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## Developmentally upregulated transcriptional elongation factor a like 3 suppresses axon regeneration after optic nerve injury

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### Abstract

Projection neurons of the mammalian central nervous system (CNS) do not spontaneously regenerate axons which have been damaged by an injury or disease, often leaving patients with permanent disabilities that affect motor, cognitive, or sensory functions. Although several molecular targets which promote some extent of axon regeneration in animal models have been identified, the resulting recovery is very limited, and the molecular mechanisms underlying the axonal regenerative failure in the CNS are still poorly understood. One of the most studied targets for axon regeneration in the CNS is the mTOR pathway. A number of developmentally regulated genes also have been found to play a role in CNS axon regeneration. Here, we found that Transcriptional Elongation Factor A Like 3 (Tceal3), belonging to the Bex/Tceal transcriptional regulator family, which also modulates the mTOR pathway, is developmentally upregulated in retinal ganglion cell (RGCs) projection CNS neurons, and suppresses their capacity to regenerate axons after injury.

### Keywords

Axon regeneration; Retinal ganglion cell; Optic nerve; RNA-seq

### 1. Introduction

The mature mammalian central nervous system (CNS), including that of humans and rodents, fails to spontaneously regenerate the axons of projection neurons after they have been severed by an injury or neurodegenerative disease [1,2]. Many studies in recent decades have developed approaches for promoting various extents of axonal regeneration in the CNS, for example, after traumatic optic neuropathy modeled by optic nerve crush (ONC) in animal models [2-9]. However, even in the most successful approaches, which target the

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CRedit authorship contribution statement

AL, JK, BAR, JX, AH, EM, AD, and TS performed the investigations. EFT conceptualized the study and wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

PI3K/mTOR intracellular signaling pathway [10], only a small subset of the axons reach their post-synaptic targets and form functional synapses [11-13]. Although a number of studies provided clues into the molecules involved in the signaling pathway through which targeting the PI3K/mTOR promotes axonal regeneration, for example Thrombospondin-1 [14] and Gsk3-Crmp2 [15], the complete downstream molecular mechanism remains unclear [7,14,16-22]. Bex3 was recently shown to modulate the mTOR signaling pathway [23]. Bex3 belongs to the family of transcriptional regulators, Brain Expressed X-Linked (Bex)/Transcription elongation factor A-like (Tceal) [23]. The Bex/Tceal gene cluster protein sequences are relatively divergent [23,24]. These genes originated by transposon and gene duplication in mammals, and its genes integrated into existing signaling pathways involved in cell-cycle regulation, cancer, development, and function of the mammalian CNS [23,25-30]. A cluster of the Bex/Tceal family genes is also expressed in the retinal ganglion cells (RGCs), in which Bex1 and Bex2 accumulate in the cytoplasm after ischemic optic nerve injury [31]. Because the Bex/Tceal gene family can regulate mTOR signaling and is expressed in the CNS, we investigated its role in axon regeneration, using bioinformatics, the ONC animal model, and gene therapy targeted specifically to the RGCs.

## 2. Results

We analyzed which of the Bex/Tceal genes are expressed in the RGCs and are developmentally regulated, because axon regeneration capacity declines in the CNS during neuronal maturation [32,33], and several developmentally regulated genes have been found to play a role in axon regeneration [8,9,34-36]. For this analysis, we used single cell RNA-seq (scRNA-seq) atlases of embryonic RGCs [37], neonatal RGCs [38], and adult RGCs [39]. Each of these published datasets is comprised of thousands of RGC transcriptomes, and thus provide a reliable resource for comparing the developmental changes of gene expression in the RGCs as they mature. We found that, Bex3, Bex5, Tceal2, and Tceal4 were not expressed, and the expression of Bex6 and Tceal7 in RGCs was in the noise range. Amongst the significantly expressed Bex/Tceal genes, Tceal 1, 3, 5, and 6 displayed the steepest developmental upregulation, whereas only one Bex member, Bex2, was developmentally upregulated, but not as profoundly as the Tceals (Fig. 1A, B). The members of the Klf transcription factors family, Klf4 and Klf9, which are developmentally upregulated in RGCs, were found to suppress axon regeneration, as silencing their expression promoted axon regeneration [8,9,34]. Likewise, we hypothesized that silencing the expression of the developmentally upregulated Tceals may promote axon regeneration. Tceal 1, 3, 5, and 6 were all steeply upregulated in the maturing RGCs (Fig. 1A). However, the expression levels of Tceal 1, 5, and 6 were downregulated after optic nerve injury, whereas the expression of Tceal3 increased (Fig. 1A). Tceal3 is also expressed similarly between most of the RGC subtypes, before and after optic nerve injury [39] (Fig. 1C, D). Thus, Tceal3 is a plausible candidate for functioning as a developmentally upregulated suppressor of axon regeneration, and therefore we tested whether knockdown (KD) of Tceal3 in RGCs could promote axon regeneration after optic nerve crush (ONC) injury.

