The inappropriate proliferation of retinal capillaries derived from preexisting vessels (retinal neovascularization) is a significant complication of many ocular conditions constituting the major causes of blindness. The current ability to prevent retinal neovascularization is severely limited and relies on ablation of functional retina using laser photocoagulation or cryotherapy.

Neovascularization is usually accompanied by retinal degeneration and may actually be secondary to it, because conditions of oxidative stress linked to diabetes or hyperoxgenation lead to retinal capillary damage. This results in retinal ischemia and secretion of vascular endothelial growth factor (VEGF) by astrocytes. In parallel, pathologic changes of apoptotic nature occur in retinal neurons.

Little is known about the actual mechanisms that induce apoptosis in retinal diseases, and none of the treatments proposed for ischemic proliferative retinopathy seem to alter the course of degeneration in the retina. A role for oxygen-derived free radicals in mediating hyperoxia-induced vaso-obliteration is supported by studies showing that the administration of exogenous antioxidants can attenuate retinopathy in certain animal models and possibly in humans as well. In addition, experimental oxygen-induced retinopathy is associated with the formation of lipid peroxides in the retina.

Two regulatory factors worth mentioning in the context of retinopathy are p53 and hypoxia inducible factor (HIF-1). Apoptosis induced by retinal ischemia in rats is associated with increased expression of p53 mRNA, whereas mice with a reduced expression of p53 show resistance to ischemia-induced retinal ganglion cell death, confirming a functional role for this protein. p53 is known to trigger the expression of several genes implicated in cellular redox control that ultimately contribute to p53-mediated apoptosis through the mitochondrial apoptotic cascade.

We have recently reported the identification and cloning of a novel direct transcriptional target of HIF-1, RTP801. Later on, RTP801 (REDD1) was demonstrated to be a shared transcriptional target of p53 and p63. We detected strong up-regulation of RTP801 by hypoxia both in vitro, in numerous cell lines, and in vivo, in an animal model of ischemic stroke. The gene was overexpressed, both in hypoxia-affected neurons within the penumbra region and in endothelial cells within the ischemic core, hence hinting of its potential functional role in diseases associated with these two types of cells. Inducible overexpression of RTP801 promoted the apoptotic death of differentiated neuron-like PC12 cells and drastically increased their sensitivity to ischemic injury and oxidative stress. Moreover, Ellis et al. detected that overexpression of RTP801 leads to the generation of reactive oxygen species (ROS).

Taking into account the specificities of regulation of RTP801 transcription (HIF-1 and p53 responsiveness), its expression pattern in disease (neurons and endothelial cells) and the consequences of its overexpression (increased generation of ROS and apoptosis of neuron-like cells), RTP801 seems an ideal candidate to study for involvement in retinopathy.
In this study, to assess the role of RTP801 in the disease’s pathogenesis, we used mice with the gemline disruption of RTP801 in a model of retinopathy of prematurity (ROP). The model is produced in newborn mouse pups (postnatal day P17) by exposure to 75% oxygen for 5 days and subsequent recovery in a room-air environment. The retinal disease that develops in the model of ROP combines many features that are also characteristic of other types of retinopathy. The pathogenesis is triggered by hypoxia-induced obliteration of vulnerable retinal blood vessels of newborn mice. After the transfer of pups to the normoxic conditions (P12), the developing relative retinal hypoxia leads to secretion of angiogenic factors, such as VEGF,15,16 and induction of abnormal vasoproliferation10 (usually measured at P17), as well as to neuronal apoptosis occurring in the inner nuclear cell layer of the retina (maximum at P14).17–19

Our results indicated that lack of RTP801 expression significantly reduces retinal pathologic neovascularization and apoptosis in the mouse model of ROP. This points to the important role that activation of RTP801 plays in the pathogenesis of retinopathy.

