Supplementary Materials and Methods

Animals

The mice used in this study were housed in the animal facility of the Sichuan Provincial People's Hospital. The *rd1* mouse line was obtained from the Jackson Laboratory (USA) and bred with C57Bl6/J mice. Only pigmented mice were used in this study. The animals were handled following the guidelines of the Association for Research in Vision and Ophthalmology for the use of animals in research. All experimental procedures were approved by the Animal Care and Use Committee of the Sichuan Provincial People's Hospital.

Preparation of rapamycin and Torin 1 emulsion

For the preparation of the rapamycin emulsion, rapamycin powder (Selleckchem, USA) was dissolved in DMSO (Sigma, USA) and the concentration was adjusted to 10 mmol/L The 10 mmol/L rapamycin was diluted in 0.9% saline to a concentration of 100 μmol/L. An equal volume of 100 μmol/L rapamycin and 20% intravenous fat emulsion (similar to Intralipid®) was mixed. The emulsion was prepared as described previously (Bittner et al., 2014), with some modification. Briefly, 1 mL of 100 μmol/L rapamycin and 1 mL of fat emulsion was drawn into a 3 mL syringe. This syringe and an empty 3 mL syringe were attached to a three-way connector. The plungers of the two syringes were alternately pushed. Thus, the mixture in one syringe was expelled into the other alternatively. The depression frequency for each syringe was roughly 10 times per minute. After 20 min, the emulsion with 50 μmol/L

rapamycin was used for intravitreal microinjection directly. The 50 μmol/L rapamycin emulsion were also further diluted in 20% fat emulsion to a concentration of 10 μmol/L or 25 μmol/L before microinjection. The Torin 1 (Selleckchem, USA) emulsion was prepared similarly.

Intravitreal microinjection

The eyelids of P10 *rd1* mice were cut open using ophthalmic scissors. A 30 G needle was used to poke a tiny hole through the pars plana. A 5 μL microsyringe (Hamilton, USA) with a 33 G blunt-end needle was used to inject 1 μL of rapamycin or Torin 1 emulsion with or without mixing with 250 μmol/L hydroxychloroquine into the vitreous space. Ophthalmic erythromycin ointment was applied to the wound site to prevent infection.

Immunoblotting

Retinas were dissected from P10 or P11 mice and lysed in 1×SDS loading buffer supplemented with the protease cocktail by brief sonication. The proteins in the lysate were resolved by 8%, 10%, or 15% PAGE gels and transferred to a nitrocellulose membrane or a polyvinylidene difluoride membrane (Millipore, USA). After blocking with 10% non-fat milk in TBST for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4 ℃. Primary antibodies included anti-mTOR (1: 1 000, Cell Signaling Technology, USA), p-mTOR (1: 2 000, Cell Signaling Technology, USA), S6K (Proteintech, China), p-S6K (Ser411) (1:2 000, HuaBio, China), caspase 3 (1: 1 000, Cell Signaling Technology, USA), cleaved caspase 3 (1: 500, Cell Signaling Technology, USA), SQSTM1

(1:500, Santa Cruz Biotechnology, USA), ATG13 (1:1 000, Proteintech, China), LC3B (1:1 000, Cell Signaling Technology, USA), rhodopsin (1:10 000, custom-made (Li et al., 2020)), and GC2 (1:2 000, a gift from Dr. Baehr at the University of Utah). Secondary antibodies included horseradish peroxidase (HRP)-conjugated goat-anti-rabbit (1:5 000) or HRP-conjugated goat-anti-mouse (1:5 000). Signals were visualized using an enhanced chemiluminescence (ECL) kit (4A Biotech, China).

