Connolly, L., and He, Z. (2009). SOCS3 deletion promotes optic nerve regeneration in vivo. Neuron 64, 617–623.

Smith, R.P., Lerch-Haner, J.K., Pardinas, J.R., Buchser, W.J., Bixby, J.L., and Lemmon, V.P. (2011). Transcriptional profiling of intrinsic PNS factors in the postnatal mouse. Mol. Cell. Neurosci. 46, 32–44.

Starkey, M.L., Davies, M., Yip, P.K., Carter, L.M., Wong, D.J., McMahon, S.B., and Bradbury, E.J. (2009). Expression of the regeneration-associated protein SPRR1A in primary sensory neurons and spinal cord of the adult mouse following peripheral and central injury. J. Comp. Neurol. 513, 51–68.

Swiech, I., Heidenreich, M., Banerjee, A., Habib, N., Li, Y., Trombetta, J., Sur, M., and Zhang, F. (2015). In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. Nat. Biotechnol. 33, 102–106.

Tan, I., and Leung, T. (2009). Myosin light chain kinases: division of work in cell migration. Cell Adhes. Migr. 3, 256–258.

Templeton, J.P., and Geisert, E.E. (2012). A practical approach to optic nerve crush in the mouse. Mol. Vis. 18, 2147–2152.

Toy, D., and Namgung, U. (2013). Role of glial cells in axonal regeneration. Exp. Neurop. Biol. 2013, 68–76.

Tran, N.M., Shekhar, K., Whitney, I.E., Jacob, A., Benhar, I., Sung, G., Yan, W., Adiconis, X., Arnold, M.E., Lee, J.M., et al. (2019). Single-cell profiles of retinal ganglion cells differing in resilience to injury reveal neuroprotective genes. Neuron 104, 1039–1055.e12.

Tsujino, H., Kondo, E., Fujukawa, T., Dai, Y., Tokunaga, A., Miki, K., Yonenobu, K., Ochi, T., and Noguchi, K. (2000). Activating transcription factor 3 (ATF3) induction by axotomy in sensory neurons of the central nervous system. Acta Neuropathol. 99, 1–10.

Toy, D., and Namgung, U. (2013). Role of glial cells in axonal regeneration. Exp. Neurop. Biol. 2013, 68–76.

Wang, Z.Q., Fung, M.R., Barlow, D.P., and Wagner, E.F. (1994). Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. Nature 372, 464–467.

Wang, K.C., Koprivica, V., Kim, J.A., Sivasankaran, R., Guo, Y., Neve, R.L., and He, Z. (2002). Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417, 941–944.

Wang, X., Duffy, P., McGee, A.W., Hassan, O., Gould, G., Tu, N., Harel, N.Y., Huang, Y., Carson, R.E., Weinzimmer, D., et al. (2011). Recovery from chronic spinal cord contusion after Nogo receptor intervention. Ann. Neurol. 70, 805–821.

