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siRNA-Mediated Knockdown of the mTOR Inhibitor RTP801 Promotes Retinal Ganglion Cell Survival and Axon Elongation by Direct and Indirect Mechanisms

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Purpose. To investigate, using in vivo and in vitro models, retinal ganglion cell (RGC) neuroprotective and axon regenerative effects and underlying mechanisms of siRTP801, a translatable small-interfering RNA (siRNA) targeting the mTOR negative regulator RTP801.

Methods. Adult rats underwent optic nerve (ON) crush (ONC) followed by intravitreal siRTP801 or control siRNA (siEGFP) every 8 days, with Brn3a$^+$ RGC survival, GFAP$^+$ reactive gliosis, and GAP43$^+$ regenerating axons analyzed immunohistochemically 24 days after injury. Retinal cultures, prepared from uninjured animals or 5 days after ONC to activate retinal glia, were treated with siRTP801/controls in the presence/absence of rapamycin and subsequently assessed for RGC survival and neurite outgrowth, RTP801 expression, glial responses, and mTOR activity. Conditioned medium was analyzed for neurotrophin titers by ELISA.

Results. Intravitreal siRTP801 enabled 82% RGC survival compared to 45% with siEGFP 24 days after ONC, correlated with greater GAP43$^+$ axon regeneration at 400 to 1200 μm beyond the ONC site, and potentiated the reactive GFAP$^+$ Müller glial response. In culture, siRTP801 had a direct RGC neuroprotective effect, but required GFAP$^+$ activated glia to stimulate neurite elongation. The siRTP801-induced neuroprotection was significantly reduced, but not abolished, by rapamycin. The siRTP801 potentiated the production and release of neurotrophins NGF, NT-3, and BDNF, and prevented downregulation of RGC mTOR activity.

Conclusions. The RTP801 knockdown promoted RGC survival and axon elongation after ONC, without increasing de novo regenerative sprouting. The neuroprotection was predominantly direct, with mTORC1-dependent and -independent components. Enhanced neurite/axon elongation by siRTP801 required the presence of activated retinal glia and was mediated by potentiated secretion of neurotrophic factors.

Keywords: neuroprotection, axon regeneration, RNA interference

Traumatic optic neuropathy, resulting from ocular or head injuries that directly or indirectly damage the optic nerve (ON), is associated with retinal ganglion cell (RGC) death and ensuing visual loss.[1] Although spontaneous visual improvement occurs in a minority of patients, permanent visual impairment is frequent and currently there are no clinically effective treatments.[2–4]

The RGC death, which commences within 5 to 6 days after ON injury with more than 90% RGC loss by 14 days,[5] is preventable by either downregulation of proapoptotic caspases[6–8] or upregulation of antiapoptotic mediators, such as Bcl-2[9,10] and Bcl-XL.[11] However, axon regeneration is not an inevitable correlate of RGC survival, as independent factors mediate both events.[7,12] For example, protection against axon growth cone collapse (mediated through the Ras homolog gene A/rho-associated kinase [RhoA/ROCK] pathway[12,13]) does not promote significant axonal regeneration in the absence of neurotrophic factor (NTF) stimulation, which drives axon growth by inducing axogenic protein synthesis and supporting growth cone dynamics.[12,14–16] The neurotrophin family includes nerve growth factor (NGF), brain-derived NTF (BDNF), neurotophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), which are produced by activated astrocytes and Müller glia after injury, and act on tyrosine kinase (Trk) receptors to modulate growth-promoting cellular signaling pathways.[17–19] In addition, other NTFs, such as ciliary neurotrophic factor (CNTF), glia-cell-line–derived neurotrophic factor (GDNF), and basic fibroblast growth factor (FGF2), also promote RGC survival and axon regeneration and, in combination with axogenic neurotrophins, act synergistically to stimulate RGC axon growth.[17,20] Activated-glial–derived NTFs are essential for promoting RGC survival and axon regeneration after ON injury,[12,19,21–25] and therefore modulation of the glial response after injury is likely

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to be beneficial for the development of an effective translatable neuroprotective and axogenic treatment strategy.

A reduction in intrinsic axon growth potential during development also contributes to the failure of axon regeneration in the mature central nervous system (CNS) after injury.[26] Key intrinsic determinants of axogenesis include cyclic AMP (cAMP), Krüppel-like transcription factors (KLF), and the serine-threonine kinase mammalian target of rapamycin (mTOR).[27–29] The contribution of mTOR to axogenic signaling has been the focus of much recent research interest. Although active during development, mTOR is downregulated in the mature CNS, probably through the progressive upregulation of phosphatase and tensin homolog (PTEN), a negative regulator of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway.[30] Deletion of PTEN promotes activation of mTOR signaling and inactivation of GSK3β, enabling RGC survival and axon regeneration after ON crush (ONC).[29,31,32] Deletion of PTEN similarly enhances the regenerative potential of spinal corticospinal tract and peripheral sensory axons.[33–35] The activity of mTOR is further downregulated after ONC, as evidenced by reduced expression of phosphorylated ribosomal S6 protein (pS6), a widely used downstream indicator of mTOR activity.[31] Deletion of PTEN prevents ONC-induced suppression of pS6 expression in up to 10% of surviving RGCs, correlated with axon regeneration in 8% to 10% surviving RGCs, confirming pS6 expression as a possible key marker of intrinsic axon regenerative ability.[29,31] However, limited axon regeneration is still observed in the presence of the mTOR complex 1 (mTORC1) inhibitor rapamycin,[31] suggesting that mTORC1-independent factors may also contribute, such as via mTOR complex 2 (mTORC2) or other pathways.

Hypoxia, energy deprivation, DNA damage, and reduced nutrient availability all inhibit mTOR-mediated protein synthesis,[36] and the TSC1/2 complex is a key nodal point in the negative regulation of mTOR function. In the canonical PI3K/Akt-signaling cascade, the TSC1/2 complex is phosphorylated and inhibited by Akt, thereby allowing accumulation of Rheb-GTP and subsequent activation of mTOR.[37] Cells deficient for TSC1/2 fail to suppress mTOR activity in conditions of energy deprivation.[38,39] Hypoxia and cellular stress promote the rapid induction of RTP801 (also known as DDIT4/Redd1), which activates the TSC1/2 complex to downregulate mTOR activity.[37,40] Moreover, AMP-activated kinase, which mediates mTOR downregulation in response to impaired cellular energy production via TSC1/2, activates RTP801 to reduce mTOR activity.[41] RTP801 is closely linked to regulation of neuronal development and viability, is a negative regulator of neurogenesis during embryologic development, and RTP801 knockdown accelerates cell cycle exit by neural progenitors and their differentiation into neurons.[42] Upregulation of RTP801 is associated with the induction of neuronal apoptosis,[43] and suppression of RTP801 has demonstrated neuroprotective effects in retinal neurons in vitro and in vivo.[44,45] Therefore, targeting the stress-induced factor RTP801 is a potentially viable strategy to promote mTOR activity after injury, and enhance CNS neuroprotection and axogenesis.

This study investigated the RGC neuroprotective and ON axon regenerative effects and underlying mechanisms of siRTP801, a therapeutically translatable small-interfering RNA (siRNA) that effectively knocks down the mTOR negative regulator RTP801 and is currently in clinical development for nonneuroprotective indications.[46–50] We show that siRTP801 supports RGC survival and elongation of regenerating axons, a response that is accompanied by potentiated reactive gliosis after ONC in vivo in the absence of nonspecific proinflammatory activity. In retinal cultures, RGC neuroprotection is directly induced by siRTP801 through both mTORC1-dependent and independent pathways, whereas the neurite elongation effect of siRTP801 is indirect, requiring the presence of activated glial fibrillary acidic protein ($GFAP^+$) retinal glia–derived NTF. Moreover, siRNA-mediated knockdown of RTP801 prevents downregulation of pS6 expression in RGCs, although the latter is not an absolute requirement for neurite growth. Our results suggest that siRTP801 is RGC neuroprotective and supports elongation of regenerating axons via direct and indirect mechanisms and may have potential for the treatment of traumatic optic neuropathy.

Methods

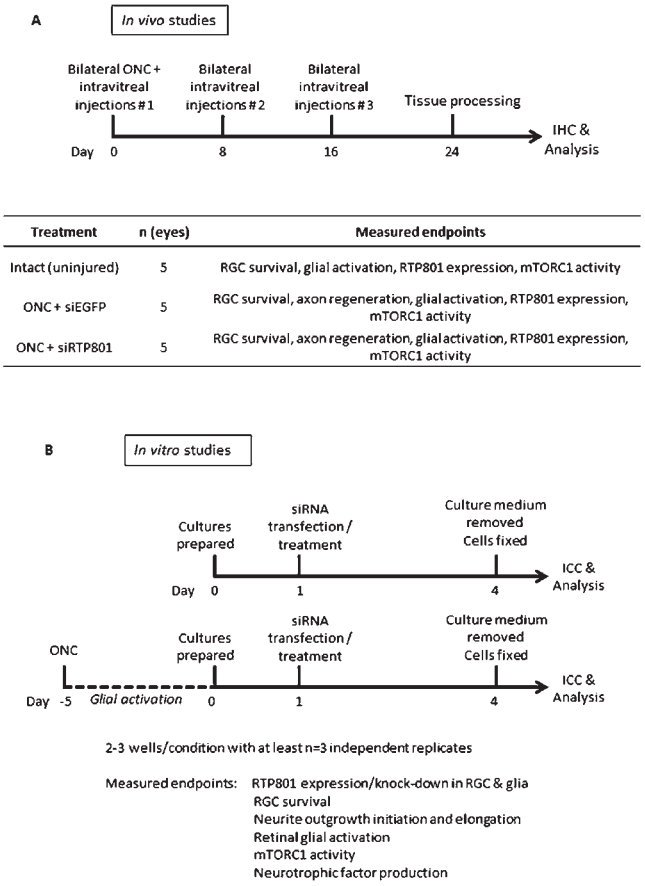
Experimental Design

An initial series of in vivo studies was undertaken to evaluate various combinations of siRNA targeting aspects of cellular signaling, and siRTP801 was identified as a suitable candidate for further study and used without combination in the results presented here. Specific details of methodology and results pertaining to siRTP801 in RGC survival, axon regeneration, and glial activation in these initial studies are given in Supplementary Tables S1 through S3 to support the present data. The siRTP801 target specificity and the lack of nonspecific proinflammatory activity in the retina after intravitreal injection were confirmed by RNA ligase–mediated rapid amplification of cDNA ends (RLM-RACE) and monitoring expression of interferon-inducible genes, respectively. In vivo and in vitro experiments were designed to evaluate RGC neuroprotection and axon regeneration after the siRTP801-mediated activation of mTOR (Fig. 1). After ONC, siRTP801 was intravitreally injected in one eye and control siEGFP to the fellow eye 0, 8, and 16 days later. Tissue was harvested at 24 days for immunohistochemistry and analysis of RGC survival, axon regeneration, glial activation, RTP801 expression, and mTORC1 activity (pS6 expression). A group of uninjured animals was used as intact controls (Fig. 1A), as a benchmark for determining the effects of siRTP801 compared with inactive control siRNA against enhanced green fluorescent protein (siEGFP) after ON injury.