To address this question, we used a well-established axon regeneration assay [6,8,36,40], in which genes are selectively KD or overexpressed in rodent RGCs via intravitreal injection of

adeno-associated virus serotype 2 (AAV2) vectors, which preferentially transduce the RGCs. In this assay, ONC is performed 2 weeks after AAV2 delivery, and axon regeneration is assayed 2 weeks following ONC. Thus, AAV2 vectors, expressing anti-Tceal3 shRNA or scrambled shRNA control, were injected intravitreally in adult mice which were randomly assigned to experimental or control conditions, and ONC was performed 2 weeks later. The transduction efficiency was approximately 30%, which is similar to prior reports that also used AAV2 to target RGCs. To visualize the regenerating axons or their absence, anterograde axonal tracer, cholera toxin subunit B (CTB), was intravitreally injected 1 day before animals were euthanized 2 weeks following ONC. The number of regenerating axons was quantified in longitudinal sections of the optic nerve, and RGC survival was quantified in retinal flatmounts (see Methods for details; experimental timeline in Fig. 2A).

We found that KD of Tceal3 promoted significant ( $p < 0.01$ ) axon regeneration, up to approximately 1.5 mm from the injury site, compared to only minor axonal sprouting, as expected, in control animals (Fig. 2B, C). No spared axons were detected in either group. There was no apparent difference in RGC survival between the experimental and control groups (Fig. 2D). These data support our hypothesis that Tceal3 is a developmentally upregulated suppressor of CNS axon regeneration. We then asked whether KD of Tceal3 involves the PI3K/mTOR downstream effector ribosomal protein S6 (S6) [10,41], which was affected in the Pten KD-promoted axon regeneration [7]. Inhibiting Pten activates the mTOR kinase, which targets ribosomal S6 kinase 1 (S6K1), that phosphorylates S6. In most adult RGCs S6 is not phosphorylated, and ONC reduces its phosphorylation in the remaining RGCs, which is rescued by Pten knockout (KO) [7]. Thus, we used antibodies to phospho-S6 (p-S6) to test whether KD of Tceal3 will rescue S6 phosphorylation in the injured RGCs. However, we did not find a significant increase in the percent of RGCs that were immunopositive for p-S6 in anti-Tceal3 shRNA treated RGCs compared to control treated RGCs (Fig. 3A, B). These data suggest that Tceal3 may be suppressing axon regeneration either through a different mTOR target or through a different mechanism.

### 3. Discussion

We found that a transcriptional regulator, Tceal3, is developmentally upregulated in RGCs and is further upregulated after ONC injury, whereas other Bex/Tceal genes that are expressed in adult RGCs are downregulated after ONC injury. Other developmentally upregulated transcription factors, Klf4 and Klf9, were previously shown to suppress RGC axon regeneration [8,9,34], and we found that Tceal3 also suppresses axon regeneration. Our finding thus further highlights the significance of transcriptional regulators in physiological suppression of the intrinsic axon growth and regeneration capacity, which declines within the CNS projection neurons as they mature [9,32,33].