Immunofluorescence microscopy

Eyeballs were enucleated from P11 (one day after microinjection) or P16 (6 days after microinjection) and fixed in 4% paraformaldehyde in 1×phosphate-buffered saline (PBS) at 4 ℃ for 2 h. After cryoprotection in 30% sucrose at 4 ℃ overnight, the cornea and lens were removed, and the eyecup was embedded in optimal cutting temperature (OCT) compound. Frozen tissue was sectioned at 12 μm on a cryostat (ThermoFisher, USA). The retinal sections were immunostained using the following primary antibodies: mTOR (1:200, Cell Signaling Technology, USA), p-mTOR (Cell Signaling Technology, USA), S6K (1:200, Proteintech, China), p-S6K (Ser411) (1:200, HuaBio, China), rhodopsin (1:900, custom-made (Li et al., 2020)), and cone arrestin (1:150, a gift from Dr. Baehr at the University of Utah). Alexa488-conjugated goat-anti-rabbit secondary antibody (ThermoFisher, USA) was diluted at 1:300. The nuclei were stained with DAPI (1:2 000, Cell Signaling Technology, USA). The images were acquired on a Zeiss LSM 900 confocal microscope. Panorama images were generated by stitching multiple images using the software built in the confocal microscope.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed using an *in-situ* cell death detection kit (Roche, Germany) following the manufacturer's instructions. Retinal sections were permeabilized on ice for 5 min, followed by incubation with the TUNEL reaction solution at 37 °C for 1 h. Nuclei were stained with DAPI. The TUNEL signals were imaged on a Zeiss 900 confocal microscope.

Electroretinography

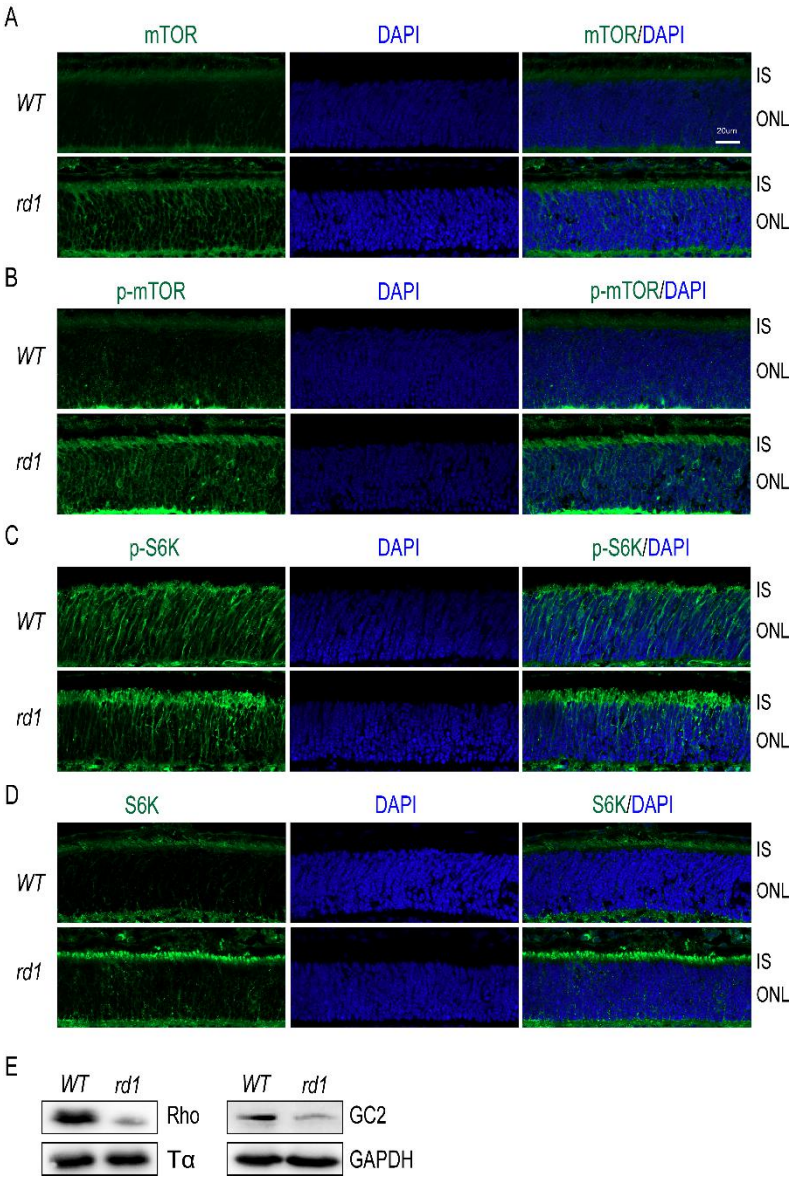
Dark-adapted P16 mice were anesthetized by an intraperitoneal injection of a ketamine (100 mg/kg body weight) and xylazine mixture (10 mg/kg body weight). ERGs were recorded using an Espion Diagnosys ERG system (Diagnosys LLC, Massachusetts, USA). As *rd1* mice have no functional rod cells, only photopic ERGs were performed in the mice to assess cone function. Prior to the recording, the mice were light-adapted under a background light of 3 cd·s/m^2 for 10 min. Photopic ERGs were recorded by presenting single flashes ranging from 0.03 cd·s/m^2 to 50 cd·s/m^2.