Wang, X., Lin, J., Arzeno, A., Choi, J.Y., Boccio, J., Frieden, E., Bhargava, A., Maynard, G., Tsai, J.C., and Strittmatter, S.M. (2015). Intravitreal delivery of human Ngf-R1/Fc decoy protein regenerates axons after optic nerve crush and protects ganglion cell survival. Invest. OphthalmoL. Vis. Sci. 56, 1357–1366.
### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Rbpm           | PhosphoSolutions | Cat#1830-Rbpms; RRID:AB_2492225 |
| Anti-Rbpm           | PhosphoSolutions | Cat#1832-Rbpms; RRID:AB_2492226 |
| Anti-Rbpm           | Proteintech | Cat#15187-1-AP; RRID:AB_2238431 |
| Anti-Brn3a          | Abcam | Cat#ab81213; RRID:AB_1640222 |
| Anti-NeuN           | Millipore | Cat#ABN91; RRID:AB_11205760 |
| Anti-GFAP           | Dako | Cat#20334; RRID:AB_10013382 |
| Anti-Iba1           | Wako | Cat#019-19741; RRID:AB_839504 |
| Anti-PDGF Receptor a| CST | Cat#3174; RRID:AB_2162345 |
| Anti-O4             | R&D Systems | Cat#MAB1326; RRID:AB_357617 |
| Anti-β-tubulin      | Promega | Cat#G712A; RRID:AB_430874 |
| Anti-Pten           | CST | Cat#9559; RRID:AB_390810 |
| Anti-II2            | Millipore | Cat#AV49523; RRID:AB_1851697 |
| Anti-p-STAT3        | CST | Cat#9145; RRID:AB_2491009 |
| Anti-DLK            | Genetex | Cat#GTX124127; RRID:AB_11170703 |
| Anti-β-actin        | CST | Cat#3700; RRID:AB_2242334 |
| Anti-β-actin        | CST | Cat#4967; RRID:AB_330288 |
| Anti-Iba1           | Abcam | Cat#ab178846; RRID:AB_2636859 |
| Anti-GFAP           | Bio-Rad | Cat#MCA1957; RRID:AB_322219 |
| Anti-GFAP           | Abcam | Cat#ab53554; RRID:AB_880202 |
| Anti-GFP            | Abcam | Cat#ab13970; RRID:AB_300798 |
| Anti-II2Ra1         | Bioss | Cat#bs-2624R; RRID:AB_10857827 |
| Anti-Atf3           | Novus Biologicals | Cat#NB1-85916; RRID:AB_11014863 |
| Anti-CD16/CD32      | BioLegend | Cat#101302; RRID:AB_312801 |
| Alexa Fluor 546     | Invitrogen | Cat#A-11035; RRID:AB_2534093 |
| Alexa Fluor 594     | Thermo Fisher | Cat#A-11058; RRID:AB_2534105 |
| Alexa Fluor 647     | Invitrogen | Cat#A21245; RRID:AB_2535813 |
| Alexa Fluor 647     | Invitrogen | Cat#A31573; RRID:AB_2536183 |
| Alexa Fluor 647     | Invitrogen | Cat#A31571; RRID:AB_162542 |
| Alexa Fluor 647     | Invitrogen | Cat#A21247; RRID:AB_141778 |
| Alexa Fluor 647     | Thermo Fisher | Cat#A-21450; RRID:AB_2735091 |
| Alexa Fluor 488     | Invitrogen | Cat#A11034; RRID:AB_2576217 |
| Alexa Fluor 488     | Invitrogen | Cat#A11073; RRID:AB_2534117 |
| Alexa Fluor 488     | Invitrogen | Cat#A11039; RRID:AB_2534096 |
| Alexa Fluor 488     | Invitrogen | Cat#SA1-72000; RRID:AB_923386 |
| Alexa Fluor 488     | Thermo Fisher | Cat#A21042; RRID:AB_2535711 |
| Alexa Fluor 555     | Thermo Fisher | Cat#A31572; RRID:AB_162543 |
| APC                 | R&D Systems | Cat#F0111; RRID:AB_573127 |
| IRDye 680           | Li-Cor | Cat#926-68072; RRID:AB_10953628 |
| IRDye 680           | Li-Cor | Cat#926-68073; RRID:AB_10954442 |
| IRDye 800           | Li-Cor | Cat#926-32213; RRID:AB_621848 |
| IRDye 800           | Li-Cor | Cat#926-32212; RRID:AB_621847 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| One Shot Stbl3 Chemically Competent E. coli | Thermo Fisher | Cat#737303 |
| **Chemicals, peptides, and recombinant proteins** | | |
| FBS | GIBCO | Cat##10437028 |
| DMEM | GIBCO | Cat##11965-092 |
| Hibernate-E | BrainBits | Cat#HE-Ca |
| Neurobasal-A | GIBCO | Cat#10880-22 |
| GlutaMAX | GIBCO | Cat#35050-061 |
| B-27 | GIBCO | Cat#17504044 |
| Penicillin/streptomycin | GIBCO | Cat##15140-122 |
| Papain | Worthington Biochemical | Cat#LS003127 |
| Dnase | Sigma-Aldrich | Cat#DN-25 |
| Benzonase nuclease | Millipore | Cat#70746-10KUN |
| T4 DNA Ligase | NEB | Cat#M0202 |
| T7 DNA Ligase | NEB | Cat#M0318 |
| EcoR I | NEB | Cat#R0101 |
| BamH I | NEB | Cat#R0136 |
| Sap I | Thermo Fisher | Cat#FD1934 |
| Polyethylenimine | Polysciences, Inc. | Cat#23966-1 |
| iQ SYBR Green Supermix | Bio-Rad | Cat#1708882 |
| DAPI | Bio-Rad | Cat#135-1303 |
| QuickExtract DNA Extraction Solution | Lucigen | Cat#QE09050 |
| Cholera Toxin Subunit B Alexa Fluor 555 Conjugate | Thermo Fisher | Cat#C34766 |
| Recombinant Murine IL-22 | Peprotech | Cat#210-22 |
| **Critical commercial assays** | | |
| Alt-R Genome Editing Detection Kit | IDT | Cat#1075932 |
| Mouse/Rat IL-22 Quantikine ELISA kit | R&D Systems | Cat#M2200 |
| RNeasy Plus Mini Kit | QIAGEN | Cat#74134 |
| iScript cDNA Synthesis Kit | Bio-Rad | Cat#1708891 |
| Taqman Gene Expression Master Mix | Applied Biosystems | Cat#4369016 |
| DNeasy Blood and Tissue Kit | QIAGEN | Cat#69504 |
| **Experimental models: cell lines** | | |
| HEK293T | ATCC | Cat#CRL-3216 |
| **Experimental models: organisms/strains** | | |
| C57BL/6J | The Jackson Laboratory | Cat#000664 |
| H11Cas9 | The Jackson Laboratory | Cat#027650 |
| **Oligonucleotides** | | |
| See Table S1 for shRNA list | N/A |
| See Table S3 for sgRNA list | N/A |
| See Table S4 for qPCR primer list | N/A |
| **Recombinant DNA** | | |
| pAAV-U6-GFP Expression Vector | Cell Biolabs | Cat#VPK-413 |
| PX551 | Swiech et al., 2015 | Addgene Plasmid #60957 |
| PX552 | Swiech et al., 2015 | Addgene Plasmid #60958 |
| **Software and algorithms** | | |
| Prism 7.04 | GraphPad | https://www.graphpad.com |
| ImageJ | NIH | https://imagej.nih.gov/ij/ |
| String | bioinformatical analysis | https://string-db.org/ |
RESOURCE AVAILABILITY