**Materials and Methods**

**Generation of RTP801-Deficient Mice**

RTP801 mutant mice were generated by Lexicon Genetics Inc. (The Woodlands, TX) as a service. The RTP801 targeting vector was constructed with the Lambda KO system previously described by Wattler et al.20 The Lambda KO phage library, arrayed over 96 superpools, was screened by PCR with one primer located in exon 1 of mouse RTP801 (RTP801-1; 5'-CTGTTGTCTCGCTGCTGCTG-3') and another primer located in exon 2 (2RTP801-11; 5'-CAGGAAAACTCGTGGAGTGAG-3') and a mouse RTP801-specific probe. Two PKO genomic clones were identified in the library screen and confirmed by sequence and restriction analysis. Gene-specific arms, (5'-CTTCCGACGGCTGCTGCTGCTG-3') and (5'-CCTCCGACGCGCCGACGACG-3') were appended by PCR to a yeast selection cassette containing the URA3 marker. One of the RTP801-containing genomic clones, pKOS90, and the yeast selection cassette were cotransformed into yeast. Clones that had undergone homologous recombination to replace a 212-bp region encompassing the RTP801 first coding exon (exon 2) with the yeast selection cassette were isolated. The yeast cassette was subsequently replaced with a LacZ/Neo selection cassette to complete the RTP801 targeting vector. The NoI linearized targeting vector was electroporated into 129/5E (Lex-1) embryonic stem (ES) cells. After selection in 0.2 mg/mL of G418 for 7 to 9 days, G418/FIAU-resistant ES cell clones were isolated, and correctly targeted clones were identified and confirmed by Southern blot analysis with the 404-bp 5’ internal 52/48 probe (amplified by using PCR primers RTP801-52; 5'-CCTCCTGGAGGTGTCCTGAC-3') and the 312-bp 3’ external 49/51 probe (amplified by using PCR primers RTP801-49; 5'-AGCCATCGTCGTCGTCGTCG-3') and RTP801-51; 5’-CCCTACGGTGGATGTTAGAC-3'). Southern blot analysis using probe 52/48 detected a 7-kb wild-type band and 5.5-kb mutant band in XbaI-digested genomic DNA, whereas probe 49/51 detected a 9-kb wild-type band and 5.5-kb mutant band in EcoRI-digested genomic DNA (not shown). Two targeted ES cell clones were microinjected into C57BL/6 blastocysts. The resultant chimeras were mated and confirmed by PCR reactions conducted with DNA polymerase (Super-Therm; JMR Holdings, London, UK) according to the manufacturer’s protocol and simultaneously included all three primers. The reactions consisted of 30 cycles: 94°C for 45 seconds, 55°C for 2 minutes, and 72°C for 1 minute, and were completed by a 10-minute incubation at 72°C.

**Rodent Model of ROP**

All animal experiments were performed in strict adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with the National Institutes of Health guidelines. Animal protocols were reviewed and approved by the Hebrew University Animal Research Committee. Oxygen-induced retinopathy was obtained in wild-type rat or mouse (C57BL/6/129) pups or in RTP801-knockout mouse pups according to a protocol previously established for the mouse model of ROP.21 In brief, at P7, rodent pups and their nursing mothers were exposed to hyperoxic conditions (75% oxygen) for 5 days in an infant incubator (Ohmeda Medical, Louisville, CO). On P12, the pups were returned to room-air (normoxic) conditions for 2 or 5 days until P14 or P17, respectively. Age-matched animals were maintained in room air (normoxic conditions) for the duration of the experiment.

**Retinal Angiography and Quantification of the Avascular Area**

Fluorescein angiography of the retina was performed as described previously.21 On P17, wild-type and RTP801-knockout mice, either control or exposed to relative hypoxic conditions, were deeply anesthetized and perfused through the left ventricle with 1 mL of 50 mg/mL fluorescein-labeled high molecular weight (2,000,000) dextran (Sigma-Aldrich, St. Louis, MO) in PBS. The mice were killed and their eyes were removed. One eye of each mouse was fixed in 10% formalin for 10 minutes. The retinas were separated from the sclera, the retinal pigment epithelium, the lens, and the cornea and fixed for an additional 2 hours. Then, each retina was cut at four to five peripheral locations and flattened on a glass slide in PBS-glycerol. The flattened retinas were photographed under a fluorescence microscope (MZFLII; Leica, Deerfield, IL, digital camera Spot RT color; Diagnostics Instruments, Inc., Sterling Heights, MI). The capillary-free area was quantified from the digital images in masked fashion (Photoshop; Adobe, Mountain View, CA).