The Bex/Tceal gene family can regulate the mTOR signaling pathway and is expressed in the brain [23,42]. The mTOR kinase targets include S6K, 4E-BP, AKT, and SGK kinases [10,41]. However, while KO of the Bex/Tceal gene family member Bex3 led to the activation of S6K1, it did not affect phosphorylation of a prominent S6K1 target S6 [23], consistent with that S6 can be regulated by compensatory mechanisms [23,43]. The mTOR pathway plays a role in axon regeneration, as Pten suppresses RGC axon regeneration

through inhibition of the mTOR pathway [7,14,16-22], whereas Pten KO activated the mTOR kinase, which targeted S6K1 that phosphorylated S6 in the injured RGCs [7]. Therefore, it is possible that, as Tceal3 is highly upregulated during RGC maturation, it may function as a physiological regulator of the developmental loss of the mTOR-gated molecular mechanism involved in axon growth and regeneration. Thus, we investigated whether Tceal3 KD-promoted axon regeneration involves rescue of S6 phosphorylation in the injured RGCs as in Pten KO, or S6 phosphorylation would not be affected as in Bex3 KO (which activates S6K1 without affecting phosphorylation of its target S6) [23]. We did not find a significant increase in the percent of injured RGCs that were immunopositive for p-S6 after Tceal3 KD. Thus, S6 may be regulated by compensatory mechanisms [23,43] after Tceal3 KD in the injured RGCs, whereas Tceal3 may be suppressing axon regeneration either through a different mTOR target or through a different mechanism. These possibilities need to be investigated in future studies. Another avenue for future studies, is to investigate a possible role of Tceal3 in regulating the mTOR pathway in the immune cells, which infiltrate the ONC site and the retina after injury and affect axon regeneration [44,45]. It would also be important to test, in future studies, whether overexpressing Tceal3 in immature RGCs will suppress their axon growth in culture, and whether co-targeting Tceal3 along with Pten [7,46,47], will lead to a synergistic effect in promoting more robust and longer distance axon regeneration, as compared to the effect of targeting Pten or Tceal3 alone.

RGC axons are rapidly and irreversibly damaged in various types of optic neuropathies, such as traumatic optic neuropathy, ischemic optic neuropathy, and different types of glaucoma (e.g., acute angle closure glaucoma), all of which lead to the loss of visual functions or blindness [5,48,49]. Therefore, success of these studies could potentially lead to the development of therapeutics [50], to help regenerate RGC axons while the RGCs are still alive for weeks and months (depending on an RGC subtype) after axonal damage, and thus could be rescued by a treatment.

## 4. Methods

### 4.1. Gene expression analyses

Raw counts of gene expression from progressive stages in RGC development were obtained as follows: For embryonic RGCs from the Gene Expression Omnibus (GEO) repository, accession GSE122466 [37]; for postnatal day 5 RGCs we generated previously [38]; and for adult uninjured and injured RGCs from the GEO repository, accession GSE137400 [39]. Seurat V4.0.0 [51] was used to merge and normalize the datasets. The function VlnPlot [51] was used to generate the violin plots for developmental gene expression. Boxplots (in Fig. 1C, D) with average normalized expression (mean and median  $\pm$  interquartile range error bars) for RGC subtypes were downloaded from the Single Cell Portal [39].

### 4.2. Animal use, surgeries, intraocular injections

All animal studies were performed at the University of Connecticut Health Center with approval of the Institutional Animal Care and Use Committee and of the Institutional Biosafety Committee, and performed in accordance with the ARVO Statement for the Use of

Animals in Ophthalmic and Visual Research. Mice were housed in the animal facility with a 12-h light/12-h dark cycle (lights on from 7:00 AM to 7:00 PM) and a maximum of five adult mice per cage. The study used wild-type 129S1/SvImJ mice (The Jackson Laboratory). Optic nerve surgeries and intravitreal injections were carried out on 8–10 weeks old mice (average body weight, 20–26 g) under general anesthesia, as described previously [6,8,12].