Lead contact
Further information for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Stephen M. Strittmatter (stephen.strittmatter@yale.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The RGC scRNA-seq adult atlas and ONC datasets analyzed in this study were previously published in Tran et al. (2019). The accession number for these datasets is GEO: GSE137400.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice and surgery
All experiments were performed on healthy mice under protocols approved by the Yale Institutional Animal Care and Use Committee. Male and female (8-12 weeks old) wild-type (C57BL/6J, The Jackson Laboratory) and Cas9 (H11Cas9; Chiou et al., 2015; The Jackson Laboratory) mice were used for this study. No notable sex-dependent differences were observed during analysis. Mice had ad libitum access to food and water and were housed under a 12 h light/dark cycle. Mice were anesthetized under isoflurane and received bilateral intravitreal AAV injections of 1.5 μL of control or targeted virus. For Il22 gain-of-function experiments, PBS or recombinant Il22 (Peprotech) at a concentration of 200 ng/μl was injected bilaterally into the vitreous cavity (Mattapallil et al., 2019). Fourteen days following AAV injection, mice were anesthetized by intraperitoneal injection of a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg). The optic nerves were exposed intraorbitally and crushed for 5 s using jeweler’s forceps (Dumont 5, Fine Science Tools) at a location 1 mm posterior to the eyeball (Wang et al., 2015). Alexa 555-CTB (cholera toxin subunit B, Thermo Fisher) was injected intravitreally 14 d post-ONC injury to anterogradely label axons. Mice were killed 3 d later by CO2 inhalation, and optic nerves and retinas were harvested. The few mice (< 3%) with macroscopic evidence for orbital infection were excluded from analysis.