**Assessment of Retinal Neovascularization Response**

Mouse eyes that were not used for angiography were fixed in 10% formalin, embedded in paraffin, and sectioned. Quantification of the neovascular response was performed in 7-μm-thick sagittal sections, 140 μm apart from each other and spanning the entire retina. After staining with periodic acid-Schiff (PAS) reagent and hematoxylin, the extent of neovascularization was determined by counting neovascular cell nuclei anterior to the internal limiting membrane (at the vitreous side). Eyes of 9 to 10 mice from each genotypetreatment group were analyzed. For each eye, 9 to 13 retinal sections were evaluated in a fully masked protocol, and the mean number of neovascular nuclei per section was determined.

**Assessment of Apoptosis In Vivo**

The same sectioning procedure that was used for the assessment of the neovascularization response was also used for the quantification of apoptotic cells. Apoptosis was detected in retinas of P14 and P17 mice by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL). The assay was performed with a peroxidase in situ apoptosis detection kit (ApopTag; Intergen, Purchase, NY) according to the manufacturer’s protocol. The sections were counterstained with methylgreen, and seven to eight mice from each genotype/.
treatment group were evaluated. For each eye, 10 to 19 sections were counted in a masked protocol and the mean number of TUNEL-positive nuclei in the retinal inner nuclear layer (INL) was determined. Some of the slides were also stained with hematoxylin-eosin to confirm apoptosis by morphologic characteristics.

**Statistical Analysis of Morphometric Data**

Because the parameters of interest were assessed in the biological material obtained in several different experimental sets, the analysis was performed by ANOVA, using factorial models (models of contrasts) to abolish the influence of interexperimental differences. The models were generated separately for evaluation of each specific parameter (neovascularization, or apoptosis).

**In Situ Hybridization**

Eyes from relative hypoxia-treated and control rodent pups were excised on either day P14 or P17, fixed in 10% formalin, sliced, and used for in situ hybridization with 35S-UTP labeled rat RTP801-specific and VEGFA-specific sense and antisense riboprobes. Radioactive riboprobes were produced from a vector (p Bluescript; Stratagene, LaJolla, CA) containing ratRTP801 cDNA encompassing the whole open reading frame of the gene, using either T7 (antisense probe) or T3 (sense probe) polymerases, as previously described. In situ hybridization was performed according to a previously published protocol. The exposed slides were developed (D-19 developer; Eastman Kodak, Rochester, NY), fixed, and counterstained with hematoxylin-eosin. The microphotographs were taken using a microscope (Axioscop-2; Carl Zeiss Meditec, Dublin, CA) equipped with a charge-coupled device (CCD) camera (Spot RT; Diagnostic Instruments).

**RNA Extraction from Paraffin-Embedded Mouse Eyes**

RNA from formalin-fixed, paraffin-embedded mouse eyes was extracted according to a previously published protocol. The excess of paraffin around the fixed eyes was carefully removed, and the whole samples were cut into 5-μm sections. Some of the samples had been
partially sectioned previously, and therefore the amount of sections obtained per eye varied ranging from a maximum of 400 to a minimum of 200. Every 100 freshly cut eye sections were positioned into one screw-cap tube (Eppendorf, Fremont, CA), and paraffin was extracted twice by short vortexing and incubation for 10 minutes with 1 mL xylene at room temperature. The samples were next washed twice with 100% ethanol, dried briefly on air, and resuspended in 0.5 mL lysis buffer (10 mM NaCl, 0.5 M Tris-HCl [pH 7.6], 20 mM EDTA, 1% SDS, and 0.5 mg/mL proteinase K) and incubated at 45°C overnight with shaking. RNA was extracted once with an equal volume of phenol-chloroform-isomyl alcohol (25:24:1) and twice with chloroform-isomyl alcohol (24:1). RNA was precipitated with an equal volume of isopropanol with an addition of linear polyacrylamide as a carrier. Each RNA pellet was washed twice with 70% ethanol. All RNA pellets obtained from the same eye were combined and dissolved in 15 μL double-distilled sterile water per 100 sections.