REFERENCES

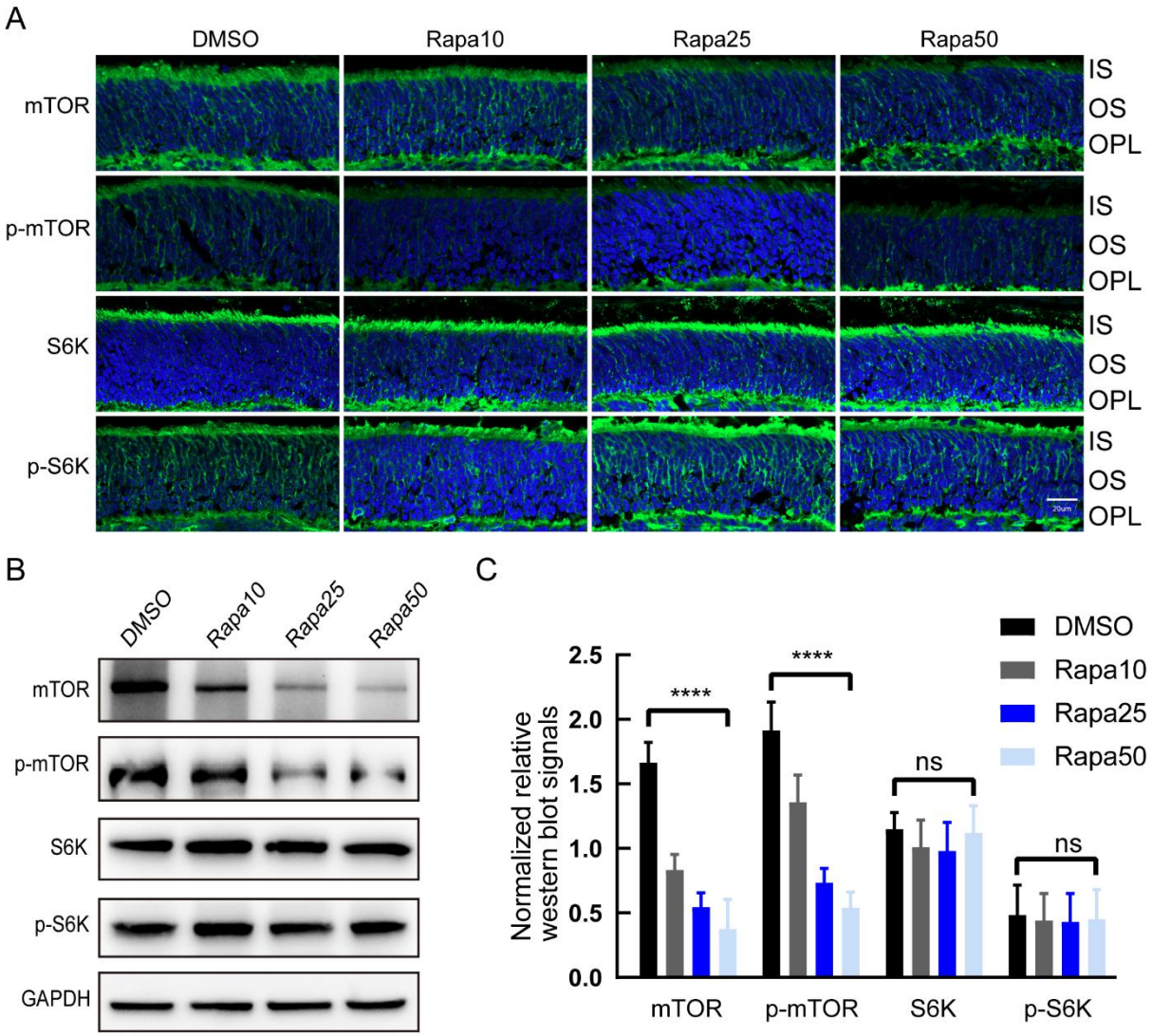
Bittner S, Afzali AM, Wiendl H, Meuth SG. 2014. Myelin oligodendrocyte glycoprotein (MOG_{35-55}) induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice. *Journal of Visualized Experiments*, (86): e51275.

