Online Supplemental Material – Supplemental Methods and Figures

Huang *et al.* REV-ERBα regulates age-related and oxidative stress-induced degeneration in retinal pigment epithelium via NRF2

Experimental Methods and Materials

Experimental Animals

Rev-erbα$^{-/-}$ mice (B6.Cg-*Nr1d1*^{tm1Ven}/LazJ) were purchased from the Jackson Laboratory ((Jackson Labs Technologies, Inc., Bar Harbor, ME, USA; Stock No: 018447). *Rev-erbα*$^{-/-}$ mouse strain (on a C57BL/6J background) has targeted disruption of part of exon 2, all of exons 3-5, and part of exon 6 of the *Rev-erbα* (*Nr1d1*) gene (*1*). Age-matched wild type (*Rev-erbα*$^{+/+}$) mice from the same breeding colony were used as controls. The *Rev-erbα*$^{flox/flox}$ strain was generated and kindly provided by Dr. Laura A Solt (Scripps Research Florida Campus, Jupiter, Florida, USA). Briefly, the *Rev-erbα*$^{flox/flox}$ strain was developed based on the same gene targeting strategy as described previously for *Rev-erbα*$^{-/-}$ mice (*1*). Embryonic stem (ES) cells were electroporated with a targeting vector where the *Rev-erbα (Nr1d1)* coding sequences from exon 2 to 5 were flanked by a loxP-FRT trap (**Fig. S11A**). A correctly targeted ES clone were injected into C57BL/6 blastocysts producing heterozygous mutants, which were further bred to give rise to *Rev-erbα*$^{flox/flox}$ mice (**Fig. S11B**). The *BEST1-cre* mice (*2*) (C57BL/6-Tg(BEST1-cre)1Jdun/J, Jackson Stock No. 017557) were kindly provided by Dr. Joshua L. Dunaief (University of Pennsylvania, Philadelphia, Pennsylvania, USA). Mice with RPE-specific deletion of REV-ERBα (*BEST1-cre:Rev-erbα*$^{fl/fl}$) were generated by breeding *Rev-erbα*$^{flox/flox}$ mice with *BEST1-cre* mice, with both *Rev-erbα*$^{flox/flox}$ and *BEST1-cre* mice used as control groups. All mice were bred and group-housed in a pathogen–free animal facility at 22°C and under a 12:12-h light–dark cycle (lights on at 7 AM, off at 7 PM). All mice had free access to water and standard chow. All experiments were performed with age-matched controls and with littermate controls when feasible. All sample collections were performed at the same time of the day (4-5 PM) to minimize circadian influence, unless stated otherwise.

RPE aging study model

Effects of aging on RPE and retinal morphology were analyzed *in vivo* in both *Rev-erbα*$^{-/-}$ and *BEST1-cre:Rev-erbα*$^{fl/fl}$ mice and their respective controls at various age points: 3-, 6-, 12-, 16- and 18-month old, unless stated otherwise. Mice were analyzed live with *in vivo* imaging of fundus and OCT, and histologically with dissected RPE/choroid/sclera flat mounts and eye cross sections, as well as by biochemical analysis of isolated RPE including protein and RNA assays.

Sodium iodate-induced RPE and retinal injury model

A chemical-induced RPE/retinal toxicity mouse model was established with one-time tail vein injection of sodium iodate ($NaIO_3$) as previously described (*3*), which induces primarily RPE damage with secondary retinal toxicity. Sodium iodate ($NaIO_3$; Sigma-Aldrich Corp., Cat#: S4007-100G) was prepared in sterile saline (0.9% sodium chloride). Eight-week-old male *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice received a single injection of $NaIO_3$ (20 mg/kg body weight) intravenously. The induced RPE and retinal damage was monitored by fundus imaging and OCT

on day 0, 3, 7 and 14 post injection. Mice were then euthanized on day 14 post $NaIO_3$ injection and assayed for histological examination. For REV-ERB agonist treatment, 8-week-old female C57BL/6J mice were pretreated with either the REV-ERB agonist SR9009 (i.p., b.i.d, 100mg/kg) or vehicle control solution for 2 days. A single injection of 40 mg/kg $NaIO_3$ was performed for all mice on day 3 and followed by 7 additional days of treatment with either SR9009 (i.p., b.i.d, 100mg/kg) or vehicle. Mouse eyes were monitored and imaged on day 0, 3 and 7 post $NaIO_3$ injection and euthanized on day 7. Eyes were collected for histological and biochemical analyses including protein and RNA. Areas of RPE damage (depigmented areas) were quantified from fundus imaging as a percentage of total fundus areas using Adobe Photoshop software.