Cell lines and cell culture
The HEK293T cell line was purchased from ATCC (Manassass, VA). Cells were maintained in DMEM (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO). Primary cortical cultures were established from P1 wild-type mice and were prepared as described previously (Sekine et al., 2018). Briefly, meninges were removed from dissected cortices in ice-cold Hibernate E medium (BrainBits) and incubated in HBSS containing 30 U/ml Papain (Worthington Biochemical), 1.5 mM CaCl2, 2.5 mM EDTA, and 2 mg/ml DNaseI (Sigma) at 37°C for 20 min. Digested tissues were triturated and suspended in Neurobasal-A (GIBCO). Cells were plated on 96 well tissue culture plates coated with poly-D-lysine at a density of 4.0 \( \times 10^4 \) cells per well in 200 μL of Neurobasal-A supplemented with B-27 (GIBCO), GlutaMAX (GIBCO), and penicillin-streptomycin.

METHOD DETAILS

DNA constructs
shRNA. The helper-free shRNA expression system (Cell Biolabs) comprised of pHelper DF6, pAAV-RC6, and pAAV-U6-GFP vectors were used. shRNA sequences (Table S1) were cloned at the BamH I and EcoR I (NEB) restriction enzyme sites of the pAAV-U6-GFP expression vector. Non-targeting shRNA was generated as a control (target sequence: CAACAGATGAAGAGACCA). sgRNA. The SpCas9 (pX551) and single guide RNA (sgRNA, pX552) expression plasmids developed by the Zhang lab (Swiech et al., 2015) were obtained from Addgene. sgRNA target sequences (Table S3) of 20-nt length were chosen using the CRISPR design tool (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design), selected to precede a 5′-NGG protospacer-adjacent motif sequence, and prioritized based on minimal off-target effects. A negative control sgRNA sequence targeting the LacZ gene from E. coli was generated using the sequence TGCGAATACGCCCAGC. To generate sgRNA-expressing constructs, pX552 was digested using SapI Fast Digest (Thermo Fisher). Annealed shRNA and sgRNA oligos were ligated using T4 and T7 DNA Ligase, respectively (NEB). Transformation was performed using One Shot Stbl3 Chemically Competent E. coli (Thermo Fisher). Following maxi prep (QIAGEN), Sanger sequencing confirmed correct shRNA and sgRNA insertion using the U6 promoter sequencing primers (5′ to 3′) TGGACCATCTCTAGAC and (5′ to 3′) GAGGGCCTATTTCCCATGATTC, respectively.

Transfection of HEK293T cells and AAV vector production
Cells were passaged the day before transfection at a density of 10^5 cells per 15-cm plate and were transfected at approximately 80% confluence. For AAV production, 18 μg of DF6 helper plasmid, 6 μg of sgRNA expression plasmid, and 6 μg of 2/2 or 2/1
serotype packaging plasmid were combined in 3 mL of serum-free DMEM. After addition of 150 μL of the transfection reagent polyethylenimine (PEI, Polysciences Inc.), the DNA:PEI transfection mixture was incubated at room temperature for 15 minutes before adding it to HEK293T cells contained in supplemented DMEM. Lower titer AAV particles using 2/1 serotype plasmid were produced for cortical neuron transduction as described in Konermann et al. (2013). Briefly, 72 h following transfection, the supernatant was collected, filtered using a 0.22 μm cellulose acetate filter (Coming), and stored at –80°C. Concentrated AAV particles for in vivo retinal cell infection were obtained using 2/2 serotype plasmid (Pang et al., 2008). Seventy-two hours after HEK293T cell transfection, cells were collected, subjected to repeated freeze-thaw cycles to release viral particles into the supernatant, treated with DNaseI (10 U/ml, Sigma-Aldrich) and benzonase (50 U/ml, Millipore) for 30 min at 37°C, and centrifuged at 3,000 x g for 20 min at 4°C to remove cell debris. AAVs were then purified using iodixanol density gradient ultracentrifugation (Hermens et al., 1999). Viral titers were determined using IQ SYBR Green Supermix (Bio-Rad) and quantitative PCR (Bio-Rad CFX96). Samples were compared against a standard curve derived from a virus of known titer diluted from 10^{13} to 10^8 copies per ml. Average viral titers for cortical neuron transduction experiments were 10^8-10^{10} copies per ml, and 10^{10}-10^{13} copies per ml for in vivo infection.