Li L, Lin YQ, Yang F, Zou TD, Yang JL, Zhang HB. 2020. An improved method for preparation of mouse retinal cryosections. *European Journal of Histochemistry*, **64**(3): 3154.

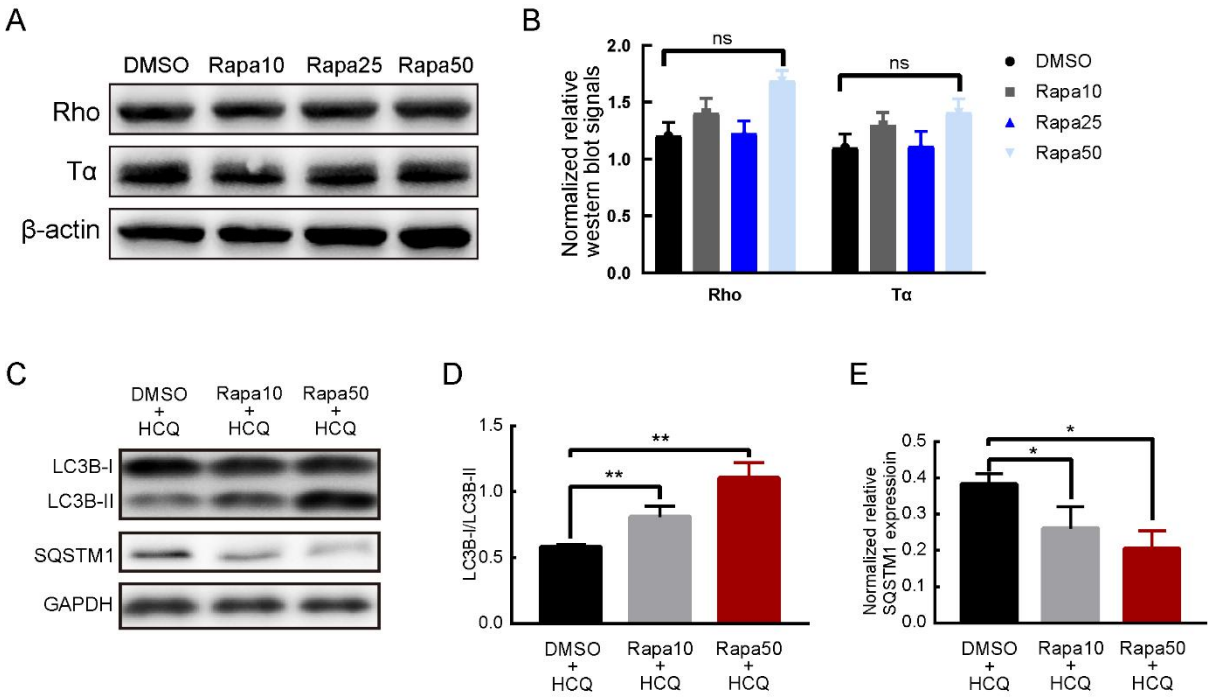
Supplementary Figures and Figure Legends