Primary mouse RPE cell isolation and culture

Primary mouse RPE isolation for cell culture. Primary mouse RPE cells were isolated from young *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice using protocols optimized from previous publications (*4, 5*). Briefly, 8-10 weeks old mice were euthanized and eyes were immediately enucleated and placed in 1x phosphate buffer saline (PBS) with 1x antibiotic-antimycotic (Thermo Fisher Scientific, Cat#: 15240062) for no more than 30 min, followed by rinse with sterile 1x PBS without antibiotic-antimycotic to remove the antibiotic-antimycotic. Eyes were then dissected under microscope to remove the cornea, iris, lens and retina. The remaining parts of the eye (RPE/choroid/sclera complex) were temporarily placed in buffer (5% BSA in sterile 1X PBS) until all eyes were dissected, then transferred to cell dissociation solution with 0.25% trypsin, 0.02% EDTA (Gibco, Cat#: 25-200-072) and incubated for 45 min at 37°C. After incubation, RPE sheets were separated from the choroid/sclera complex with brief pipetting. After removal of choroid/sclera complexes, the remaining RPE sheets in trypsin-EDTA solution were then transferred to the seeding medium of DMEM: F-12 Medium (ATCC, Cat#: 30-2006) with 20% heat-inactivated fetal bovine serum (HI FBS, Gibco, Cat#: 10082147) and 1x antibiotic-antimycotic (at 1 to 9 ratio) to neutralized the trypsin enzymatic activity. The medium was centrifuged at 1,000 rpm for 5 min to pellet RPE cell sheets. RPE cell sheets were then resuspended and incubated in fresh 1 ml 0.25% Trypsin-EDTA solution for 5 min followed by brief pipetting to breakdown RPE sheets, then filtered by cell strainers with 70 μm mesh size (Corning Falcon, Cat#: 08-771-2) to form a single RPE cell suspension. After centrifugation and resuspension in the seeding medium, single RPE cells were seeded in 24-well plates at a density of 4 eyes/well, and incubated under 37°C and 5% CO_2 for 48 hours with no disturbance. Afterwards the seeding medium was then replaced by the culture medium (DMEM: F-12 Medium with 1% HI FBS and 1x antibiotic-antimycotic). Formation of primary RPE cell monolayer were validated by morphological hexagonal characterization and confirmed by transendothelial electrical resistance (TEER) value (when seeded on Transwells) (*5*). Primary RPE cells were assayed at passage 0 (P0) 3-4 weeks after seeding when the cells restored pigmentation and cell-cell contact. Alternatively, primary RPE cells were sub-cultured in 96-well plates and assayed for Seahorse analysis at P1 (3 weeks after sub-culturing). Each experiment was repeated with at least three repeats of primary RPE cell culture, each with at least 3 wells/group.

Primary mouse RPE isolation for mRNA extraction. Primary mouse RPE cells were isolated from *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice using a previously published protocol with adaptation and optimization (*6*). RPE/choroid/sclera complexes were dissected as described above and then incubated in the RNAprotect cell reagent (Qiagen, Cat#: 76526) for 15 min in room temperature

with gentle shaking. Afterwards, RPE cells were separated and removed from choroid/sclera complexes, and pelleted by centrifugation at 2,500 rpm for 15 min at 4°C. RNAprotect cell reagent was then carefully aspirated. Total RNA was extracted from the RPE cell pellets using the PureLink RNA Mini Kit (Thermo Fisher Scientific, Cat#: 12183025) following manufacture's instruction.

Primary mouse RPE isolation for protein extraction. Major procedures and materials were adapted and optimized from a previously published protocol (*7*). RPE/choroid/sclera complexes were dissected then incubated in RIPA Lysis and Extraction Buffer (Thermo Scientific, Cat#: 89900) for 15 min on ice with gentle shaking. After incubation, choroid/sclera complexes were gently removed. RPE cells were continuously incubated in the RIPA buffer on ice for another 30 min and followed by centrifugation at maximum speed for 15 min at 4°C. The supernatant of total protein lysate was then assayed for protein concentration and Western Blotting.