**Optic nerve tissue processing and regeneration analysis**

Crushed optic nerves were fixed in 4% PFA for 1 h and chemically cleared with BABB (1 part benzyl alcohol, 2 parts benzyl benzoate) using an adapted protocol from Ertürk et al. (2011). Briefly, nerves were rinsed in dd{H}_2{O} and then incubated in 50%, 80%, and 100% tetrahydrofuran for 20 min, 20 min and 1 h, respectively, followed by 20 min in 100% dichloromethane. Nerves were then submerged in BABB at 4°C for 1 h until mounted and coverslipped. The number of regenerating axons were counted from each image of approximately 30-40 z stacks using an epifluorescence microscope (Zeiss Axio Imager M2) with a 10x lens. Positive axon counts extended 500 μm distal to the crush site. Representative z stack images were taken at 20x using a confocal microscope (Zeiss LSM 710).

**RGC survival**

Eyes were removed and fixed in 4% PFA for 1 h at room temperature. Intact retinas were isolated and post-fixed in 4% PFA for 15 min and washed in PBS. Following a 1 h incubation in PBS containing 5% NDS, retinas were incubated with a RbpmS primary antibody (1:1000; PhosphoSolutions, #1830-Rbpms) for 3 d at 4°C. Retinas were subsequently washed in PBS, incubated with an AF555 secondary antibody (1:400; Thermo Fisher) for 1 h at room temperature, and washed in PBS. Four incisions were made from the retinal periphery halfway to the optic nerve to produce a flat preparation, and the whole retinas were mounted on microcopy slides with antifade mounting medium (Vectorshied). Cell counts were performed on an Axio Imager M2 (Zeiss) with a 10x objective, and representative images were taken at 40x on a confocal microscope (Zeiss LSM 710). For each retina, 3 fields of equal dimensions were sampled from each of the 4 retinal quadrants (12 fields total). Each field was run though ImageJ’s FFT bandpass filter, auto-thresholded, and converted to a binary image. After removing outliers, RGCs were counted using the cell counter. The total number of RbpmS+ RGCs were summed from each field, averaged across 4-5 retinas, and presented as a percentage of RbpmS+ RGCs from uninjured retina.

**Primary cortical neuron culture**

On DIV17, uninjured cortical neuron cultures were fixed in 4% PFA, and incubated overnight at 4°C with the following antibodies: anti-NeuN (1:500, Millipore), anti-GFAP (1:500, Dako), anti-Iba1 (1:1000, Wako), anti-PDGFR Receptor α (1:500, CST), anti-O4 (1:500, R&D Systems). Secondary antibody incubation was performed for 1 h at room temperature using AF488 or AF555 IgG (1:1000, Invitrogen), or AF488 IgM (1:1000, Thermo Fisher). For editing and regeneration studies, cortical neurons were co-transduced with sgRNA-AAV and Cas9-AAV on DIV3. AAV serotype 2/1 was used for efficient transduction of cortical neurons (Hammond et al., 2017). Neurons were collected on DIV17 for DNA, RNA, and protein assays. The axon regeneration assay was performed as described previously with modifications (Huebner et al., 2011). On DIV17, 96-well cultures were scraped using a floating pin tool with FP1-WP pins (V&P Scientific) and allowed to regenerate for another 8 d before fixation with 4% PFA. Regenerating axons in the scrape zone were visualized using an anti-βIII-tubulin antibody (1:2000, Promega) and AF647 secondary antibody (1:1000, Invitrogen). DAPI (Bio-Rad) was used to label nuclei in all experiments. Images were taken on a 10x objective in an automated high-throughput imager (ImageXpress Micro XLS, Molecular Devices) under identical conditions. Image thresholding and quantitation were automated using an ImageJ script.