**Quantification of RTP801 and VEGF Expression in the Retina**

Relative amounts of RTP801 and VEGF-A mRNAs and that of a control gene (rhodopsin) were quantified by real-time PCR analysis (LightCycler system; Roche Diagnostics, Indianapolis, IN). Of each RNA sample obtained from paraffin-embedded mouse eyes, 5 μL was reverse transcribed in a 20-μL reaction mixture containing 0.35 nM random hexamer primers (Roche Diagnostics) with reverse transcriptase (Superscript II; Invitrogen, San Diego, CA) and the supplied enzyme buffer. RNA and the primer were preannealed at 72°C for 2 minutes and then combined with the remaining reaction components on ice. Reverse transcription (RT) was performed at 25°C for 10 minutes and at 42°C for 1 hour and was terminated by heating at 95°C for 5 minutes. Each RT reaction was diluted 1:25 and 1:50 in double-distilled water, and 2.5 μL of each dilution was added to 7.5 μL of a reaction mix (LightCycler FastStart DNA Master SYBR Green I; Roche Diagnostics), containing 5 pM of PCR amplification primer pairs (final concentration). All runs were performed in duplicate. The reactions were run with the following parameters: denaturation at 95°C for 7 minutes and 35 amplification cycles (95°C for 10 seconds, 55°C for 5 seconds, and 72°C for 8 seconds). Relative standard curves were generated for each primer set so that the input amount from the samples could be calculated. The following PCR primers were used for amplification: mouse RTP801, 5'-GCCGGAGGAGGAGTCTCCCT-3' and 5'-GCTGCACTAGGTGGCACAC-3'; mouse VEGFA, 5'-GCAGGCTGTGCTACTAGGATGAA-3' and 5'-TCCGCATGATGCTGATGTTGTA-3'; and mouse rhodopisin, 5'-TTGGCCACACTTGAGGGTGAA-3' and 5'-ACCACACGCAG- TAGGGCCTAAT-3'. Each primer pair was verified to generate a single specific amplified DNA fragment, and each RNA sample was verified to yield no PCR bands in the absence of reverse transcriptase, thus excluding DNA contamination. Because in situ hybridization analysis detected RTP801 and VEGF induction exclusively in the retinas and because some of the paraffin blocks were partially sectioned, we included a rhodopsin-specific PCR reaction as a normalization control. In addition, the results were normalized to the initial RNA concentrations.

**RESULTS**

**Generation of RTP801-Deficient Mice**

To determine the role of RTP801 in various pathologic conditions, we established a mouse strain with a germline disruption of RTP801. As illustrated in Figure 1A, the targeting vector was designed to replace the second exon, containing the initiation ATG, of RTP801 with the LacZ/neomycin resistance cassette. The targeting vector was linearized and electroporated into LEX-1 ES cells. Two cell clones containing the disrupted RTP801 allele were identified and injected into C57BL/6 blastocysts to generate chimeric mice. Germline transmission of the mutant allele was obtained. Genotyping was performed by PCR analysis with template DNA from tail biopsy specimens (Fig. 1B). Expression deficiency of RTP801 was confirmed by Northern blot analysis with a full-length RTP801 cDNA probe (Fig. 1C). As opposed to the wild-type mice, no RTP801 expression was detected in any of the tissues tested in the knockout mice (heart, spleen, kidney, muscle, brain, and ovary).

The absence of RTP801 had no obvious consequences for prenatal and postnatal development and growth of the mice. Mice homozygous for the RTP801 deletion were born normal, and the mutant allele was transmitted in a Mendelian manner. They were weaned at the same age as the wild-type mice, had body weights and physical features indistinguishable from normal, and were normally fertile. General morphologic examination and histologic assessment of retinas, specifically, did not reveal any pathologic changes.