ARPE-19 cell line

The ARPE-19 cell line was purchase from ATCC (CRL-2302) at P20. Cells were cultured, sub-cultured and maintained strictly following ATCC instructions. Polarity of cultured cell was validated with transepithelial electrical resistance (TEER) measurement using an Epithelial Volt/Ohm Meter (EVOM2, World Precision Instruments) and cells validated with expression of RPE marker (RPE65). All assays were conducted before 30 passages.

Experimental procedure details

***In vivo* mouse retinal imaging with fundus photography and optical coherence tomography (OCT)**

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and pupils were dilated by topical administration of Cyclomydril drops (Alcon Laboratories, Fort Worth, TX). Fundus and OCT images were taken with a rodent retinal imaging microscope (Micron IV, Phoenix Research Laboratories, Pleasanton, CA) by following the manufacturer's instructions and using the vendor's image acquisition software.

Quantification of fundus lesions

For the aging model, white/yellowish fundus lesions were counted manually using the Adobe Photoshop 2020 (Adobe Systems, San Jose, CA). For the $NaIO_3$-induced RPE injury model, RPE lesion areas with hypopigmentation were manually selected and quantified by the Adobe Photoshop 2020 Histogram. During quantification, the contrast of fundus images was temporarily adjusted in Photoshop to improve visualization of the lesions (whitish dots or areas), with a fixed threshold of color range set throughout all images for quantification purpose. Representative fundus images are shown in the figures with no contrast adjustment.

RPE/choroid/sclera flat mounts

Mice were euthanized with CO_2 inhalation and followed by cervical dislocation. Eyes were enucleated immediately after euthanasia, and fixed in 4% paraformaldehyde (PFA, diluted from 32% Paraformaldehyde aqueous solution, EMS, Cat#: 50-980-495) for 30 min, then transferred

to 1× PBS solution. Extraocular muscles and connective tissues attached to the eyeball were dissected away as much as possible. The anterior segment of the eyes and the retina were removed. Posterior eyecups with RPE/choroid/sclera complexes were fixed in 4% PFA for an additional 10 min and subsequently washed with 1× PBS. Fixed eyecups were permeabilized and blocked in 5% serum (from the same host species of secondary antibodies) with 0.1% Triton X-100 (Invitrogen, Cat#: 85112) for 1 hour. Eyecups were then incubated in primary antibodies overnight in 1% FBS with 0.1% Triton X-100 and washed in 1× PBS, followed by incubation at room temperature with secondary antibodies and DAPI (Invitrogen, Cat#: D1306) in 1% FBS with 0.1% Triton X-100 for 1 hour. After final washes in 1× PBS, eyecups were placed on glass slides, flattened with 4–6 radial incision, and coverslipped using Fluoro-Gel (EMS, Cat#: 17985-10) as a mounting medium. Flat mounts were imaged using a confocal microscopy (LSM 880; Zeiss, Oberkochen, Germany).

Immunohistochemistry

Immunofluorescence. Mouse eyes were embedded and frozen in optimal cutting temperature (OCT) compound. Cross sections (12 μm) were cut by a cryostat (Leica Biosystems, Wetzlar, Germany) and placed on positively charged microscope slides (VWR, Cat#: 16004-406). Sections were completely air dried and then fixed in 4% paraformaldehyde for 15 min and followed by 3x washing in 1x PBS. Sections were then blocked in 5% serum (from the same host species of secondary antibodies) in 1X PBS with 0.1% Triton X-100 (PBST) for 1 hour then incubated with primary antibodies diluted in 1% serum in PBST overnight at 4°C. Sections were incubated at room temperature with secondary antibodies and DAPI, and then mounted using Fluoro-Gel. Stained sections were imaged using a Zeiss LSM 880 confocal microscopy. For 8-OHdG Staining, fixed eye cross sections were pre-treated with proteinase K (5ng/ml) for 15 min at 37°C, then followed by standard protocols for immunofluorescence staining with anti-8-OHdG antibodies. Antibodies used in the immunofluorescence experiments were described in **Supplementary Table S1**.

TUNEL staining. Apoptotic cells in fixed eye cross sections were detected using TdT In Situ Apoptosis Detection Kit – Fluorescein kit (R & D systems, Cat#: 4812-30-K) per manufacturer's instructions. Specifically, 1:200 dilution of proteinase K was used for sample permeabilization for 15 min at 37°C, and Mn^{2+} was used in the labeling reaction. The numbers of fluorescent positive apoptotic RPE or retinal cell nuclei were quantified as a percentage of total RPE or retinal cell counts from selected images within or around the lesion areas.