Retinal culture studies were undertaken to evaluate the cellular mechanisms underlying the effects seen in vivo (Fig. 1B). Dissociated mixed cell cultures were obtained from adult rat retinae, either uninjured or at 5 days after ONC to activate retinal glia. Cultured cells were transfected with either siRTP801 or control siEGFP or culture medium alone, all in the presence or absence of the mTORC1 inhibitor rapamycin and incubated for 3 days. Cultures were immunostained for analysis of RGC survival, neurite outgrowth, siRTP801-target knockdown, reactive glial marker expression, and mTORC1 activity. Culture medium from siRTP801-transfected and control (siEGFP and culture medium alone) wells, both from uninjured animal retinal cultures and after glial activation, was assayed for neurotrophin titers by ELISA. All reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

Small-Interfering RNA

All siRNA molecules used in this study were stabilized by alternating 2′O-methylation as previously described,[51] and were chemically synthesized at BioSpring (Frankfurt, Germany). The siRTP801 had sequence 5′-GUGCCAACCUGAUG CAGCU-3′ (sense strand); siRNA against EGFP was as previously described.[52] Previous in vivo studies using a similar siRNA approach demonstrated detectable siRNA levels in the retina up to 28 days after intravitreal injection, with half-life of approximately 7 days.[6]



Treatment	n (eyes)	Measured endpoints
Intact (uninjured)	5	RGC survival, glial activation, RTP801 expression, mTORC1 activity
ONC + siEGFP	5	RGC survival, axon regeneration, glial activation, RTP801 expression, mTORC1 activity
ONC + siRTP801	5	RGC survival, axon regeneration, glial activation, RTP801 expression, mTORC1 activity

FIGURE 1. Experimental design for in vivo and in vitro experiments. (**A**) Timeline of in vivo study protocol, and treatment groups with measured endpoints (IHC, immunohistochemistry). (**B**) Timeline of in vitro studies, detailing retinal cultures obtained from uninjured adult rats and 5 days after ONC to activate retinal glia, with measured endpoints (ICC, immunocytochemistry). Treatment conditions included siRTP801, siEGFP, and sNBA culture medium alone both in standard and glial-activated cultures. Specific wells were also treated with the mTORC1 inhibitor rapamycin.

In Vivo Experiments

Animals and Surgical Procedures. All studies using experimental animals were conducted in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult male Wistar rats (Charles River, Kent, UK), weighing 200 to 250 g at the commencement of experiments, were housed at 21°C/55% humidity in a 12-hour light and dark cycle with ad libitum access to food and water. All surgeries performed at University of Birmingham and Neuregenix Ltd. were carried out under the UK Home Office regulations for the care and use of experimental animals and the UK Animals (Scientific Procedures) Act 1986, and approved by the University of Birmingham Ethical Review Committee. Inhalational anesthesia was induced with 5% isoflurane with 1.5 L/min O_2 (National Veterinary Supplies, Stoke, UK) and maintained at 3% throughout surgery. After surgery, lubricating Carbomer Gel 0.2% eye lubricant (Blumont Healthcare, Grantham, UK) was applied, and animals recovered from surgery in heated cages under supervision until the emergence of normal behavior, when they were returned to home cages. All animal procedures performed at the facilities of Quark Pharmaceuticals, Inc., were reviewed and approved by the Hebrew University Animal Research Committee in Israel.

Optic Nerve Crush. After analgesia with subcutaneous injection of buprenorphine (0.1 mL/100 g; National Veterinary Supplies), animals were anesthetized, secured in a head-holding frame, and underwent bilateral ONC as described previously.[53,54] Briefly, after a midline cutaneous incision, the ON was exposed through a superior orbital approach and crushed with forceps 2 mm from the lamina cribrosa, within the dural sheath to sever all RGC axons while preserving retinal vascular supply.

Intravitreal Injections and Treatment Schedule. Directly after ONC, intravitreal injections were performed using glass micropipettes inserted immediately posterior to the limbus, without damaging the lens. Animals were bilaterally injected with of 20 μg siRNA, dissolved in 10 μL sterile PBS, with intravitreal delivery of siRTP801 to one eye and control siRNA (siEGFP) to the contralateral eye ($n = 5$ animals). Repeat siRNA injections were delivered 8 and 16 days after ONC, and tissue harvested at 24 days after ONC for processing and analysis. An additional group of uninjured animals was used as intact controls ($n = 5$ eyes) (Fig. 1A).

Tissue Preparation. At 24 days after ONC, animals were killed by overdose of CO_2 and perfused transcardially with PBS followed by 4% paraformaldehyde (PFA; TAAB, Reading, UK). Eyes and ONs were dissected and postfixed in 4% PFA for 2 hours at 4°C. Tissue was cryoprotected sequentially in 10%, 20%, and 30% sucrose solutions in PBS for 24 hours at 4°C, embedded in optimal cutting temperature embedding medium (Thermo Shandon, Runcorn, UK), frozen rapidly on dry ice, and stored at −80°C. Radial sections of eyes and longitudinal ON sections were cut at 15 μm on a cryostat (Bright, Huntingdon, UK), mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA), and stored at −20°C until analysis. Sections of ON with a clearly demarcated ONC site, and radial ocular sections taken through the ON head were selected for analysis.

Immunohistochemistry. Eye and ON sections were washed in rinsing buffer (0.1% Triton X-100 in PBS) for 3 × 5 minutes. Excess rinsing buffer was aspirated and a hydrophobic pen used to draw around each section. A 10% normal goat/donkey serum (Vector Laboratories, Peterborough, UK) and 3% BSA blocking solution was applied to each section to eliminate nonspecific protein binding, and incubated in a humidified chamber for 1 hour at room temperature. Blocking buffer was removed and sections were then incubated with primary antibodies (100 μL/section, Supplementary Table S4) in antibody diluting buffer (ADB; 3% BSA in PBS) in a humidified chamber overnight at 4°C. Additional sections were prepared as described, but in the absence of primary antibody to act as negative controls (not shown). The following day, sections were washed in rinsing buffer (3 × 5 minutes) and incubated in a humidified chamber for 1 hour in the dark at room temperature with appropriate secondary antibodies (100 μL/section; Supplementary Table S4). Slides were then washed in rinsing buffer (3 × 5 minutes) and coverslip mounted using Vectashield with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and stored at 4°C before microscopic analysis as detailed below.

Assessment of RGC Survival and Axon Regeneration. All sections were viewed under an upright Axioplan-2 fluorescence microscope and ×200 magnification photomicrograph images captured with Axiovision software (both Carl Zeiss Ltd., Hertfordshire, UK). Survival of RGCs was analyzed as previously described.[55] Briefly, RGCs were identified and counted in a standard 250-μm linear strip of the ganglion cell layer (GCL) in radial sections on either side of the ON head (four radial sections/retina, $n = 5$ eyes/treatment group), using

the RGC phenotypic antibody marker Brn3a,[56] and results were expressed as the mean number of RGCs per 250-μm GCL. Survival analysis of RGCs in the initial in vivo studies used βIII-tubulin as the RGC phenotypic marker (Supplementary Table S1), which was superseded with the more specific Brn3a for the studies reported here. Estimation of RGC numbers using Brn3a immunostained retinal sections detects quantitatively similar RGC loss compared with Brn3a-stained retinal whole-mounts or FluoroGold (FG) back-labeled retinal whole-mounts, while avoiding the additional surgery required for FG studies. Retinal analysis is therefore simplified compared with whole-mount preparations and enables both RGC survival and axon regeneration analyses from the same animals, thereby reducing animal usage and associated costs.[56] Quantification of axon regeneration in longitudinal ON sections was undertaken using a method described previously.[24] Briefly, composite images were constructed from individual ×200 magnification ON sections in Photoshop CS3 (Adobe Systems, Inc., San Jose, CA, USA). The ONC site was identified by laminin$^+$ staining and the number of GAP43$^+$ regenerating axons extending 100, 200, 400, 800, and 1200 μm from the center of the ONC site counted (three sections/ON, $n = 5$ eyes/group). We used GAP43 to label regenerating axons because this is the gold standard method of quantifying RGC axon regeneration in the distal segment of the rat ON.[57] In addition, a recent study from our laboratory reported that the number of GAP43$^+$ regenerating axons in the distal ON segment correlated with the number of axons detected by the anterograde tracer Rhodamine B.[23] The cross-sectional width of the ON was measured at the point at which axon counts were taken, and used to calculate the number of axons/mm ON width. This value was used to derive Σad, the total number of axons extending distance d in an ON with radius r using the following formula:

$$\Sigma ad = \pi r^2 x(average\ number\ of\ axons/mm\ width)/$$

$$(section\ thickness\ 0.015\ mm)$$

Assessment of Glial Activation. Astrocytes in the GCL/nerve fiber layer (NFL) and Müller glial processes and somata were detected using GFAP and S100β immunohistochemistry. Müller glial processes and somata were quantified using a method described previously.[24] Radial retinal sections through the ON head were immunostained for GFAP, an activated astrocyte/Müller cell antibody marker. The number of GFAP$^+$ Müller cell processes intersecting a 250-μm horizontal linear sampling line passing through the inner plexiform layer (IPL) was counted at the midpoint between the GCL and inner nuclear layer (INL) (four sections/retina, $n = 5$ eyes/group). Retinal sections were also immunostained for the S100β protein, expressed by both activated and quiescent astrocytes/Müller glia.[58] The number of S100β$^+$ Müller glia somata in the INL was also quantified along a 250-μm sample line (four sections/retina, $n = 5$ eyes/group).

Assessment of RTP801 Expression and mTORC1 Activity. The RTP801 protein was detected in retinal sections by immunohistochemistry. The RTP801$^+$ RGCs were identified by coimmunostaining with the RGC phenotypic marker Brn3a, and reported as a percentage (±SEM) of the total RGC count in the GCL sample. The RTP801$^+$/Brn3a$^-$ nonneuronal cells of the GCL were counted in the same 250-μm GCL linear sample as described. Activity of mTORC1 was evaluated by immunohistochemical detection of pS6 in retinal sections, and pS6$^+$ RGC reported as a percentage (±SEM) of the total RGC count in the GCL sample.