**Expression of RTP801 in the Model of ROP**

Before the functional assessment of the role that RTP801 may play in ROP, we studied whether the gene’s expression is specifically induced in this model. In situ hybridization analysis with an RTP801-specific antisense riboprobe was performed on retinal sections from P14 mouse (not shown) and rat (Figs. 2A–D) pups either subjected to ROP or grown under normal conditions. Sections of control normoxic retina displayed a very weak background hybridization signal in all retinal layers (Fig. 2B). RTP801-specific hybridization signal was significantly elevated in hypoxic retinas, with the maximum concentration in the outer portion of the INL (Figs. 2C, 2D). The sense probes gave no hybridization signal (not shown). To further substantiate our findings, we performed a quantitative real-time RT-PCR with mouse RTP801-specific primers and RNA extracted from formalin-fixed, paraffin-embedded wild-type mouse eyes enucleated on P14 from the treated and control pups. This quantitative analysis confirmed the in situ hybridization results (Fig. 2E). Thus, we concluded that RTP801 is upregulated in hypoxic retinal cells in the model of perinatal retinopathy both in rats and mice.

**Retinal Vaso-obliteration and Neovascularization in the ROP Model in RTP801-Deficient Mice**

To determine the role that RTP801 plays in the pathogenesis of retinopathy, wild-type and RTP801-knockout mice were subjected to the model of ROP. Simultaneous availability of wild-type and knockout age-matched litters was assured by establishment of mating between mice heterozygous for the RTP801 null allele. The structure of the retinal blood vessel microvasculature was assayed in the ROP Model in RTP801-deficient mice (Fig. 3D, 3F). However, the signs of pathologic changes were significantly less affected (Figs. 3D, 3F). Therefore, the signs of central vasoobliteration and of neovascularization response were still present (compare Fig. 3D with 3B). Retinal angio-
grams of control age-matched wild-type and knockout mice kept in normoxic conditions were indistinguishable from each other (Figs. 3A, 3B). The quantitative comparison of the non-perfused retinal areas (using angiographic image analysis as previously described) between wild-type and knockout mice revealed a statistically significant reduction in the latter genotype ($P < 0.04$, one-tailed t-test; Fig. 3G).

For quantitative assessment of the vasoproliferation response, we embarked on counting the nuclei of endothelial cells in the new blood vessels extending into the vitreous beyond the internal limiting membrane. The nuclei were observed in PAS/hematoxylin-stained retinal sections (Fig. 4A) and an average of 230 sections for each genotype/treatment group were assessed. No neovascular nuclei were found in retinas of either wild-type or RTP801-knockout mice grown in normoxic conditions, whereas both wild-type and knockout retinas showed a neovascular response in the ROP model. However, there was a significant difference ($P < 0.0001$) between the number of retinal neovascular nuclei in the wild-type and RTP801-deficient mice (Fig. 4B). Whereas the average number of neovascular nuclei in hypoxic wild-type retinas was 43, the average number of nuclei counted in retinas of similarly treated RTP801-null mice was only 16. Thus, the absence of RTP801 expression in the model of ROP significantly attenuated the neovascularization response.

**Neuroretinal Apoptosis in RTP801-Deficient Mice in the Model of ROP**

Occurrence of TUNEL-positive cells in the INL of retinas of wild-type and RTP801-knockout mice grown in room air or in the ROP model, was estimated at days P14 and P17. At P14, both groups of room-air–raised control mice had a similar ($P > 0.8$) low number of TUNEL-positive cells in the INL (Fig. 5B). When assessed at P14 after exposure to ROP model, both wild-type and knockout mice had a significantly elevated number of apoptotic INL cells ($P < 0.0001$ and $P = 0.015$, respectively). However, the average numbers of TUNEL-positive cells in the knockout mice were significantly reduced ($P < 0.0001$) compared to the wild-type ones: 16 vs. 28 (Figs. 5). The INL TUNEL-positive cells exhibited typical signs of apoptosis, including pericentric clumps of chromatin, pyknotic nuclei, and eosinophilic cytoplasm, thus supporting the apoptotic nature of the DNA breaks detected by the TUNEL technique (Fig. 5A). At P17, the number of INL TUNEL-positive cells in both wild-type and RTP801-knockout mice in the ROP model was significantly reduced and approximated the corresponding numbers detected in the control animals at P14 ($P = 0.15$ and $P = 0.65$, respectively; Fig. 5B). Thus, we concluded that the absence of RTP801 expression in the model of ROP significantly atten-
ated the hypoxia-induced apoptotic response of the INL cells.