MitoTracker Staining. MitoTracker (Invitrogen/Molecular Probes, Cat#: M7512) solution (1 mM) was prepared in DMSO. ARPE-19 cells were seeded and cultured on sterile glass coverslips in a 24-well plate. When the cells were ready for assay, 1 μl stock MitoTracker solution was added to 10ml culture solution, giving a final concentration of 100 nM, and incubate at 37°C for 5 minutes. Cells were then fixed in 4% PFA in PBS, followed by standard protocols for immunofluorescence staining. The numbers of mitochondrial cluster per cell were quantified with Image J.

Histology: hematoxylin and eosin (H&E) staining

Frozen sections. Frozen eye cross sections in OCT were air dried and stained with hematoxylin solution (Sigma-Aldrich Corp, Cat# HHS32-1L) for 3 min. Sections were then rinsed in ddH_2O

and developed in tap water for 5 min, followed by 10 dips in acid alcohol (0.5% hydrochloric acid in 70% ethanol) to remove excess stain. Sections were then stained with eosin Y solution (Sigma-Aldrich Corp, Cat#: 1098441000) for 30 seconds and rinsed in ddH_2O. The section slides were dehydrated in a graded series of ethanol (50%, 70%, 95% and 100%) and incubated with xylene for at least 15 min. Slides were mounted with Permount Mounting Medium (Fisher Scientific, Cat#: SP15-100) and air dried before imaging.

Paraffin sections. Eyes from 6-, 12-, and 18-month-old *Rev-erbα*$^{-/-}$ were enucleated and fixed in 4% formaldehyde for 24 hours before embedded in paraffin. Sagittal sections of 5 μm thickness were cut from cornea to optic nerve. At least 10 slides from each eye were examined for presence of sub-retinal lesions. Paraffin sections were deparaffinized in xylene and rehydrated in a graded series of ethanol (50%, 70%, 95% and 100%) and water, and stained with H&E as described above.

Histology: Oil Red O staining

Fixed frozen eye cross sections were air dried and rinsed with 60% isopropanol and stained with freshly prepared Oil Red O staining working solution (0.3% w/v in 60% isopropanol and 40% dH_2O). After another rinse with 60% isopropanol, sections were counterstained with hematoxylin per standard protocols and imaged.

Transmission Electron Microscopy (TEM)

Major procedures of EM were described previously (*8*), and performed at Harvard Medical School EM core facility. Briefly, mouse eyes were enucleated and fixed in 4% PFA and 5% glutaraldehyde solution for 1 hour, then followed by 4% PFA fixation for 24 hours. After removal of the anterior segment and lens, eye cups with intact retina and RPE/choroid/sclera were then dissected and cut into 50-μm-thick free-floating sections using a vibrotome. Sections were then stained with osmication and uranyl acetate, dehydrated and embedded in Taab 812 Epoxy Resin (Marivac Ltd., Nova Scotia, Canada). Ultrathin sections (80nm) were cut with an ultracut microtome (Leica), placed on copper grids, and stained with lead citrate. Sections were viewed and selected subretinal areas including photoreceptors and RPE were imaged under a JEOL 1200Ex Electron Microscope.

Quantification of Bruch's membrane thickness

The thickness of Bruch's membrane (BrM) from 6-month-old mice was measured and quantified from TEM images with Adobe Photoshop 2020. A transparent grid was superimposed onto the micrograph, with RPE basement membrane aligned with the horizontal line. Three mice (1 eye from each mouse) from each genotype were analyzed, and at least 5 images were captured for each eye at a direct magnification of 4,000X. Ten random measurements were made on each image. Areas with considerably thickened outer collagenous layer were excluded. The thickness of BrM was determined by averaging all measurements of each group (each dot in the plot represents the average of 10 random measurements from each image).

Toluidine blue staining

Fixed eye cups embedded in epoxy resin for TEM processing were cut into semi thin sections (1μm) with a microtome and collected on glass slides. After dehydration, semi thin sections were

stained with 1% toluidine blue and 2% borate in dH_2O, followed by imaging with a light microscope (LSM 880; Zeiss, Oberkochen, Germany).