**DNA isolation, T7 endonuclease I (T7EI) assay and indel detection**

Cortical neuron DNA was isolated using QuickExtract DNA Extraction Solution (Lucigen) 14 d following transduction with Cas9-AAV and sgRNA-AAV targeting LacZ, Pten-1 or Pten-3. The following primers were used to amplify the Pten locus containing the sgRNA sequences (5'–3'): Pten-1F AGTCTTACATGGGTTGTATAG; Pten-1R CTCCTCCTCCCAAAGTGTATCT; Pten-3F GCCTTGTTATGGGTTGTATAG; Pten-3R TTTGAAGGCCTCCTCTTCTC. Indel detection was performed with Alt-R Genome Editing Detection Kit (IDT) according to the manufacturer’s instructions. Editing efficiency was calculated by measuring the percent reduction of the band intensity of the full-length treated (+T7EI) amplicon relative to the full-length untreated (-T7EI) amplicon.
qPCR
Retinas were harvested and frozen 3 d post-ONC. Total RNA was prepared using RNeasy Plus Mini Kit (QIAGEN) and subjected to RT-PCR using iScript cDNA Synthesis Kit (Bio-Rad). cDNA was used for real-time quantitative PCR with TaqMan gene expression master mix (Applied Biosystems) and prevalidated TaqMan gene expression assays (Applied Biosystems; Table S4) on a Bio-Rad CFX Connect real-time PCR detection system using standard cycles. Each sample was analyzed in duplicate. Relative expression was determined using the Comparative Ct Model ($\Delta\Delta$Ct) with Gapdh as the internal control.

Immunoblotting
Tissue or primary cortical neurons were homogenized using a RIPA Lysis Buffer System (SCBT), and protein concentrations were determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Protein homogenates were analyzed by SDS-PAGE in 4%-20% Tris-Glycine gels (Bio-Rad). Membranes were blocked in TBST containing 5% BSA for 1 h at room temperature, and subsequently incubated overnight at 4°C with the following antibodies: anti-Pten (1:1000, CST), anti-Iil22 (1:1000, Millipore), anti-p-STAT3 (1:2000, CST), anti-DLK (1:1000, Genetex), anti-\(\beta\)-actin (1:2000, CST). Following primary antibody incubation and washing, secondary antibodies (Odyssey IRDye 680 or 800) were applied for 1 h at room temperature. Membranes were then washed and visualized using the LI-COR Odyssey IR imaging system.

ELISA
Retinal concentrations of Il22 were measured using a commercially available kit (R&D Systems). Total protein was isolated from retinas 14 d after ONC, and samples were prepared according to the manufacturer’s instructions. Standards, controls, and experimental samples were assayed in duplicate.

Histology and immunostaining
Eyes were harvested and fixed for 1 h in 4% PFA. Following cryopreservation in 10%, 20%, and 30% sucrose at 4°C for 30 min, 2 h and overnight, respectively, eyes were embedded in Tissue-Tek OCT compound (Electron Microscopy Sciences) and sectioned at 10 μm using a cryostat. The following primary antibodies were incubated with tissue sections overnight at 4°C: anti-Iba1 (1:200, Abcam), anti-CD68 (1:250, Bio-Rad), anti-GFAP (1500, Abcam), anti-DLK (1:100, Genetex), anti-Atf3 (1:200, Novus Biologicals), anti-GFP (1:500, Abcam), anti-Iil22Ra1 (1:200, Bioss), anti-Rpmps (1:100; PhosphoSolutions), anti-\(\beta\)-tubulin antibody (1:500, Promega). Following primary antibody application and washing, tissue was subsequently incubated in AF594, AF546, AF647, or AF488 secondary antibodies (1:400; Invitrogen, Thermo Fisher) for 1 h. DAPI (1:1000; Invitrogen) was used to label nuclei. Images were captured using an Axio Imager M2 (Zeiss), a LSM 880 confocal (Zeiss), or a TCS SP8 confocal (Leica). To visualize AAV injection in wholemount retinas, tissue was stained with an anti-GFP (1:500, Abcam) antibody for 3 d at 4°C. Retinas were subsequently washed in PBS, incubated with an AF488 secondary antibody (1:400; Invitrogen) for 2 h at room temperature, and washed in PBS.