Confirmation of RTP801-Specific RNA Interference Following Intravitreal Administration of siRTP801. Specificity of siRTP801 was confirmed by detection of siRTP801-specific RTP801 mRNA cleavage by RLM-RACE in the rat retina in vivo. Adult male 180- to 220-g Sprague-Dawley rats ($n = 4$/treatment group) were given a 20-μg intravitreal injection of either siRTP801 or siEGFP in 10 μL PBS per eye. Four hours later, retinae were harvested and extracted RNA (EZ-RNA extraction kit; Biological Industries, Beth HaEmek, Israel) was analyzed using RLM-RACE to detect siRTP801-produced mRNA cleavage product using GeneRacer Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA samples extracted from cultured PC12 cells transfected with siRTP801 were used as a positive control. The RACE products were separated on 2% agarose gel, blotted, and hybridized to the RACE product-specific oligonucleotide probe (5′-GGAGTAGAAATGATGCAGCT-3′) labeled with γ-^{33}P-ATP. After hybridization, the membrane was washed and exposed to a KODAK BioMax film (Kodak, Rochester, NY, USA). The following primers were used for the amplification of the RTP801-RLM-RACE-product: 5′-GGCTGTAACCAGGGACCAAG-3′ (RTP801-specific RT primer); 5′-ATGCCAGGCGCAG GAGTTCCTT-3′ (RTP801-specific reverse PCR primer); 5′-AGGAGTTCCTTGCCCACCT-3′ (RTP801-specific nested reverse PCR primer); 5′-CGACTGGAGCACGAGGACACTGCAT-3′ (adapter-specific forward PCR primer); and 5′-GGACACTG CATGGACTGAAGGAGTA-3′ (adapter-specific nested forward PCR primer).

Confirmation of the Lack of Nonspecific Proinflammatory Effects of Intravitreal siRTP801 Administration. Sprague-Dawley rats ($n = 6$/treatment group) were injected intravitreally with either 20 mg per eye of poly(I:C) (200–1000 bp; Invivogen, Toulouse, France) in 10 mL PBS or 35 mg siRTP801 in 10 mL PBS (19 bp; ~30-fold molar excess compared with poly [I:C]). Control animals were injected with 10 mL PBS (the vehicle). Four or 24 hours after injection, eyes were enucleated and retina/choroid complexes isolated for RNA extraction (EZ-RNA extraction kit; Biological Industries) and assessment of expression of IFN-responsive genes using quantitative real-time RT-PCR (qPCR). The following amplification primers were used (official gene symbols are shown in parentheses): rat cyclophilin A (*PPIA*): forward: 5′-CGACTGTGGACAGCTCTAAT-3′; reverse: 5′-CCTGAGCTACA GAAGGAATG-3′; rat beta-actin (*ACTB*): forward: 5′-AGAGC TATGAGCTGCCTGAC-3′; reverse: 5′-AATTGAATGTAGTTT CATGGATG-3′; rat interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*): forward: 5′-GCCTACGTGAGA CACCTGAA-3′; reverse: 5′-AGGTCACCAGGCTTCTCTTG-3′; rat 2′-5′ oligoadenylate synthetase 1B (*OAS1B*): forward: 5′-TGATGTGCTTCCAGCCTATG-3′; reverse: 5′-TGCGCTCACT GATGAGATTG-3′.

In Vitro Experiments

Adult Rat Retinal Cultures. Animals were killed by CO_2 overdose, and retinae dissociated into single cells using a papain dissociation kit in accordance with the manufacturer's protocols (Worthington Biochemicals, Lakewood, NJ, USA), as described previously.[19,54] Retinal cells were plated at a density of 125,000 cells per well into 8-well chamber slides (BD Biosciences, Erembodegem, Belgium) precoated with poly-D-lysine and laminin, in 300 μL/well Neurobasal A with B27 supplement and gentamicin (sNBA; all from Invitrogen, Paisley, UK). To assess the effect of siRTP801 on RGC survival and neurite outgrowth in the presence of activated retinal glia, additional cultures were prepared from animals 5 days after ONC (as described above).[25] Cultures were incubated at 37°C and 5% CO_2 overnight and subsequently transfected with siRNA in the presence or absence of inhibitors.

Transfection of Retinal Cultures With siRNA and Treatment With Rapamycin. Mixed retinal cell cultures were transfected with siRNA using Lipofectamine 2000

transfection reagent (Invitrogen) in accordance with the manufacturer's guidelines, as previously reported.[59] In brief, siRNA and transfection reagent were each diluted in sNBA culture medium (without antibiotics) and incubated for 5 minutes at room temperature. The two solutions were combined, gently mixed, and incubated for a further 25 minutes at room temperature to form siRNA-reagent complexes. Complexes were diluted to the desired concentrations in medium, added to the cells, and transfected for 5 hours before addition of sNBA to a final volume of 500 µL per well, and incubated at 37°C and 5% CO_2 for 3 days. Culture medium alone and Lipofectamine reagent without siRNA were used as controls. A dose-response assay was undertaken initially, with both siRTP801 and siEGFP tested at 10-nM, 20-nM, and 50-nM concentrations, confirming that a concentration of 20 nM was optimal for RGC neuroprotection (see Results) and used for further experiments. Specific wells were also treated with rapamycin (10 nM), added at the end of the siRNA transfection period. In vitro experiments composed of two to three wells per treatment condition, repeated with retinae from at least three independent animals.

Immunocytochemistry. After incubation for 3 days, cell culture medium was removed from wells and cells were fixed in 4% PFA in PBS for 10 minutes before immunostaining. In each well, cells were washed three times in rinsing buffer (0.1% Triton X-100 in PBS), and nonspecific protein binding blocked with 150 µL blocking buffer (10% normal goat/donkey serum and 3% BSA in PBS) for 1 hour at room temperature. Primary antibodies (150 µL/well in ADB; Supplementary Table S4) were added to wells and cells incubated for 1 hour at room temperature. Cells were then washed again (3 × 5 minutes) in rinsing buffer and incubated with secondary antibodies (150 µL/well in ADB; Supplementary Table S4) for 1 hour at room temperature in the dark. After further washes, chamber well walls were removed from slides that were then coverslip mounted with Vectashield containing DAPI (Vector Laboratories) and viewed under fluorescence microscopy (Carl Zeiss Ltd.).

Assessment of RGC Survival and Neurite Outgrowth. Surviving RGCs were identified by βIII-tubulin immunostaining (Supplementary Fig. S1). Each well was divided into a grid of 9 squares, and 4 photomicrographs (×200 magnification) were captured within each square, giving a total of 36 images per well. Counts of RGCs were undertaken from these images and cell numbers per well determined from these samples. The βIII-tubulin$^+$ RGC extending neurite(s) over a length greater than the diameter of somata were identified (Supplementary Fig. S1). The longest neurite per RGC was measured using Image Pro 6.2 (Media Cybernetics, Bethesda, MD, USA). The RTP801$^+$/βIII-tubulin$^+$ RGCs were expressed as a percentage of the total βIII-tubulin$^+$ RGC population. The GFAP$^+$/S100β$^+$glia were identified immunocytochemically (Supplementary Fig. S1; Supplementary Table S4) and quantified as described for RGCs.

Neurotrophic Factor ELISA. To evaluate NTF production after siRNA treatment of cultured retinal cells, conditioned medium was removed from wells for ELISA after 3 days of incubation, immediately before fixing cells for immunocytochemistry. Enzyme-linked immunosorbent assays for NGF, neurotrophin-3 (NT3; both R&D Systems, Abingdon, UK), and brain-derived neurotrophic factor (BDNF; Promega, Southampton, UK) were undertaken according to the manufacturers' instructions. Briefly, test samples of conditioned medium were run at graded concentrations (n = 3 independent samples/condition) and neurotrophin titers extrapolated from a standard curve generated from the standard samples provided.

Statistics. Statistical tests were undertaken with SPSS 19.0 (IBM SPSS, Inc., Chicago, IL, USA) and data presented as means ± SEM. All experimental results were derived from a minimum of three independent replicates. For analysis of axon regeneration after ONC in vivo, the Student's t-test was used to compare treatment (siRTP801) with control (siEGFP) conditions. All other comparisons between groups were undertaken with ANOVA, followed by Dunnett or Tukey post hoc tests. Statistical significance was considered at P less than 0.05.

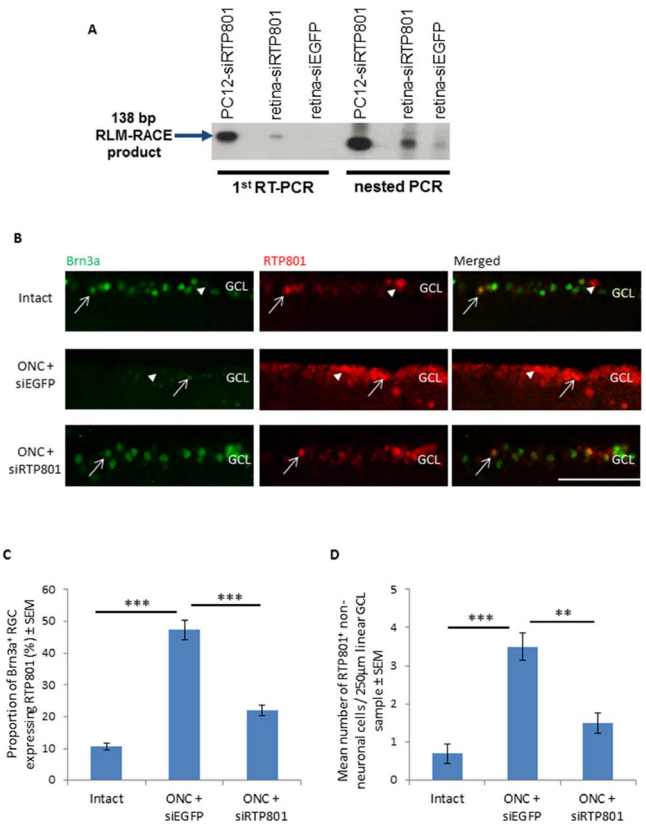


Figure 2. Confirmation of siRNA specificity and RTP801 expression/knockdown in vivo. (**A**) Detection of siRTP801-specific RTP801 mRNA cleavage by RLM-RACE in the retina in vivo. (**B**) Retinal sections immunostained for Brn3a (*green*) and RTP801 (*red*) in the GCL of intact retinae and 24 days after ONC + siEGFP and ONC + siRTP801 (*scale bar*: 100 µm). Note the presence of RTP801$^+$ RGC (*arrows*) and also RTP801 expression in nonneuronal cells (*arrowheads*). (**C**) Quantification of RTP801$^+$ RGC in a 250-µm GCL linear region of the animals described in (**B**) (n = 5 retinae/group; $^{***}P < 0.001$), indicating injury-induced RTP801 upregulation in RGC and target knockdown by siRTP801. (**D**) Quantification of RTP801 activity in Brn3a$^-$ nonneuronal cells of the GCL for the groups described in (**B**) ($^{**}P < 0.01$, $^{***}P < 0.001$), indicating injury-induced RTP801 upregulation and target knockdown by siRTP801.