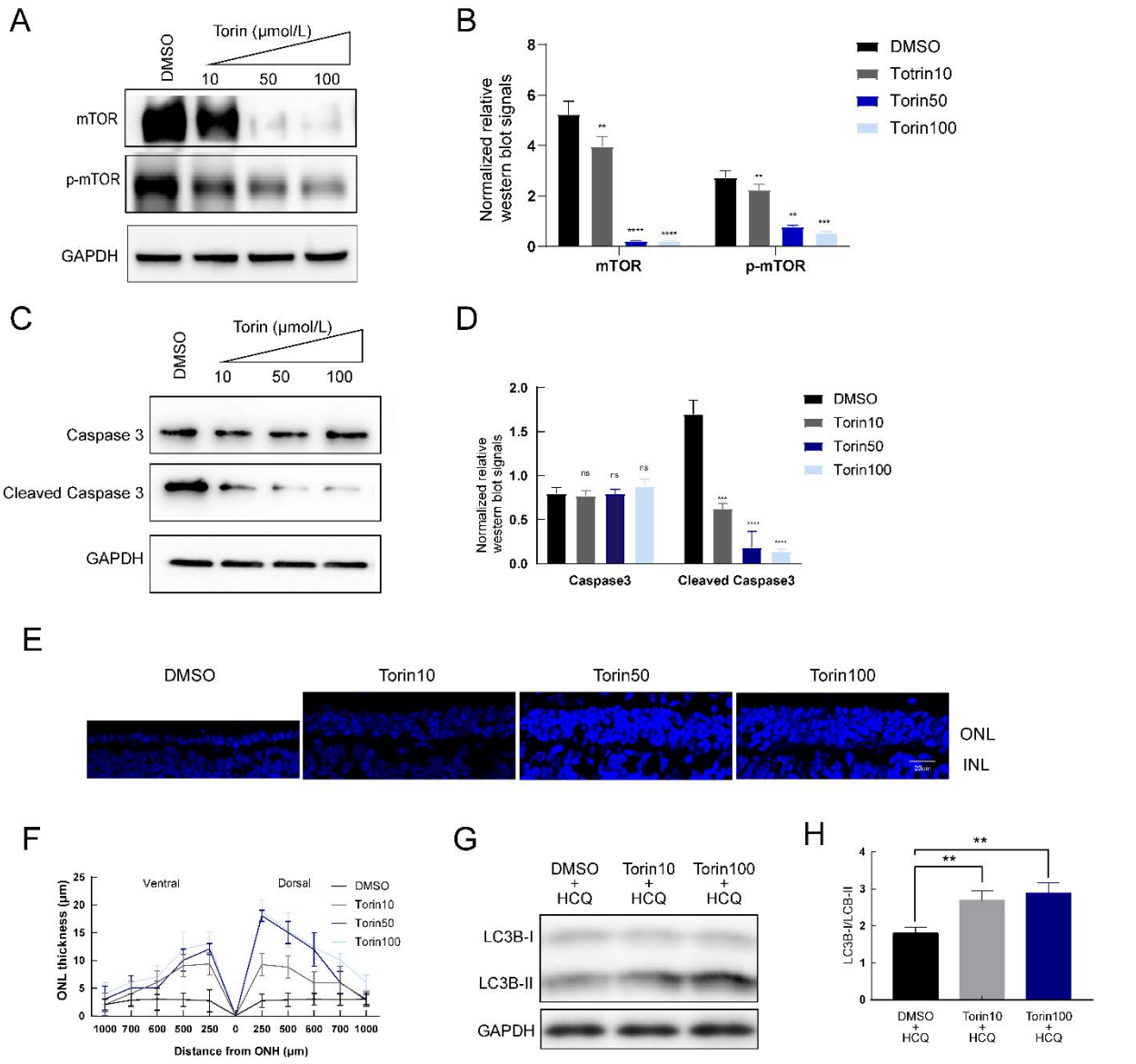
Supplementary Figure S1. Activation of mTOR signaling pathway in *rd1* photoreceptors. A-D: Representative images showing P10 wild-type (*WT*) (*n*=4) and *rd1* (*n*=4) mouse retinal sections immunostained with mTOR (A), p-mTOR (B), S6K (C), and p-S6K (D) antibodies and counterstained with DAPI. IS, inner segment; ONL, outer nuclear layer. Scale bar, 20 μm. E: Immunoblots showing expression of rhodopsin (Rho) and guanylate cyclase 2 (GC2) in *WT* (*n*=3) and *rd1* (*n*=3) retinas. GAPDH was used as a loading control.