**Expression of VEGF in Wild-Type and RTP801-Deficient Mice in the ROP Model**

We next investigated the retinal expression of VEGF-A by in situ hybridization in wild-type and RTP801-knockout mice in the ROP model. Despite the observed differences between the genotypes in their neovascularization response, the expression of VEGF mRNA was upregulated in P17 retinas of ROP-subjected mice, regardless of their genotype (Fig. 6A). Similar VEGF upregulation was also observed at P14 in both wild-type and knockout retinas, as detected by both in situ hybridization analysis (not shown) and by quantitative real-time PCR (Fig. 3).
6B). These results indicate that the mode of VEGF expression did not substantially differ between wild-type and RTP801-knockout mice.

**DISCUSSION**

In ocular diseases such as diabetic retinopathy, ROP, and retinal arterial or vein occlusions, hypoxic conditions developing as a consequence of vascular occlusion often lead to pathologic angiogenesis and neuroretina degeneration, which in some cases cause severe visual loss.

Our data demonstrate that RTP801, a novel HIF-1-responsive gene, is not only upregulated in the INL in the rodent model of ROP but also functionally contributes to the development of retinal disease in this model. RTP801-knockout mice in the model of ROP clearly demonstrated a significant attenuation in the development of major pathologic features usually documented for this model: retinal vaso-obliteration, retinal neovascularization, and apoptosis of INL cells. Because of the complexity of ROP pathogenesis and the mutual influence and
interconnection of its different stages, at present it is difficult to pinpoint the primary event that is affected by the absence of RTP801. Previous studies\textsuperscript{11,12} have demonstrated that overexpression of RTP801 may lead to apoptosis in target cells. It is conceivable that a lack of RTP801 may protect endothelial cells (notably, induction of RTP801 expression in the endothelial cells, e.g., within a poststroke necrotic zone, has been reported\textsuperscript{13} from hyperoxia-induced apoptosis resulting in reduced retinal vaso-obliteration. This, in turn, may lead to a lesser subsequent relative hypoxia after transfer to normal air. Lesser hypoxia may lead to the reduction of compensatory VEGF secretion and neovascularization and of apoptosis occurring in the INL during the hypoxic stage of retinopathy.\textsuperscript{25}

However, this view may indeed be too simplistic, as in reality the mechanism of protective effect of RTP801 deficiency appears to be more complex, reflecting its direct involvement at all the stages of the pathogenesis. First, it seems that whereas there was a clear direct correlation between the size of nonperfused retinal area and the number of neovascular nuclei in the wild-type mice, this correlation was not so obvious in the RTP801 knockout animals (Fig. 7), indicating that relationships between these two pathologic processes may be perturbed in the absence of RTP801. Second, significant reduction of vasoproliferative response in the knockout mice was not accompanied by a significant reduction in retinal VEGF expression, as confirmed by both in situ hybridization and quantitative RT-PCR analyses. It is important to indicate, however, that although VEGF is considered a major factor triggering neovascularization in the hypoxic retina,\textsuperscript{18,20} it acts together with other factors, being by itself insufficient to induce retinal neovascularization.\textsuperscript{2} Expression of some of these factors may be influenced by RTP801, and it will be interesting to identify them in the future. Third, the absence of RTP801 may directly prevent the occurrence of neuroretinal apoptosis in the hypoxic phase of ROP rather than the protection from apoptosis being secondary to the overall reduced vascular retinal disease. This view is supported by a substantial amount of data. Thus, we have previously demonstrated that overexpression of RTP801 in differentiated neuron-like PC12 cells promotes their apoptotic death and sensitization to hypoxic conditions in vitro.\textsuperscript{14} Moreover, it was recently found that the expression of antisense RTP801 RNA protected neuroblastoma cells from amyloid-β toxicity.\textsuperscript{29} Finally, our preliminary data indicate that primary cortical neurons from RTP801-knockout mice are twice as resistant as control cells in conditions of oxidative stress created by hydrogen peroxide treatment in vitro.