Results

In Vivo Experiments

Characterization of Specific and Nonspecific Activity of siRTP801 in the Retina Following Intravitreal Injection. Detection of siRTP801-specific RTP801 mRNA cleavage by RLM-RACE in the retina after intravitreal siRTP801 delivery confirmed the specific activity of this siRNA in vivo (Fig. 2A). The lack of induction of retinal expression of interferon-responsive genes after intravitreal administration of siRTP801 confirmed the lack of proinflammatory (mediated by Toll-like receptor [TLR] activation)[60,61] effects of this compound (Supplementary Fig. S2). There was a low level of RTP801 immunoreactivity in the GCL of retinal sections from intact

uninjured animals (Fig. 2B). Increased RTP801 protein was detected in GCL cells of retinal sections 24 days after ONC and intravitreal siEGFP, which was attenuated in sections from ONC + siRTP801 animals. Quantitatively, RTP801 positivity was detected in 10.6% ± 1.1% Brn3a$^+$ RGC in intact controls, and significantly increased to 47.3% ± 3.1% ($P < 0.001$) of remaining RGCs after ONC + siEGFP. Treatment with siRTP801 significantly reduced the proportion of RTP801$^+$ RGC compared with siEGFP-injected eyes (21.9% ± 1.6%, $P < 0.001$) (Fig. 2C). In addition to RGCs, RTP801 localized to Brn3a$^-$ cells of the GCL of intact animals, probably astrocytes/amacrine cells (0.7 ± 0.3 cells/250 μm GCL), with increased RTP801 detection in these cells after ONC + siEGFP (3.5 ± 0.4 cells, $P < 0.001$), which was reduced after siRTP801 treatment (1.5 ± 0.3 cells, $P = 0.001$) (Fig. 2D). Together, these findings indicated a low level constitutive RTP801 activity in the retina of uninjured animals, increased RTP801 levels after ONC injury in RGC and non-RGC cells, and attenuation of RTP801 immunoreactivity after siRTP801 treatment.

siRTP801 Promoted RGC Survival Up to 24 Days After ONC. Compared with uninjured intact control animals (20.4 ± 0.6 RGCs/250 μm GCL), there was a significant reduction ($P < 0.001$) in Brn3a$^+$ RGC survival at 24 days after ONC and intravitreal control siEGFP (9.2 ± 0.6 RGCs) (Figs. 3A, 3B) or PBS (not shown; see Supplementary Table S1). Intravitreal treatment with siRTP801 promoted a significant increase ($P < 0.001$) in Brn3a$^+$ RGC survival (16.9 ± 0.6 RGC) compared with animals receiving siEGFP at 24 days after ONC (Figs. 3A, 3B). Treatment with siRTP801 allowed survival of 82% RGCs at 24 days after ONC, whereas RGC survival after siEGFP was 45% compared with intact eyes. These results suggested that targeted siRNA-mediated knockdown of RTP801 was RGC neuroprotective after ONC. The results of two supporting initial in vivo experiments are summarized in Supplementary Table S1.

siRTP801 Promoted Longer Growth of Regenerating Axons Up to 1200 μm Beyond the Injury Site Without Affecting Initiation of Axon Growth After ONC. After ONC, the number of GAP43$^+$ regenerating RGC axons was significantly greater at 400 μm ($P < 0.05$), 800 μm ($P < 0.05$), and 1200 μm ($P < 0.01$) distal to the crush site after treatment with siRTP801 (87.1 ± 21.1, 68.9 ± 11.2, 55.8 ± 11.5 axons/nerve, respectively) compared with siEGFP (44.9 ± 23.0, 28.6 ± 15.8, 15.4 ± 9.9 axons/nerve, respectively) (Figs. 3C, 3D). There was no significant difference in the number of GAP43$^+$ RGC axons in the ON at 100 μm and 200 μm distal to the center of the ON crush site between siRTP801- and siEGFP-injected animals. These results showed that siRTP801 and control siEGFP treatments led to sprouting of similar numbers of axons about the injury site, but siRTP801 treatment sustained significantly longer growth of regenerating axons after ONC. The results of two supporting initial in vivo experiments are summarized in Supplementary Table S2.

siRTP801 Potentiated GFAP$^+$ Glial Activation Without Cellular Proliferation After ONC In Vivo. In the retina, there was subdued GFAP$^+$ (a marker of activated glia[62]) astrocyte immunoreactivity in the NFL and GFAP$^+$ Müller glial processes were absent from the IPL in intact animals (Fig. 4A). After ONC, GFAP$^+$ immunoreactivity increased in the NFL, and GFAP$^+$ Müller glial processes traversed the IPL (Fig. 4A). The number of GFAP$^+$ Müller glial processes in the IPL was significantly ($P < 0.001$) increased at 24 days after ONC in retinae treated with siRTP801 (14.9 ± 0.7/250 μm) compared with siEGFP (10.2 ± 0.5) after the same injury (Figs. 4A, 4B). The number of S100β$^+$ Müller glia somata (a marker of Müller glia cells[58,62,63]) in the INL was unchanged after ONC in siEGFP controls (16.6 ± 0.4/250 μm) and siRTP801-treated retinae (17.3 ± 0.4) compared with intact retinae (16.7 ± 0.5) (Figs. 4C, 4D). These data suggested that siRTP801 enhanced

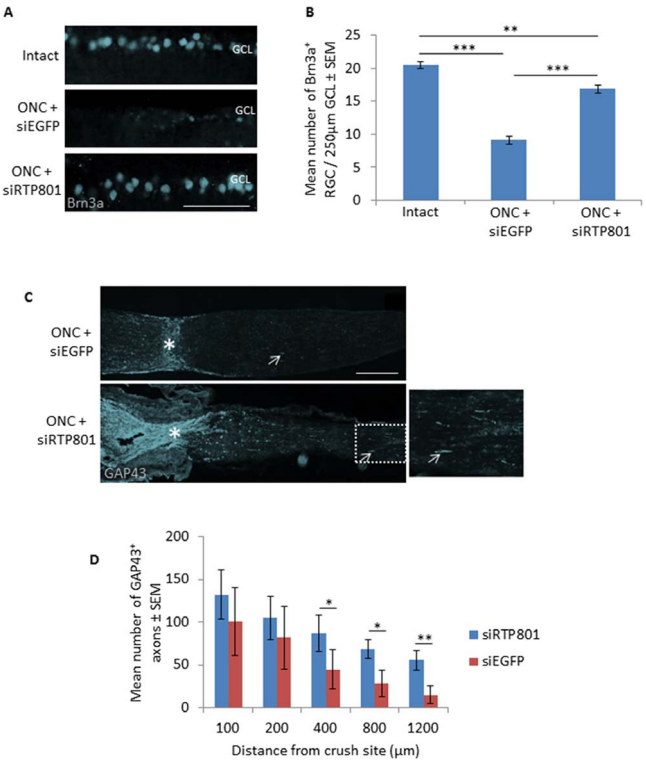


Figure 3. Retinal ganglion cell survival and axon regeneration after targeted knockdown of RTP801. (**A**) The Brn3a$^+$ immunostained RGCs in the GCL in intact retinae and 24 days after ONC + siEGFP and ONC + siRTP801 treatments. *Scale bar*: 100 μm. (**B**) Quantification of Brn3a$^+$ RGC in a 250-μm linear region of the GCL in animals described in (**A**) ($n = 5$ retinae/group; $^{**}P < 0.01$, $^{***}P < 0.001$). (**C**) Longitudinal ON sections immunostained to demonstrate GAP43$^+$ axons (*arrows*) after ONC + siEGFP– and ONC + siRTP801–treated rats. *Scale bar*: 200 μm. The *asterisk* demarcates ONC sites and the *boxed area in the lower panel* represents a magnified area. Note that the increased number of axons in the proximal ON segment (*lower panel*) correlates with enhanced RGC survival. (**D**) Quantification of GAP43$^+$ axons at 100, 200, 400, 800, and 1200 μm beyond the ONC site after ONC + siRTP801 (*blue bars*) and ONC + siEGFP (*red bars*) ($n = 5$ nerves/group; $^*P < 0.05$, $^{**}P < 0.01$).

activation of GFAP$^+$ glia after ONC, without significantly increasing Müller glial proliferation. The results of a previous supporting independent evaluation of GFAP$^+$ Müller glial activation are summarized in Supplementary Table S3.

Injury-Induced Reduction in RGC mTORC1 Activity Was Prevented by siRTP801. Immunohistochemical detection of pS6 was observed in the GCL of intact animals, although was limited to a minority of Brn3a$^+$ RGCs (15.4% ± 1.7% RGC, Fig. 5). After ONC + siEGFP, there was a significant reduction in pS6 activity, localizing to only 5.9% ± 0.5% surviving RGCs ($P < 0.01$) at 24 days after ONC. Treatment with siRTP801 was associated with pS6 expression in 13.7% ± 1.4% RGCs at the same time point after ONC, not significantly different from the intact controls. Thus, the expression of pS6, a marker of mTORC1 activity, was reduced by ONC injury and maintained at preinjury levels by siRTP801 treatment.