Viruses (3  $\mu$ l per eye) were injected intravitreally, avoiding injury to the lens, in 8-week-old mice, 2 weeks prior to ONC surgery. This lead time allowed for sufficient transduction and expression of the shRNA to knockdown expression of Tceal3 in RGCs at the time of ONC. The viruses included AAV2 expressing anti-Tceal3 (target sequences are as follows: 5'-AAGTATCTACCAGGGAAAGCG-3', 5'-ATATCTGCGCACTCTTCATC-3', 5'-TTTACAATGTACATGCACATG-3', and 5'-CTTCTTGTCTGGCTTCTCGT-3') and an mCherry reporter (titer  $1 \times 10^{13}$  GC/mL; Vector-Builder, Inc.), and AAV2 expressing scrambled shRNA control sequences (5'-TCGAGGCGACTTAACCTTAG-3') and an mCherry reporter (titer  $1 \times 10^1$  GC/mL; VectorBuilder, Inc.).

Cholera toxin subunit B (CTB) conjugated to Alexa Fluor 488 dye (C34775, ThermoFisher Scientific) was injected (1% in 3  $\mu$ l PBS) intravitreally one day prior to sacrifice, at 2 weeks after ONC, in order to visualize the regenerating axons. Investigators performing the surgeries and quantifications were masked to the group identity by another researcher until the end of the experiment. A few animals that developed a cataract in the injured eye were excluded.

#### 4.3. Tissue processing

Standard histological procedures were used, as described previously [6,8,12,47]. Briefly, anesthetized mice were transcardially perfused with isotonic saline followed by 4% paraformaldehyde (PFA) at 2 weeks after ONC, the eyes and optic nerves were dissected, postfixed 2 h, the retinas were dissected-out, and optic nerves were transferred to 30% sucrose overnight at 4 °C. The optic nerves were then embedded in OCT Tissue Tek Medium (Sakura Finetek), frozen, cryostat-sectioned longitudinally at 14  $\mu$ m, and then mounted for imaging on coated glass slides. Free-floating retinas were immunostained in 24-well plate wells and, after making 4 symmetrical slits, flat-mounted on coated glass slides for imaging. For immunostaining, free-floating retinas were blocked with the appropriate sera, incubated overnight at 4 °C with primary anti- $\beta$ III-Tubulin (1:500, rabbit polyclonal; Abcam, #Ab18207) and anti-p-S6 (1:200, rabbit; CTS, #2211S) antibodies, then washed three times, incubated with appropriate fluorescent secondary antibodies (1:500; Alexa Fluor, ThermoFisher Scientific) 4 h at room temperature, washed three times again, and mounted for imaging. Images of the regenerating axons and surviving RGCs were acquired using fluorescent microscope (Zeiss, AxioObserver.Z1); images of p-S6 positive cells were acquired using confocal microscope (Zeiss Confocal, LSM800).

#### 4.4. Quantification of regenerated axons, RGC Survival, and p-S6 positive RGCs

Axons were visualized at 2 weeks after ONC by Alexa Fluor 488-conjugated CTB dye (C34775, ThermoFisher Scientific), which was injected intravitreally 1 day prior to sacrifice. Sections were examined for possible axon sparing; no spared axons were found in control

and no evidence of axon sparing was found in experimental conditions (i.e., at 2 weeks after injury, no axons were found at distal from the injury region of the optic nerve). Regenerated axons (defined as fibers continuous for > 100  $\mu\text{m}$ , which are absent in controls and are discernible from background puncta and artefactual structures), were counted manually using a fluorescent microscope (Zeiss, AxioObserver.Z1) in at least 4 longitudinal sections per optic nerve at 0.5 mm, 1 mm, and 1.5 mm distances from the injury site (identified by the abrupt disruption of the densely packed axons near the optic nerve head, as marked by a rhombus in Fig. 2B), and these values were used to estimate the total number of regenerating axons per nerve, as described [6,8,12].

RGC survival was quantified as described [8,12,47], by immunostaining with an antibody to  $\beta\text{III-Tubulin}$  (1:500, rabbit polyclonal; Abcam, #Ab18207), taking advantage of the selective expression of  $\beta\text{III-tubulin}$  in RGCs. ImageJ software was used to count  $\beta\text{III-Tubulin}^+$  cells from images (Zeiss, AxioObserver.Z1) taken at 1–2 mm from the optic nerve head in four directions, then averaged to estimate overall RGC survival per  $\text{mm}^2$  of the retina. P-S6-positive RGCs were quantified using Image J software and Cell Counter Plugin. P-S6 immunopositive cells were quantified only in the mCherry + RGCs, and represented for the analysis as percent of average p-S6+/mCherry + vs. p-S6-/mCherry + RGCs per retina.