The specific molecular mechanism by which the absence of RTP801 expression attenuates retinal disease in the model of ROP remains unknown. RTP801 has been shown to modulate cellular VEGF secretion and neovascularization and of apoptosis occurring in the retina (WT) and three knockout (KO) mice in the ROP model, for which we have previously demonstrated that overexpression of RTP801 attenuates retinal disease in the model of ROP.\textsuperscript{31} Thus, it is tempting to speculate that RTP801 may be functionally involved in these processes.

The identification of RTP801, as a downstream target of both p53 and HIF-1\textsuperscript{11,12}, suggests that it may function in concert with other redox regulatory genes, known to be induced by these two transcription factors. Both p53 and HIF-1 were shown to be involved in oxygen-induced retinopathy and to constitute an important part of the control of neovascularization.\textsuperscript{6,32} A recent study indicates that in hypoxic conditions, HIF-1α regulates p53 activity at the levels of stability and nuclear export through interactions with Mdm2.\textsuperscript{33} Furthermore, in hypoxic neurons HIF-1α and p53 conspire to promote a pathologic sequence resulting in cell death.\textsuperscript{34} The direct

![Figure 6](image_url)

**Figure 6.** VEGF expression in the retinas of RTP801 knockout and wild-type mice in the model of ROP. (A) Bright-field images of retinal sections derived from wild-type (+/+) and RTP801 knockout (−/−) mice, either grown in normal air conditions (N; normoxia) or in the model of ROP (H; hypoxia) and analyzed at P17. Retinal sections were hybridized in situ to a VEGF-A-specific riboprobe. The hybridization signal was evident as black dots. Scale bar, 50 µm. (B) Relative normalized amounts of VEGF-A RNA at P14 quantified by real-time PCR in the retinas of seven control and five treated wild-type mouse pups in the ROP model. $P = 0.18$ (two-tailed, two samples equal variance $t$-test).

![Figure 7](image_url)

**Figure 7.** Dependence of retinal neovascularization response on the severity of retinal vaso-obliteration. The analysis was performed as a scatterplot (Excel; Microsoft, Redmond, WA) and data from four wild-type (WT) and three knockout (KO) mice in the ROP model, for which both parameters (average number of neovascular nuclei per section [Fig. 4]) and percent of capillary-free area [Fig. 4]) were available. WT: correlation = 0.94; KO: correlation = −1.56575E-16.
transcriptional control of RTP801 by both p53 and HIF-1 stresses that both pathways involved in redox regulation merge at a certain stage.

Many pathologic features developing in the rodent model of ROP are common in a more frequent type of proliferative retinopathy, diabetic retinopathy. These include ROS-induced vaso-obliteration, reactive neovascularization, and neuronal apoptosis in the INL. Stress that both pathways involved in redox regulation merge transcriptional control of RTP801 by both p53 and HIF-1.

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Apoptosis in the INL.35,36 Suppression or attenuation of ischemic proliferative retinopathy would be a highly effective therapeutic option for many retinal disorders. The current treatment of proliferative retinopathies is panretinal laser photocoagulation, which in many cases is effective but not optimal. In addition to possible recurrence and progression of the disease, requiring repeated laser treatments, there are also significant side effects, which include the loss of peripheral and night vision. Several experimental alternative approaches have been designed to curtail the development and/or progression of retinal neovascularization. These approaches include targeting growth factors,18,37 cell surface receptors,38 or proteinases.39 Our studies demonstrate that RTP801 may be regarded as a novel therapeutic target for treatment of proliferative retinopathies. Inhibition of RTP801 function may lead to the attenuation of both vasoproliferation and neurodegeneration symptoms. Currently, several VEGF-inhibiting drugs are being developed for the treatment of retinal diseases.40,41 Because the lack of RTP801 seems to attenuate retinopathy without influencing VEGF production (at least in the model of ROP), whereas a certain residual retinal disease is still present in the knockout mice, simultaneous targeting of both genes may yield a more profound synergistic therapeutic effect. The potential use of RTP801 as a drug target for the treatment of ischemic proliferative retinopathy thus warrants further study.

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