In Vitro Experiments

Expression of RTP801 Was Knocked Down Both in RGCs and Glia by siRTP801. The proportion of βIII-tubulin$^+$ RGC expressing RTP801 in vitro was significantly reduced ($P < 0.001$) from 88.3% ± 5.9% and 79.0% ± 5.5% in sNBA and siEGFP control wells, respectively, to 26.3% ± 8.8% after treatment with

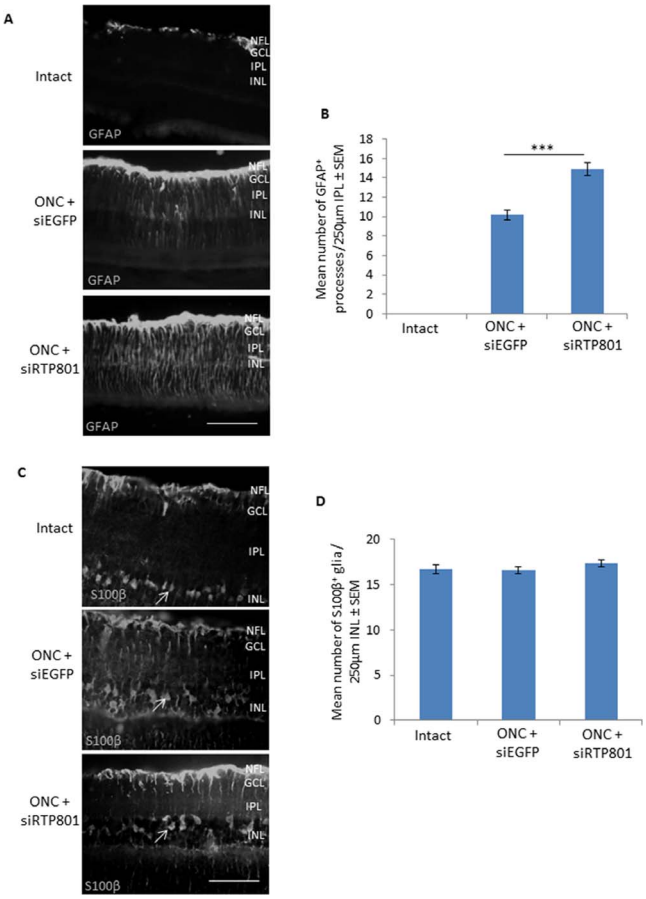


Figure 4. Retinal astrocyte/Müller glial response 24 days after ONC. (**A**) Immunostained retinal sections showing GFAP$^+$ glia in retinal sections of intact uninjured retinae and in sections after ONC + siEGFP and ONC + siRTP801. *Scale bar*: 100 μm. (**B**) Quantification of GFAP$^+$ Müller glia processes in a 250-μm linear region of the IPL in groups described in (**A**) ($n = 5$ retinae/group; $^{***}P < 0.001$). (**C**) Immunostained retinal sections showing S100β$^+$ Müller glia for the same conditions as described in (**A**) (*arrows* indicate Müller glial somata; *scale bar*: 100 μm). (**D**) Quantification of S100β$^+$ Müller glia somata in a 250-μm linear sample of the INL ($n = 5$ retinae/group).

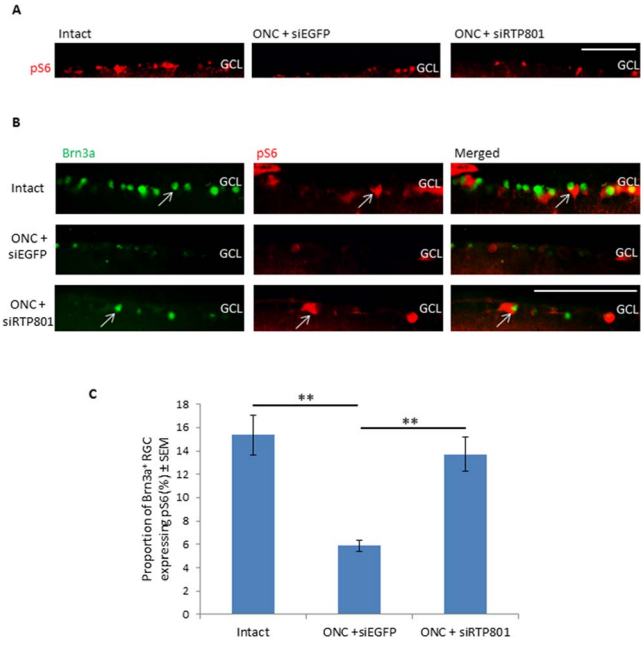


Figure 5. Maintenance of RGC mTORC1 activity (pS6 expression) after ONC in vivo. (**A**) Radial sections of the retina showing GCL immunostained for pS6 in uninjured intact animals (*left*), and after ONC + siEGFP (*middle*) and ONC + siRTP801 (*right*). *Scale bar*: 100 μm. (**B**) Higher magnification sections of GCL from groups described in (**A**), immunostained for Brn3a (*green*) and pS6 (*red*), demonstrating pS6$^+$ RGC (*arrows*). *Scale bar*: 100 μm. (**C**) Quantification of pS6 expression in Brn3a$^+$ RGC in retinal sections from groups described in (**A**), demonstrating low-level constitutive mTORC1 activity, injury-induced pS6 downregulation, and maintenance of RGC pS6 expression after siRTP801 treatment ($n = 5$ retinae/group; $^{**}P < 0.01$).

siRTP801, indicating knockdown of RTP801 expression in RGCs (Figs. 6A, 6C). In addition to RGCs, RTP801 expression was also observed in a population of nonneuronal βIII-tubulin$^-$ cells. The observed number of RTP801$^+$/βIII-tubulin$^-$ cells was significantly reduced ($P < 0.001$) after siRTP801 treatment (85.6 ± 15.3 cells/well) compared with sNBA (312.3 ± 21.8) and siEGFP (420.3 ± 17.9) controls, demonstrating target knockdown in cells other than RGCs (Figs. 6B, 6C).

The siRTP801 Was RGC Neuroprotective, but Neither Initiated Neurite Growth nor Increased Neurite Length in the Absence of Activated Retinal Glia. In primary retinal cultures established from intact rat eyes, 3-day siRTP801 treatment at concentrations of 10 nM, 20 nM, and 50 nM significantly increased the survival of βIII-tubulin$^+$ RGCs from 327.7 ± 15.6 RGCs/well in untreated sNBA controls to 528.7 ± 23.9, 620.7 ± 15.6, and 533.3 ± 23.3 RGCs, respectively ($P < 0.001$, at each test concentration) (Figs. 6C, 6D). There was no significant difference in RGC survival after incubation of retinal cultures with the siRNA transfection agent Lipofectamine alone (294.3 ± 13.9 RGC), or with siEGFP controls at concentrations of 10 nM (264.3 ± 13.9 RGCs), 20 nM (259.3 ± 23.6 RGCs), and 50 nM (266.7 ± 22.6 RGCs) (Fig. 6D). Treatment of retinal cultures with siRTP801 at concentrations of 10 nM, 20 nM, and 50 nM neither increased the proportion of RGC-extending neurites, nor the length of RGC neurites compared with controls (Figs. 6C, 6E, 6F).

There were no GFAP$^+$ activated glia present after incubation of retinal cell cultures prepared from intact eyes in the presence of sNBA culture medium alone, siEGFP, or siRTP801 (Fig. 7A), demonstrating an absence of activated astrocytes per Müller glia in this primary cell culture model. However, S100β$^+$ Müller glia were present in cultures prepared from intact eyes and there was no significant difference in S100β$^+$ glial number among sNBA-, siEGFP-, and siRTP801-treated cultures (Figs. 7B, 7C). Of the observed S100β glia, 87.7% ± 5.2% and 84.3% ± 3.7% were also RTP801$^+$ after sNBA and siEGFP treatments, respectively (Figs. 7B, 7D), and treatment with siRTP801 significantly reduced the proportion of S100β$^+$/RTP801$^+$glia to 36.7% ± 11.8% ($P < 0.01$ versus sNBA control, $P < 0.05$ versus siEGFP control), indicating target knockdown in astrocytes and Müller glia (Figs. 7B, 7D).

Treatment of retinal cultures with siRTP801 in vitro therefore enhanced RGC survival in the absence of activated retinal glia, but had no significant effect on either the initiation of neurite outgrowth or the length of neurites. The data also confirmed that treatment with siRNA was nontoxic in several retinal cell populations, and did not lead to nonspecific glia activation, as expected from the in vivo data demonstrating the lack of TLR activation with subsequent induction of interferon response (Supplementary Fig. S2).

The siRTP801 Reduced RTP801 Expression in Activated Retinal Glia but Did Not Promote Glial Proliferation. Although absent from primary retinal cultures prepared from intact animals, GFAP$^+$ retinal glia were observed in cultures prepared from retinae harvested at 5 days after astrocyte/

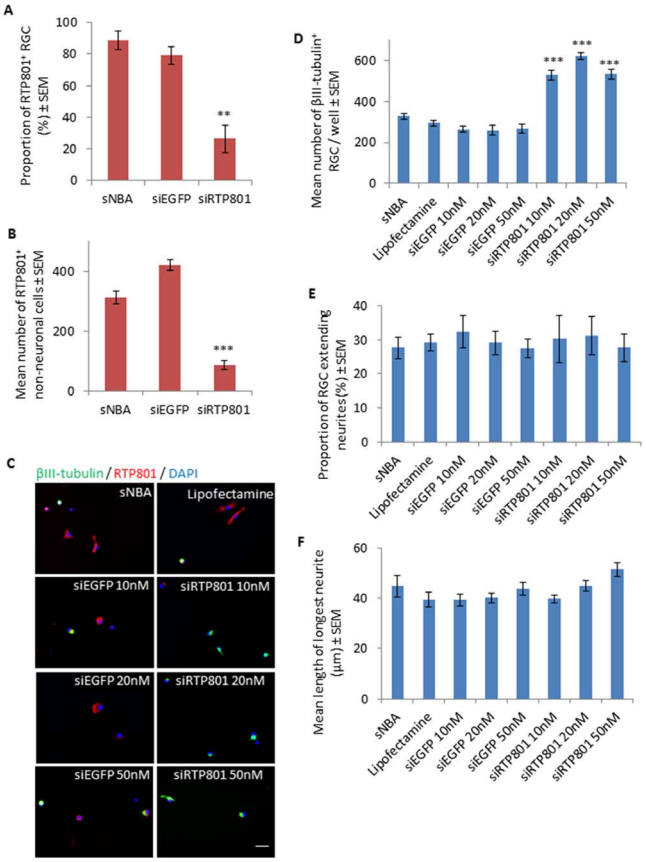


Figure 6. Knockdown of RTP801, RGC survival, and neurite growth in cultured retinal cells in the absence of activated retinal glia. (**A**) Proportion of βIII-tubulin$^+$ RGC expressing RTP801 after treatment with 20 nM siRTP801 compared with controls ($^{**}P < 0.01$ versus sNBA control). (**B**) Quantification of RTP801$^+$ βIII-tubulin$^-$ nonneuronal cells ($^{***}P < 0.001$ versus sNBA control). (**C**) Retinal cultures immunostained for βIII-tubulin (*green*) and RTP801 (*red*) after 3 days in culture. *Scale bar*: 20 μm. (**D**) Quantification of surviving βIII-tubulin$^+$ RGC after 3 days in culture ($^{***}P < 0.001$ versus sNBA control). (**E**) Proportion of surviving βIII-tubulin$^+$ RGC extending neurites. (**F**) Length of longest neurites in βIII-tubulin$^+$ RGC.