#### 4.5. Statistical analyses

All tissue processing, quantification, and data analysis were done masked throughout the study. Sample sizes were based on accepted standards in the literature and our prior experiences. Sample size ( $n$ ) represents total number of biological replicates in each condition. All experiments included appropriate controls. No cases were excluded in our data analysis, although a few animals that developed a cataract in the injured eye were excluded from the study, and their tissues were not processed. The data was analyzed (as specified in the applicable Figure legends) by MANOVA, or by ANOVA with Repeated Measures and a posthoc LSD, or by an independent samples t-test, 2-tailed (SPSS). Data are presented as means  $\pm$  SEM. All differences were considered significant at  $p < 0.05$ .

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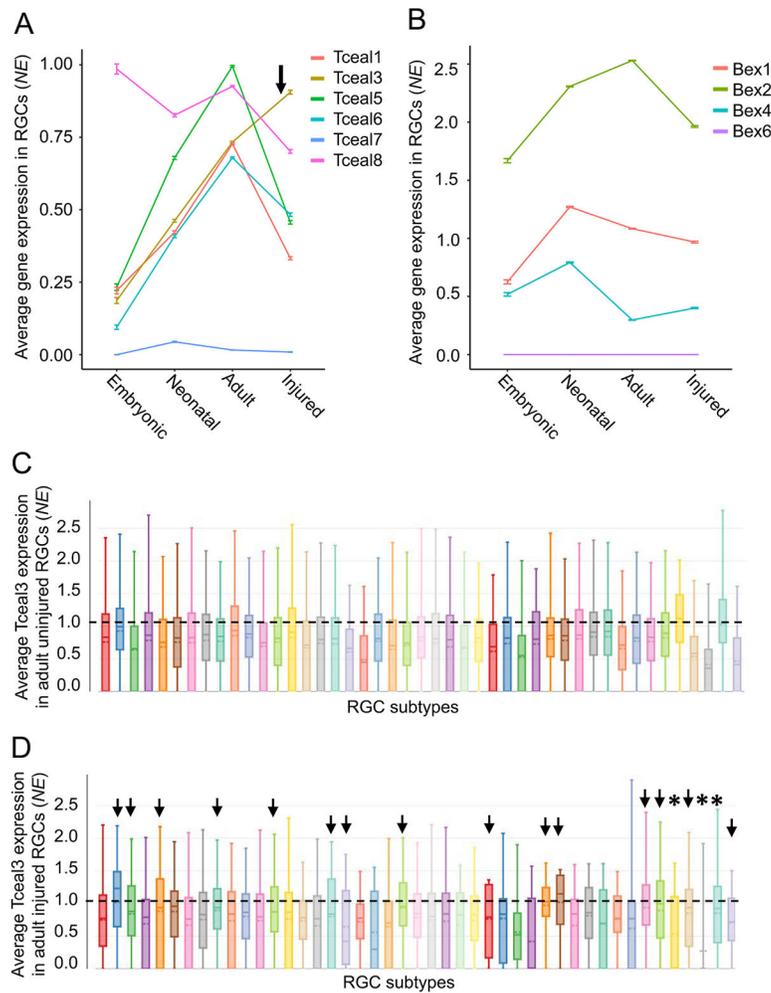
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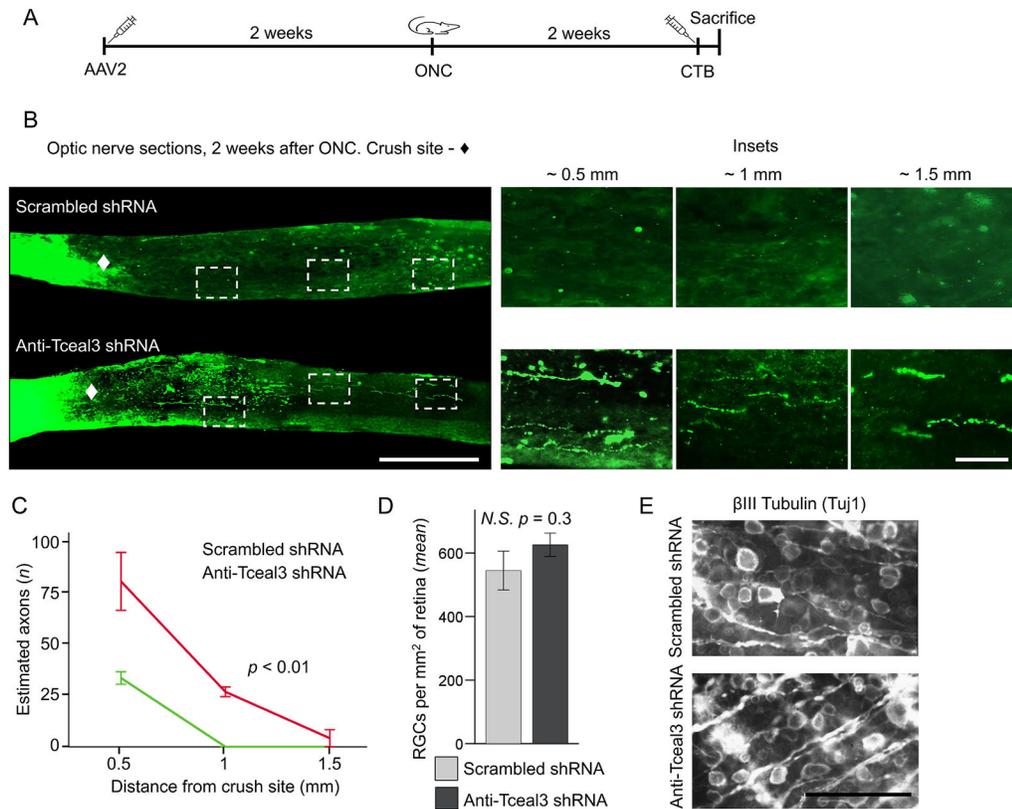
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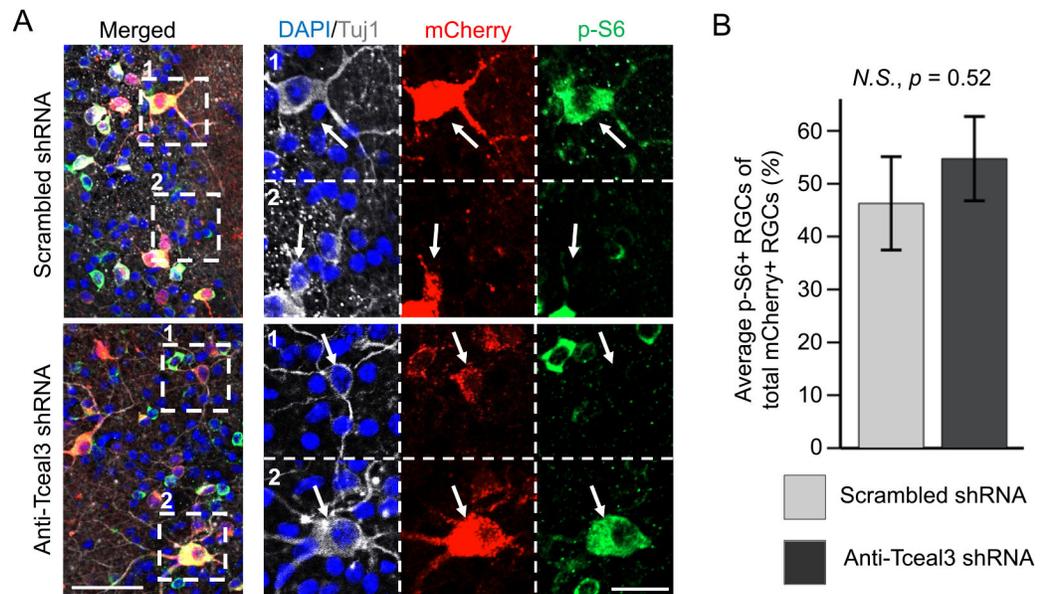
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**Fig. 1.** Differential expression of Bex/Tceal genes in RGCs during development and after injury. (A, B) Expression of Tceal (A) and Bex (B) genes in embryonic (E15), neonatal (P5), adult (10 weeks old), and injured (2 weeks after ONC) RGCs, based on scRNA-seq profiling and averaging gene expression from all the cells (mean — SEM shown). Significance of differential expression at  $p < 0.01$  determined by MANOVA with posthoc LSD pairwise comparisons for all the genes except Bex6 ( $n > 1,200$  RGCs per group). Arrow (in A) points to Tceal3, as the only Tceal/Bex gene that was upregulated after injury. (C, D) Boxplots with average (mean and median  $\pm$  interquartile range error bars shown) Tceal3 expression in subtypes of adult uninjured (C) and injured (D) RGCs show that, Tceal3 is expressed overall similarly across most subtypes (C), and that many subtypes upregulate (arrows in D), whereas only a few subtypes downregulate (asterisks in D), Tceal3 after injury. The dashed line at 1 NE (indicating a moderate level of gene expression in these datasets) is for relative comparison of the clusters in C-D. Downloaded from the Single Cell Portal (see Methods). NE, normalized expression.