Supplementary Figure S2. Inhibition of mTOR signaling pathway in *rd1* photoreceptors by rapamycin. A: P10 *rd1* mice were treated by intravitreal injection of 10 μmol/L (Rapa10) (*n*=3), 25 μmol/L (Rapa25) (*n*=3), 50 μmol/L (Rapa50) (*n*=3) rapamycin, or vehicle control (DMSO) (*n*=3), respectively. Mouse retinas were analyzed 20 h after injection. Representative images showing retinal sections immunostained by mTOR, p-mTOR, S6K, and p-S6K antibodies (green). Nuclei were labeled by DAPI (blue). IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar, 20 μm. B: Western blot analysis of rapamycin-treated *rd1* retinas using mTOR, p-mTOR, S6K, and p-S6K antibodies. GAPDH was used as a loading control. C: Quantification of western blot signals shown in (B). Data are mean±SD. ****P<0.0001; ns, no significance P>0.05.

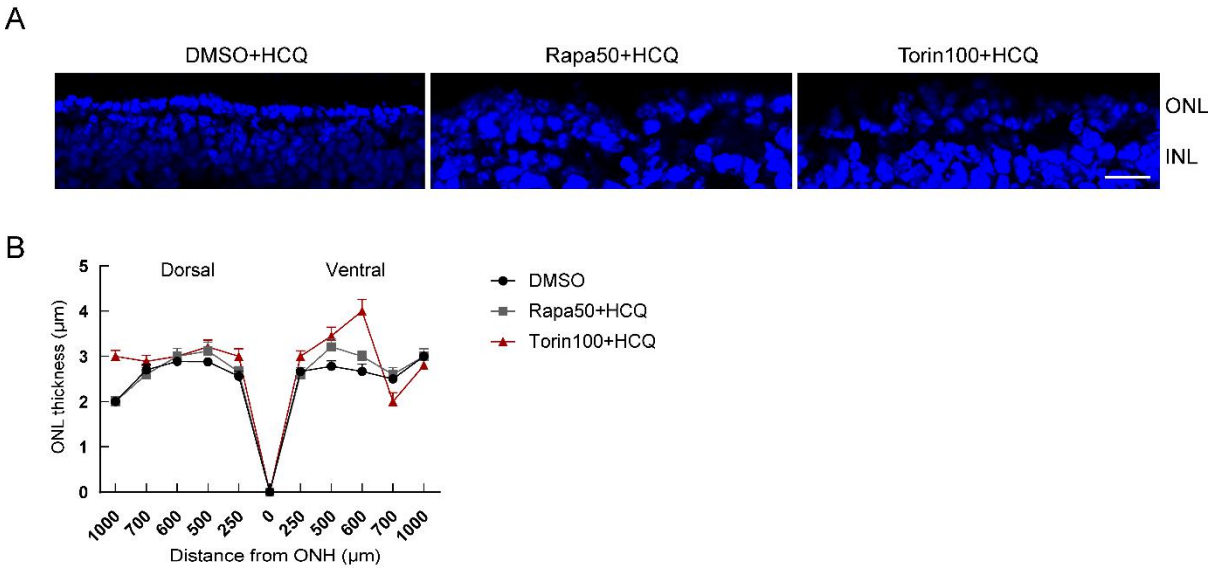


Supplementary Figure S3. Activation of autophagy in *rd1* retinas by rapamycin treatment. A, B: P10 *rd1* mice were treated by intravitreal injection of 10 μmol/L (Rapa10) (*n*=3), 25 μmol/L (Rapa25) (*n*=3), or 50 μmol/L (Rapa50) (*n*=3) rapamycin, respectively. DMSO was used as vehicle control. Mouse retinas were analyzed 20 h after injection by western blotting using representative phototransduction and autophagy antibodies. A: Western blotting was used to detect expression of phototransduction proteins rhodopsin (Rho) and transducin α (Tα). β-actin was used as a loading control. B: Quantification of western blot signals. C: Western blotting was used to detect expression of autophagy proteins LC3B and SQSTM1 in retina 20 h after P10 *rd1* mice received intravitreal co-injection of 250 μmol/L hydroxychloroquine (HCQ) with 10 μmol/L rapamycin (Rapa10+HCQ), 50 μmol/L rapamycin (Rapa50+HCQ), or vehicle control (DMSO+HCQ). GAPDH was used as a loading control. *n*=3 for each concentration of rapamycin. D, E: Quantitative analysis of western blot signals shown in (C). Western blot quantification data are expressed as mean±SD. **P*<0.05; ***P*<0.01; ns, no significance *P*>0.05.