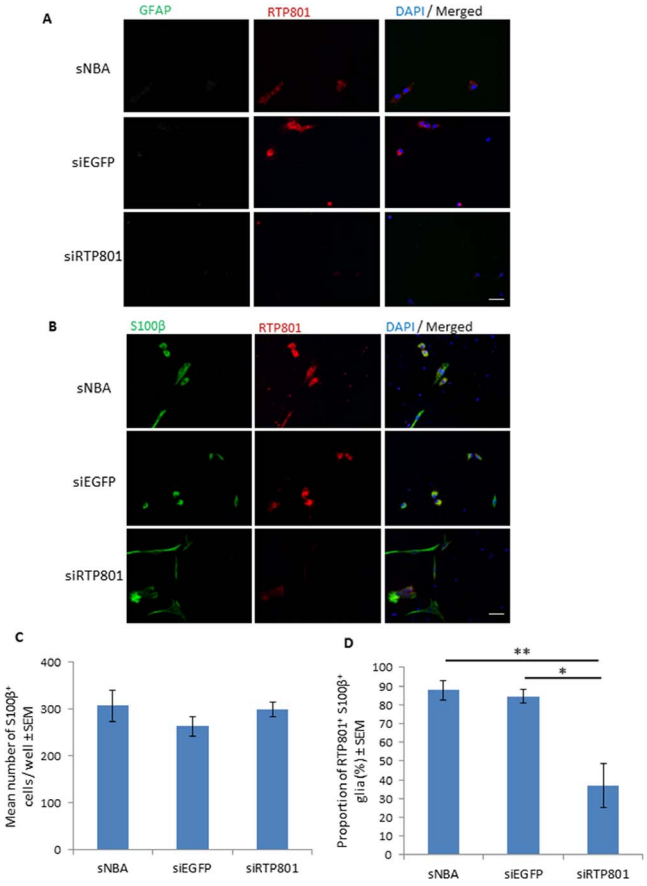


Figure 7. Expression of glial antibody markers in nonactivated retinal cultures and RTP801 knockdown in retinal glia. Retinal cultures established from uninjured animals immunostained for (**A**) GFAP (*green*) and RTP801 (*red*), and (**B**) S100β (*green*) and RTP801 (*red*) after 3 days in culture with sNBA culture medium, siEGFP, and siRTP801, indicating RTP801 expression in glia, and knockdown in siRTP801-treated wells; DAPI (*blue*) was used as a nuclear counterstain. *Scale bar*: 20 μm. (**C**) Quantification of S100β$^+$ cells in retinal cultures described in (**A**), indicating no significant glial proliferation or toxicity between treatment groups. (**D**) Proportion of S100β$^+$ glia expressing RTP801 after siRTP801 treatment compared with controls ($^*P < 0.05$, $^{**}P < 0.01$).

Müller glial activation by ONC in vivo, although there was no significant difference in total cell numbers among siRTP801-treated wells (568.1 ± 24.1 cells/well), sNBA controls (518.6 ± 42.6), and siEGFP controls (537 ± 26.1) (Figs. 8A, 8B) 3 days after siRNA transfection. The expression of RTP801 in activated GFAP$^+$ glia was significantly reduced ($P < 0.001$) by siRTP801 treatment (40.2% ± 5.4%) compared with sNBA (81.2% ± 3.9%) and siEGFP-treated controls (84.4% ± 4.3%) (Fig. 8C). These data showed that RTP801 was expressed in activated astrocytes and Müller glia, and that siRTP801 induced target knockdown in these cells without affecting the total number of GFAP$^+$ activated glia.

The siRTP801 Was RGC Neuroprotective and Promoted Neurite Elongation in the Presence of Activated Retinal Glia. In the presence of activated retinal glia in vitro, the number of surviving βIII-tubulin$^+$ RGCs significantly increased ($P < 0.001$) from 299.5 ± 27.9 and 264.7 ± 28.9 in sNBA and siEGFP control wells, respectively, to 605.3 ± 31.3 cells after siRTP801 treatment (Figs. 9A, 9B). These data are comparable to cultures established from intact eyes (Fig. 6), indicating both that significant RGC death had not yet occurred in vivo 5 days after ONC, and also that the presence of activated retinal glia in culture did not affect the pro-survival effect of siRTP801. There was no statistically significant difference in the proportion of neurite-bearing RGCs between siRTP801-treated wells (37.2% ± 4.9%) and sNBA- (26.8% ± 5.2%) or siEGFP-treated (24.8% ± 5.5%) controls (Fig. 9C), comparable to observations in the absence of activated retinal glia. However, whereas the mean length of the longest neurite extending from RGCs was 36 ± 8 μm and 34 ± 6 μm for sNBA and siEGFP controls, respectively, it was significantly longer (78 ± 12 μm; $P < 0.05$) after siRTP801 treatment (Figs. 9A, 9D). Thus, similar to in vivo data, siRTP801 promoted both RGC survival and neurite elongation, but not neurite initiation, in the presence of activated retinal glia.

The contribution of mTORC1 signaling to siRTP801-induced RGC neuroprotection and neurite elongation in the presence of activated glia was examined in retinal cultures in the presence of rapamycin, which completely abolished cellular expression of pS6 (Supplementary Fig. S3). The RGC neuroprotective effect of siRTP801 in culture was significantly reduced ($P < 0.05$) in the presence of rapamycin (452.0 ± 36.1), but not abolished, indicating both mTORC1-dependent and mTORC1-independent mechanisms contrib-

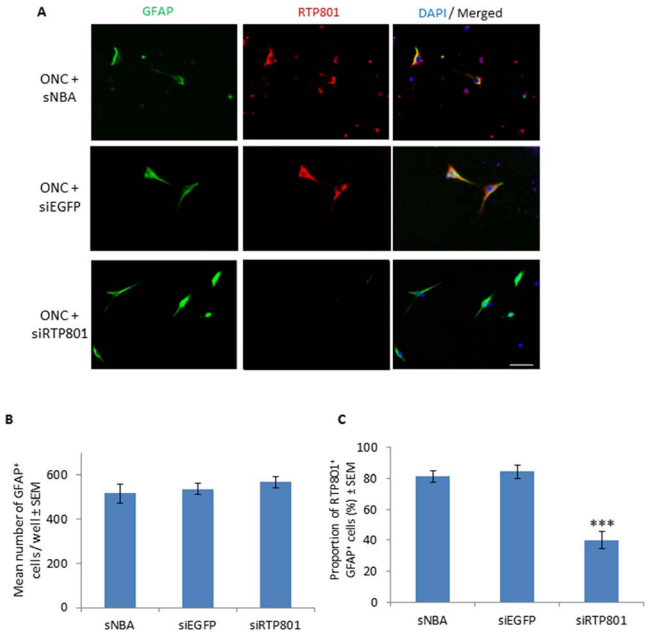


Figure 8. The GFAP$^+$ activated glia and RTP801 knockdown in activated glia in retinal cultures established 5 days after ONC. (**A**) Immunostaining of cultured retinal cells by GFAP (*green*) and RTP801 (*red*) after treatment with siRTP801 and controls; DAPI (*blue*) was used as a nuclear counterstain. *Scale bar*: 20 μm. (**B**) Quantification of GFAP$^+$ cells in glial-activated retinal cultures after 3 days in culture. (**C**) Proportion of GFAP$^+$ glia expressing RTP801 after siRTP801 treatment compared with controls after 3 days in culture (***$P <$ 0.001).

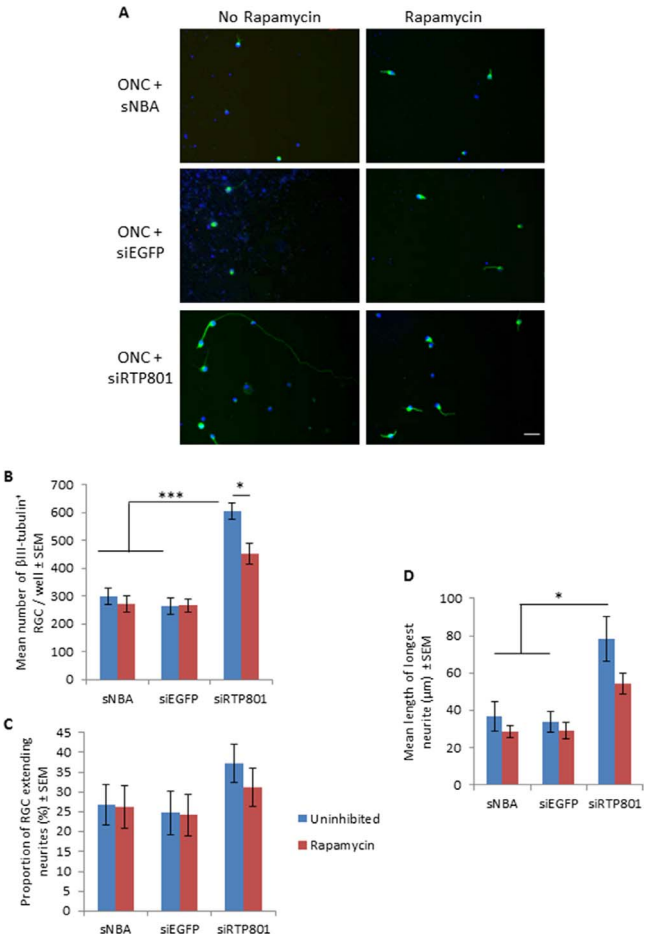


Figure 9. Retinal ganglion cell survival and neurite growth in the presence of activated glia in vitro, and effects of mTORC1 inhibition with rapamycin. (**A**) βIII-tubulin$^+$ RGC (*green*) after siRTP801 treatment and in controls in the presence of activated retinal glia, in the absence of inhibitor (*left column*) and in the presence of 10 nM rapamycin (*right column*); DAPI (*blue*) was used as a nuclear counterstain. *Scale bar*: 20 μm. The numbers of surviving βIII-tubulin$^+$ RGC (**B**), the proportion of βIII-tubulin$^+$ RGC extending neurites (**C**), and the length of longest RGC neurites (**D**) after 3 days in culture in the absence of inhibitor (*blue bars*), and in the presence of rapamycin (*red bars*) (*$P <$ 0.05, ***$P <$ 0.001).

uting to the siRTP801-mediated RGC survival effect (Figs. 9A, 9B). Rapamycin did not reduce the number of βIII-tubulin$^+$ RGCs in control wells, suggesting that rapamycin was not RGC neurotoxic in this model (Figs. 9A, 9B). Rapamycin also had no significant effect on RGC neuritogenesis (Figs. 9A, 9C), but did reduce mean neurite length in siRTP801-treated wells (54 ± 6 μm) (Figs. 9A, 9D), although the latter result did not reach statistical significance (Fig. 9D), suggesting that siRTP801-induced neurite elongation was partially mTORC1 independent.

Glial Activation and the Production of Neurotrophins Were Both Potentiated by siRTP801. In medium from retinal cell cultures in which activated retinal glia were absent, titers of NGF, NT-3, and BDNF were very low or undetectable (Fig. 10, blue bars). After glial activation by previous ONC, titers of BDNF, NGF, and NT-3 were increased (Fig. 10, red bars), and significantly higher levels of NGF ($P < 0.001$), NT-3 ($P < 0.01$), and BDNF ($P < 0.001$) were present after siRTP801 treatment when compared with sNBA and siEGFP treatments (Fig. 10, red bars). These data suggested that activation of retinal glia by ONC led to the production and release of NTF, an effect that was potentiated by siRTP801 treatment but not initiated by it.