FACS sorting, next-generation sequencing and indel detection
Fourteen days after AAV injection with either LacZ or Il22 sgRNA-AAV, single retinas were enzymatically digested in 0.125% collagenase for 1 h at 37°C. Following mechanical digestion and single-cell suspension, cells were washed in FACS buffer (PBS, 1% BSA) and blocked with a monoclonal antibody to CD16/CD32 (1:500, Biolegend) for 10 min at 4°C. Cells were incubated with primary antibodies against Rpmps (1:100, Proteintech) and Brn3a (1:100, Abcam) for 2 h at 4°C, washed, incubated with APC-conjugated secondary antibody (10 μl/10^6 cells, R&D Systems) for 1 h at 4°C, washed, resuspended in FACS buffer, and then sorted and collected using a BD FACS:Aria II (BD Biosciences) based on expression of GFP, Rpmps, and Brn3a. All events were gated based on viable single cells. DNA was extracted from collected cells using DNeasy Blood and Tissue Kit (QIAGEN), and a 302 bp amplicon that spanned the sgRNA-targeted region in the Il22 locus was generated (5'-3': F: GAACTCATACTCTTTGGCTACTC, 5'-3': R: CATCAGGTAAGCAGTACCTTAG) for each sample. PCR products were submitted to Genewiz for their Amplicon-EZ service (Illumina 2x250 bp platform) to examine the incidence of indels at approximately 50,000 reads per sample.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical details of each experiment can be found in the figure legends. The number of experimental replicates (n), and the designation of n belonging to the number of tissues or cells are indicated in the figure legends or outlined in the Method details. Statistical comparisons included one- and two-way ANOVA and Student’s t test as specified in the figure legends using Prism software (GraphPad version 7.04) or Excel. Statistical significance was set at p < 0.05. Data presented in graphs are mean ± SEM unless stated otherwise. All imaging quantifications were conducted by experimenters unaware of experimental group.

scRNA-seq analysis
The RGC scRNA-seq adult atlas and ONC datasets analyzed in this study were previously published in Tran et al. (2019) and the P14 whole mouse retina scRNA-seq dataset in Macosko et al. (2015). Previous tSNE, RGC cell type designations, and processed expression matrices of atlas and post-ONC RGCs were used. Feature, violin, and dot plots were generated using custom RStudio scripts as previously described (Macosko et al., 2015; Shekhar et al., 2016; Tran et al., 2019). For the scatterplots showing the expression of
gene targets (392 queried, 248 plotted), the average number of transcripts in expressing cells was plotted against the fraction of RGCs expressing each gene in RGCs from the atlas dataset and the ONC dataset merged and averaged across all time points (0.5, 1, 2, 4, 7, 14 d post-ONC) using ggplot2. Expression level was defined as (average number of transcripts in expressing cells) * (fraction of expressing RGCs). For violin plots of the whole retina scRNA-seq dataset, data from clusters corresponding to the six major retinal neuron classes (horizontal cells (HC), bipolar cells (BP), amacrine cells (AC), retinal ganglion cells (RGC), rods and cones, and non-neuronal populations (including Muller glia, astrocytes, fibroblasts, vascular endothelium, pericytes and microglia) were merged and plotted. For these plots, expression level was defined as (average number of transcripts in expressing cells) * (fraction of expressing cell-specific type).