Supplementary Figure S4. Suppression of retinal degeneration in *rd1* retinas by mTOR signaling inhibitor Torin 1 via activation of autophagy. P10 *rd1* mice were treated by intravitreal injection of 10 μmol/L (Torin10), 50 μmol/L (Torin50), or 100 μmol/L Torin 1 (Torin100), respectively. DMSO was used as vehicle control. Mouse retinas were analyzed 20 h after injection by western blotting using representative phototransduction and autophagy antibodies. A-D, G-H: Western blot analysis of *rd1* retinas 20 h after treatment. DMSO was used as vehicle control. E-F: Morphological analysis of *rd1* retinas (P16) 6 days after treatment. For each data point, *n*=3 mice. A: Western blotting was performed using mTOR and p-mTOR antibodies. GAPDH was used as a loading control. B: Quantification of western blot signals shown in (A). C: Western blots were used to detect expression of apoptotic proteins caspase 3 and cleaved caspase 3. GAPDH was used as a loading control. D: Quantification of western blot signals shown in (C). E: Representative individual confocal images of retinal sections stained by DAPI (blue). Scale bar, 20 μm. ONL, outer nuclear layer; INL, inner nuclear layer. F: Measurement of outer nuclear layer thickness along central meridian at 10 locations on dorsal to ventral axis of mouse eyes. G: Western blots were used

to detect expression of autophagy proteins LC3B and SQSTM1 in retina 20 h after P10 *rd1* mice received intravitreal co-injection of 250 μmol/L hydroxychloroquine (HCQ) with 10 μmol/L Torin 1 (Torin10+HCQ), 100 μmol/L Torin 1 (Torin100+HCQ), or vehicle control (DMSO+HCQ). GAPDH was used as a loading control. n=3 for each concentration of Torin 1. H, I: Quantitative analysis of western blot signals shown in (G). All western blot quantification data are expressed as mean±SD. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns, no significance P>0.05.



Supplementary Figure S5. Attenuation of rescue effects of rapamycin on *rd1* photoreceptors by hydroxychloroquine (HCQ). Co-injection of 250 μmol/L HCQ with 50 μmol/L rapamycin (Rapa50+HCQ) or 100 μmol/L Torin 1 (Torin100+HCQ) in intravitreal space of *rd1* mice at P10. Retinas were analyzed at P16. A: Representative individual confocal images of retinal sections stained by DAPI (blue). Scale bar, 20 μm. ONL, outer nuclear layer; INL, inner nuclear layer. HCQ abolished rescue effects of rapamycin. B: Measurement of ONL thickness along central meridian at 10 locations on dorsal to ventral axis of mouse eyes.