The siRTP801 Protected Against Injury-Induced pS6 Downregulation, Although siRTP801-Induced Neurite Outgrowth Did Not Require S6 Phosphorylation. In retinal cultures prepared from uninjured animals, 23.1% ± 1.7% RGCs were pS6$^+$ (Figs. 11A, 11B). After ONC, the proportion of RGCs in culture expressing pS6 was reduced to 9.2% ± 3.1% and 6.7% ± 2.7% in sNBA and siEGFP-treated controls, respectively (Figs. 11A, 11B). In cultures treated with siRTP801 after ONC, pS6 expression was maintained at a level not significantly different from uninjured control cultures (23.5% ± 3.9%) (Figs. 11A, 11B), suggesting that siRTP801 treatment prevented injury-induced pS6 downregulation in RGCs, which reflects maintained mTORC1 activity. Detection of pS6 was not limited to RGCs, as βIII-tubulin$^-$ cells also expressed pS6, suggesting mTORC1 activity also in glia (Fig. 11A). Detection of pS6 was observed in 51.1% ± 9.8% and 41% ± 5.8% neurite-bearing RGCs in sNBA- and siEGFP-treated control wells, and this proportion was not significantly increased after siRTP801 treatment (52.1% ± 4.8%) (Figs. 12A, 12B). Furthermore, neurite outgrowth was not limited to pS6$^+$ RGC, as many pS6$^-$/βIII-tubulin$^+$ RGC extended neurites (Fig. 12A). In keeping with the in vivo findings, these data demonstrated that siRTP801 maintained mTORC1 activity in retinal cells after injury and neurite outgrowth was more likely in pS6$^+$ RGC, although the expression of this marker was not obligatory for RGC neurite outgrowth after glia activation.

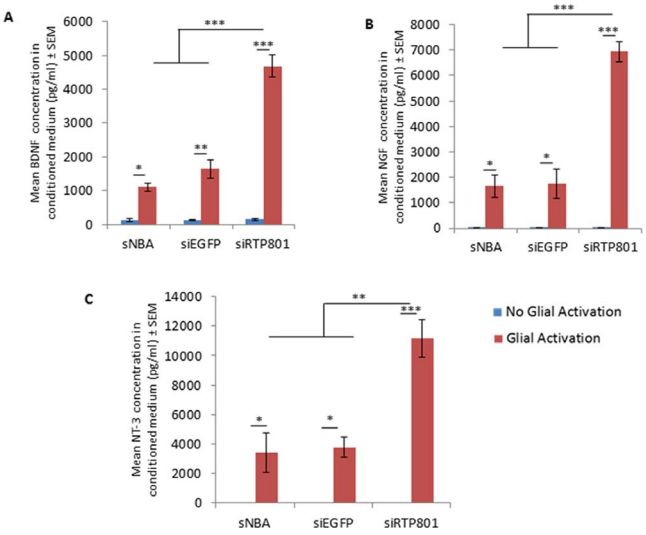


Figure 10. Neurotrophic factor titers in conditioned culture medium from retinal cultures in the absence or presence of activated retinal glia. Titers of BDNF (**A**), NGF (**B**), and NT-3 (**C**) in culture medium measured by ELISA in the absence (*blue bars*) or presence (*red bars*) of activated retinal glia, after treatment with siRTP801 or controls ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Discussion

Recent studies have implied a key role for mTOR signaling in RGC survival and axon regeneration and have suggested that this pathway is a potentially useful therapeutic target for neurodegenerative conditions. However, the most impressive results have been achieved with laboratory techniques that are largely not clinically translatable,[31,64,65] and mechanisms underpinning the neuroprotective/regenerative effects reported are not well defined. Accordingly, this study investigated the effects of a clinically translatable siRNA targeting RTP801, a stress-induced negative regulator of mTOR signaling, on RGC survival, axon regeneration, and activation of retinal glia after ONC in vivo, and used retinal cultures to investigate potential underlying mechanisms. Expression of RTP801 is not limited to RGCs, and we have observed both expression and siRNA-mediated knockdown in RGC and glia, consistent with previous findings that RTP801 is expressed in several retinal layers, and is susceptible to siRNA-mediated knockdown within 1 day after intravitreal injection.[44,47]

The major findings in vivo were that, compared with siEGFP controls, siRTP801 intravitreal treatment after ONC promoted significant RGC neuroprotection and, although the numbers of RGC axons sprouting at the lesion site and into the distal ON segment adjacent to the lesion were not increased, more axons extended 400 to 1200 μm beyond the ONC. Hence, siRTP801 treatment promoted axon elongation but did not stimulate axogenesis. Suppression of RTP801 activity also prevented injury-induced downregulation of mTORC1 activity and potentiated ONC-induced retinal astrocyte/Müller glial activation. This glial activation was probably specific in view of the lack of nonspecific proinflammatory activity of this siRNA, which, if it exists, is mediated via TLR activation known as a promoter of glia activation.[66]

Although the anatomical integrity of retinal tissues is not maintained in culture, our experiments indicate that use of in vitro studies can be informative with regard to identifying underlying cellular mechanisms. As in uninjured control animals in vivo, retinal cultures obtained from uninjured adult rats demonstrated an absence of GFAP^{+} activated glia, but

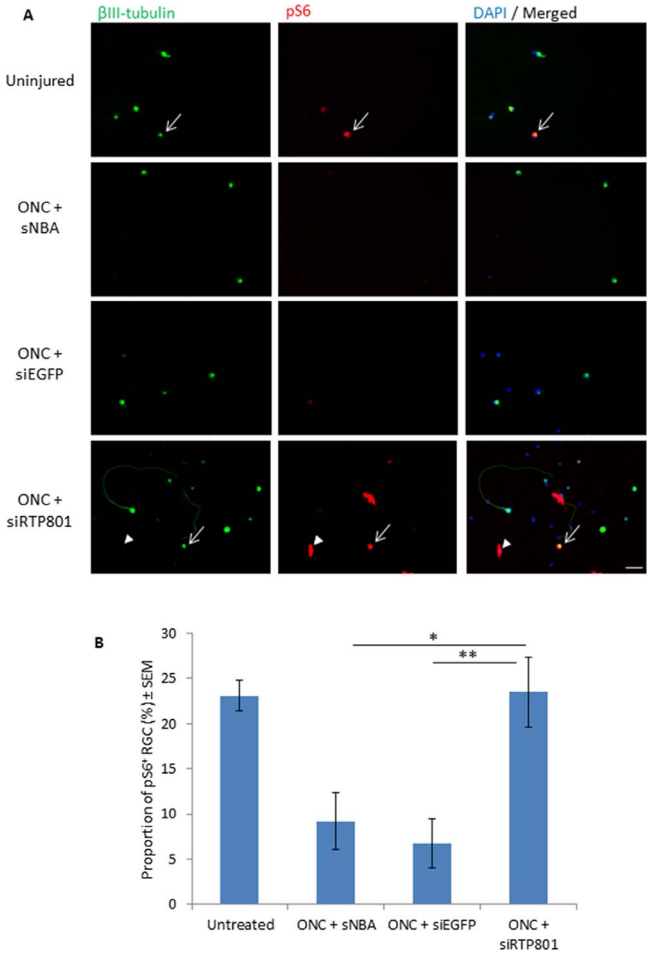


Figure 11. Activity of mTORC1 (pS6 expression) in cultured retinal cells. (**A**) Retinal cells immunostained for βIII-tubulin (*green*) and pS6 (*red*) in cultures obtained from uninjured control animals, and 5 days after ONC and then treated with siRTP801 or sNBA/siEGFP controls. Note the detection of pS6 both in βIII-tubulin^{+} RGC (*arrows*) and glia (*arrowheads*); DAPI (*blue*) was used as a nuclear counterstain. *Scale bar*: 20 μm. (**B**) The proportion of βIII-tubulin^{+} RGC expressing pS6 after 3 days in culture for conditions described in (**A**) ($^{*}P < 0.05$, $^{**}P < 0.01$).

treatment with siRTP801 promoted RGC neuroprotection in these cultures, indicating a direct effect on RGC-intrinsic mTOR signaling for cell survival. Neurite outgrowth was not affected by siRTP801 in the absence of activated retinal glia; however, consistent with previous reports, retinal cultures prepared 5 days after ONC contained GFAP^{+} activated glia,[19,23,25,67] and therefore may be considered more representative of the postinjury in vivo condition. The siRTP801 had no significant effect on initiation of neurite outgrowth, but promoted longer neurite growth, suggesting that additional factors were present in glial-activated cultures that enabled more sustained neurite outgrowth. Such factors could be neurotrophins secreted by activated glia and which production appeared potentiated by siRTP801 treatment. These in vitro data closely correlate with our in vivo findings that siRTP801 did not significantly affect the number of RGC axons sprouting at the lesion site, but did promote growth of longer axons regenerating into the distal ON segment, associated with maintenance of mTORC1 activity. Although the culture of retinal cells after ONC and subsequent in vitro siRNA treatment and analysis may be associated with potential mechanistic differences from the in vivo conditions, this approach closely

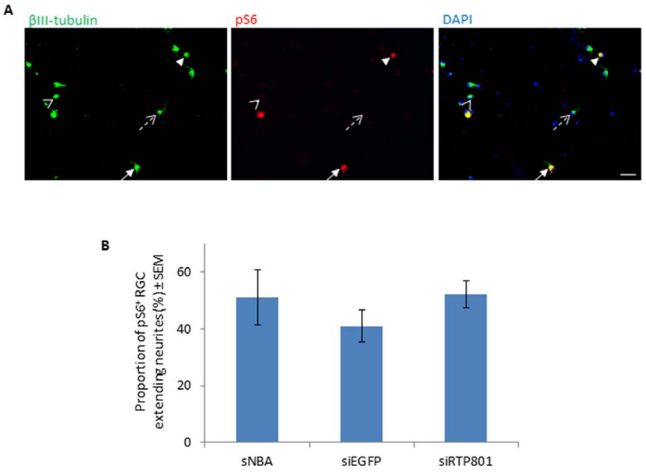


Figure 12. Expression of pS6 in both neurite-bearing and non–neurite-bearing RGCs. (**A**) Retinal cultures immunostained for βIII-tubulin (*green*) and pS6 (*red*): pS6$^+$ RGC with neurite (*closed arrow*), pS6$^-$ RGC with neurite (*dashed open arrow*), pS6$^+$ RGC without neurite (*closed arrowhead*), pS6$^-$ RGC without neurite (*open arrowhead*); DAPI (*blue*) was used as a nuclear counterstain. *Scale bar*: 20 μm. (**B**) The proportion of neurite-bearing RGCs expressing pS6 in controls and after siRTP801 treatment (no significant difference between groups).

models the glial activation seen in vivo, and enables multiple analyses of biological processes while minimizing animal usage. Moreover, the concordance between the observations in the glial-activated culture paradigm and the in vivo setting support this strategy in evaluating underlying cellular mechanisms. Thus, our data suggest that the observed RGC survival and axon/neurite elongation after siRTP801 treatment reflect both direct effects on RGC and indirect sequelae attributable to RTP801 suppression in astrocytes and Müller glia.