**Fig. 2.**

Axon regeneration and RGC survival after treatment with Anti-Tceal3 shRNA. (A) Experimental timeline: 8 weeks old mice were pre-treated with AAV2 vectors expressing anti-Tceal3 shRNA or scrambled shRNA control. Optic nerve crush (ONC) injury was performed 2 weeks later. Animals were sacrificed for histological analysis 2 weeks after ONC. Axonal tracer CTB was injected intravitreally 1 day prior to sacrifice. (B) Representative images of the optic nerve longitudinal sections with CTB-labeled axons at 2 weeks after ONC from the animals pre-treated with scrambled shRNA control or anti-Tceal3 shRNA, as marked. The edges of the tissue were optically trimmed (i.e., cropped-out) due to artefactual autofluorescence that is common at tissue edges. Insets: Representative images of the optic nerve regions proximal and distal to the injury site are magnified for better visualization of the axons or their absence. Scale bars, 500  $\mu\text{m}$  (main panels), 50  $\mu\text{m}$  (insets). (C) Quantitation of CTB-labeled regenerating axons at 2 weeks after ONC, at increasing distances from the injury site, as marked (mean  $\pm$  S.E.M shown;  $n = 4$  cases per group). Data analyzed using ANOVA with repeated measures, sphericity assumed, overall  $F = 44.1$ ,  $p < 0.01$  by posthoc LSD. (D, E) Quantitation of RGC survival in retinal flatmounts immunostained for an RGC marker  $\beta\text{III-Tubulin}$  (Tuj1 antibody) at 2 weeks after ONC did not show a significant difference between the scrambled shRNA control or anti-Tceal3 shRNA treatments (mean — SEM shown,  $n = 4$  cases per group;  $*p = 0.3$  by independent samples t-test, 2-tailed) (D), and corresponding representative images are shown; scale bar, 25  $\mu\text{m}$  (E).



**Fig. 3.**

The treatment with Anti-Tceal3 shRNA does not significantly affect the levels of p-S6 in injured RGCs. (A) Representative images of retinas at 2 weeks after ONC from the animals pre-treated with scrambled shRNA control or anti-Tceal3 shRNA, as marked. Retinas were immunostained for the anti- $\beta$ III-tubulin (gray; Tuj1) and anti-p-S6 (green) antibodies, and counterstained with DAPI (to label nuclei; blue). An mCherry reporter (red) of transduction is shown. Insets show mCherry+/Tuj1+ RGCs (white arrows) with (1) or without (2) clear p-S6 signal. Scale bars, 50  $\mu$ m (main panels), 20  $\mu$ m (insets). (B) Quantitation of percent p-S6+/mCherry+/Tuj1+ of the total mCherry+/Tuj1+ RGCs per retina, at 2 weeks after ONC, did not show a significant difference between the scrambled shRNA control or anti-Tceal3 shRNA treatments (mean  $\pm$  SEM shown,  $n = 4$  cases per group;  $*p = 0.52$  by independent samples  $t$ -test, 2-tailed).