The observed neuroprotective effect of siRTP801 in the ONC model is consistent with previous reports on the effects of RTP801 overexpression or knockdown/knockout in the nervous system. Overexpression of RTP801 in nondividing neuron-like PC12 cells promotes apoptosis,[43] and RTP801 knockout mice exhibit reduced neuronal apoptosis in a murine model of retinopathy of prematurity.[45] Expression of RTP801 is elevated in substantia nigra neurons and associated with cell death both in a murine Parkinson's disease (PD) model and in human PD postmortem brains, and knockdown of RTP801 protects cultured neurons from apoptosis in cellular PD models.[68] Furthermore, siRNA-mediated knockdown of RTP801 protects RGC-5 neurons from blue light– and cobalt chloride–induced cell death.[44] Thus, RTP801 inhibition is a viable neuroprotective strategy supported by diverse and solid experimental data.

RTP801 is rapidly upregulated in response to cellular stress/injury and acts on the TSC1/2 complex to suppress mTOR signaling.[37,40] After axotomy, a reduction in the supply of available target/glial-derived NTF, which normally promote mTOR activity via receptor-activated PI3K/Akt signaling, is compounded by RTP801 upregulation, which acts to further suppress mTOR. Our approach of inhibiting RTP801 to promote mTOR activity compares favorably with other strategies aimed at increasing PI3K pathway signaling for RGC survival. For example, RGC survival of 45% is observed after conditional deletion of PTEN at 14 days after ONC in mice,[31] but rises to 60% when PI3K-pathway upregulation is combined with adjunctive SOCS3 deletion and CNTF administration.[64] We observed RGC survival of approximately 45% with our control siRNA at 24 days after ONC, which could be associated with induction of an inflammatory response[69] that may promote neuroprotection after RGC axotomy.[22] However, we have not observed significant differences in RGC survival between siEGFP and PBS in this model (Supplementary Table S1), and siEGFP transfection of retinal cultures from intact animals not associated with the presence of GFAP$^+$ activated retinal glia, indicating a lack of immunogenicity of the control siRNA. Furthermore, in all the in vitro analyses undertaken, there were no significant differences between control siEGFP and the sNBA culture medium alone, therefore demonstrating the inactivity of control siRNA in these studies and enabling the phenotypic effects of siRTP801 to be attributed to the sequence specificity of this siRNA, rather than due to an siRNA class effect. Intravitreal delivery of siRTP801 promoted favorable RGC neuroprotection, although recent reports suggest that RGC survival after ONC may be subtype specific, with M1-RGC and αRGC preferentially surviving after ONC.[70] It remains to be investigated whether siRTP801-induced RGC neuroprotection differentially affects RGC classes after ONC, but our results indicate that RTP801 inhibition may be a useful component of future treatment strategies to prevent visual loss after ON injury.

Our findings suggested that RTP801 knockdown supported the elongation of regenerating axons rather than the initiation of axogenesis after ONC. There was, however, a marked disparity between the potent RGC neuroprotective effect of siRTP801 and the relatively limited axon regeneration, confirming that axon regeneration is not inevitable in surviving RGCs.[19,71] A typical rodent ON contains approximately 100,000 RGC axons,[72] and thus the number of regenerating axons we observed represents a regenerative response in fewer than 0.1% RGCs. Nonetheless, our results complement the increasing literature implicating the PI3K/Akt/mTOR pathway as a mediator of axon regeneration in several models of CNS injury.[29,31,34,35] Robust ON axon regeneration is reported after PTEN deletion, associated with the maintenance of RGC pS6 expression,[31] whereas RTP801 modulates the PI3K signaling pathway further downstream the pathway, acting on the TSC1/2 complex.[37,40] A more muted regenerative response is observed after deletion of TSC compared with PTEN,[31] possibly reflecting differential signaling through the GSK3β pathway.[29,31] Moreover, axon regeneration after PTEN deletion is restricted to predominantly the αRGC subtype, which is preferentially resistant to apoptosis after ONC,[70] and so the predominant weighting of RTP801 knockdown in favor of survival compared with axon/neurite growth may reflect survival of RGC subtypes in which a regenerative response is not possible, with mTOR-related protein synthesis maintaining neuronal viability and contributing to growth of axons that have initiated regeneration in response to other triggers. In agreement with our data, mTOR activity has been implicated as an important factor in the maintenance of an axon growth state,[73] whereas initiation of the axogenic program may be dependent on separate, although complementary, pathways such as CNTF/LIF-mediated JAK-STAT activity.[21–23,26,73,74]

Previous studies investigating the role of PI3K-mTOR signaling on neuronal survival and axon regeneration have focused on RGC responses alone, rather than on the contribution of glial-derived factors. Retinal glial activation/reactive gliosis has a major influence on RGC axon regeneration through the production and release of neurotrophins and other proregenerative and inflammatory cytokines, which modulate the injury response through autocrine and paracrine mechanisms.[19,23–26,75–78] Development of RGC neuroprotective/regenerative therapies must therefore acknowledge the glial response and associated cellular signaling.

Our observation of specific (in the absence of induction of nonspecific innate TLR-mediated responses) potentiated retinal astrocyte/Müller glial activation by siRTP801 is consistent with a role for activated glia in mediating neuroprotection and axon elongation. This in vivo observation was supported by in vitro data in mixed retinal cultures treated with siRTP801. Although being unable to induce glia activation directly in cultures derived from intact eyes, siRTP801 treatment of cultures comprising already activated glia (established 5 days after ONC) resulted in potentiation of the secretory phenotype of the activated glia (i.e., in significantly increased production of neurotrophins BDNF, NGF, and NT-3). Interestingly, the presence of activated glia in retinal cultures was an absolute requirement for the axon elongation effect of siRTP801, suggesting that despite activated glia promoting NTF production, axon growth may be limited by the downstream inactivation of mTOR by RTP801 in control wells. Inhibition of RTP801 therefore appears both to potentiate glia NTF production, and to disinhibit the RGC response to enable longer neurite growth. Potentiation of reactive gliosis may not be beneficial in all optic neuropathies, as aspects of the retinal glial response may be detrimental to RGC viability and axon growth in some circumstances. In glaucoma, for example, activated microglial-derived proinflammatory factors, such as TNF-α and IL-6, may induce neurotoxicity rather than neuroprotection.[79] Time-course analyses of gene expression changes during reactive gliosis found more than 1300 genes differentially expressed within 1 day of ONC, with functionally distinct groups of temporally specific genes associated with inflammatory responses, tissue remodeling, and structural alterations.[80] Modulation of retinal gliosis to optimize RGC survival and axon regeneration must therefore consider multiple tissue-specific, injury-specific, and temporal factors.

There was approximately equivalent RGC survival both in the absence and presence of activated retinal glia in vitro, indicating that siRTP801-mediated neuroprotection predominantly stemmed from a direct effect on RGCs. This effect was only partially mTORC1-dependent, as rapamycin treatment abolished pS6 expression (a correlate of mTORC1 activity), yet only partially attenuated siRTP801 neuroprotection. Both initiation of neurite sprouting and elongation of neurite growth were predominantly independent of mTORC1 activity, consistent in part with previous observations that rapamycin reduced but did not abolish axon regeneration after PTEN knockout.[31] Moreover, accelerated neurite outgrowth in the peripheral nervous system after PTEN inhibition is unaltered by rapamycin.[34] Our data are also consistent with the results of Leibinger et al.,[73] who found that, although inflammation-induced RGC neurite outgrowth is rapamycin insensitive, it does require mTORC1 activity for the maintenance of a regenerative state. It is likely, therefore, that initiation of axon/neurite growth is mTORC1 independent, but under the right cellular conditions mTORC1 has a key role in maintaining axonal growth.

Treatment with siRTP801 prevented injury-induced downregulation of mTORC1 activity, both after ONC in vivo and in the culture model. Rapamycin-sensitive mTORC1 activity seemed to contribute to, but was not obligatory for, neuroprotection and axon/neurite growth elicited by siRTP801, thus implicating alternative intracellular signaling pathways; among them, the relatively rapamycin-insensitive mTORC2 is a primary candidate.[30] The mTORC2 is also downstream to RTP801/TSC1/2 and is likely to contribute to neuroprotection/axon growth by siRTP801 in the presence of rapamycin. The mTORC2 influences cellular processes either through the downstream substrate PKCα, which is associated with actin dynamics and may influence growth cone activity,[30] or by feedback phosphorylation and activation of Akt, which in turn regulates the activity of other growth and cell survival mediators, such as GSK3β,[29,81] and components of mitochondrial apoptosis machinery.[82] Several reports provide evidence that GSK3β inhibition is associated with enhanced neuronal growth.[83–85]

In summary, siRTP801-dependent potentiation of PI3K pathway signaling in RGCs via paracrine secretion of neurotrophins by activated glia combined with siRTP801-mediated activation of both mTORC1 and mTORC2 in RGCs themselves may be responsible for the observed increased survival and longer axon/neurite growth seen after siRTP801 treatment both in vitro (mixed retinal cultures) and in vivo. The challenge remains for the development of novel neuroprotective and axon regenerative therapies that can be used for the benefit of patients.[78] Clinical trials of intravitreal siRNA treatments, including those targeting RTP801, have reported safety and efficacy for ocular diseases.[48,50,86] Despite the inherent vulnerabilities of immunofluorescence-based analyses, such as antibody specificity and limitations of sampling for neuronal/glial quantification, both our in vivo and in vitro observations yielded consistent results. Here, we show that siRNA-mediated knockdown of RTP801 promotes RGC neuroprotection, supports elongation of regenerating axons after ONC in vivo, and potentiates a proregenerative reactive glial response to injury. Furthermore, RGC neuroprotection is predominantly directly mediated, via mTORC1-dependent and independent components, whereas siRTP801-induced neurite elongation uses an indirect mechanism, requiring the presence of activated retinal glial-derived NTF and is independent of mTORC1 activity. Understanding the mechanisms underpinning the neuronal responses achieved with siRNA treatments is critical to improving their efficacy and pointing the way to exploring more defined combinatorial approaches. Importantly, this siRNA-based approach is potentially rapidly translational, and may contribute to future strategies to promote RGC survival and axon regeneration after ocular and ON injuries.

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