Phagocytosis assays

In vivo. *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice were euthanized at 9 am when RPE phagocytic activity is at the burst (*9*). RPE/choroid/sclera complexes were isolated as eye cups and stained with Alexa Fluor™ 488 Phalloidin, rhodamine-conjugated peanut agglutinin (PNA) and DAPI at room temperature for 1 hour. Eye cups were then flat mounted and imaged using a Zeiss LSM 880 confocal microscopy. The numbers of engulfed cone outer segments per RPE cell were counted and quantified from 40-50 cells using Image J software.

In vitro. Primary mouse RPE cells were cultured for 3 weeks before the assay. Cells were challenged by either FITC-labeled porcine photoreceptor out segments (POS) or microspheres (FluoSpheres™ Carboxylate-Modified Microspheres, 1.0 μm, red fluorescent (580/605), Thermofisher Scientific, Cat#: F8821). Porcine eyes were purchased from Sierra for Medical Science (Whittier, CA, USA). The procedure of porcine POS isolation and labeling was described previously (*10*). For porcine POS treatment, cells were incubated with porcine POS for 2 hours at a density of 10 POS/cell. Cells were then rinsed with 1x PBS and incubated with 0.4% trypan blue solution (Sigma-Aldrich Corp., Cat#: T8154-20ML) for 1 min to quench the fluorescent signals from unengulfed POS. Cells were then undergone standard immunofluorescence protocol and mounted for imaging. For microsphere treatment (*11*), cells were incubated with microspheres for 6 hours at a density of 10 microspheres/cell, and then rinsed and imaged as described above. The numbers of engulfed POS per RPE cell were counted and quantified from 20-30 cells using Image J software.

Cellular Reactive Oxygen Species (ROS) assay with DCFDA / H2DCFDA

Rev-erbα$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice were euthanized at 3, 6 and 12 months old. RPE/choroid/sclera complexes were incubated in 0.25% Trypsin, 0.02% EDTA solution for 30 min to separate RPE sheets from choroid. Age-matched eyes with the same genotype were pooled together to increase the cell density. RPE sheets were pelleted and washed in 1X PBS. In each sample, half of the RPE sheets were saved for genomic DNA extraction to determine the total cell volume, and the other half were assayed for the cellular ROS production using the DCFDA / H2DCFDA - Cellular ROS Assay Kit (Abcam, Cat#: ab113851) per manufacture's instruction. Particularly, RPE cells were stained by resuspended in the diluted DCFDA solution at a concentration of 4 eyes/mL and incubate at 37°C for 30 minutes in the dark. After washing with 1x buffer, stained cells were seeded on a dark, clear bottom 96-well microplate at a density of 0.5 eyes/well. The plate was measured immediately on an EnSight™ multimode plate reader (PerkinElmer, Cat# HH34000000) at Ex/Em = 485/535 nm. The fluorescence intensity was normalized to the total DNA amount of each sample.

Oxygen consumption rate analysis

Oxygen consumption rate (OCR) was measured using a Seahorse XFe96 Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA). The procedure was adapted from previously described methods (*12*) and optimized for primary mouse RPE cells. Briefly, primary mouse RPE cells were cultured on a 96 well spheroid plate at a density of 1 eye/well for 3 weeks. *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ primary RPE cells were treated with paraquat (PQ, 0.5mM) for 2 hours right before

measurements. Sequential injection of various compounds (1 μmol/L oligomycin [Oligo], 1 μmol/L carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone [FCCP], 0.5 μmol/L rotenone and Antimycin A [RAA]) was used to determine the amount of proton uncoupling (OCR independent of adenosine triphosphate production), the maximal mitochondrial respiration, and the non-mitochondrial respiration rate, respectively. Cell counts of each well were determined by CyQUANT Cell Proliferation Assay kit (ThermoFisher Scientific, Cat#: C7026) per manufacture's instruction, and OCR values were normalized to the number of cells in each sample.

Cell viability assay

The MTT assay was performed in either primary mouse RPE cells or human ARPE-19 cells to determine cell viability. For primary mouse RPE cells, cells were cultured on a 96-well-plate at a density of 1 eye/well for 3 weeks. Cells were treated with either PBS or paraquat (PQ, 0.5mM) for 4 hours, and followed by 24-hour treatment of either DMSO (as vehicle) or REV-ERBα agonists SR9009 or SR9011 (1μM or 10 μM). The tetrazolium blue chloride (Sigma Aldrich, Cat# M5655) stocking solution (5mg/ml in PBS) was diluted in the culture medium at 1:10 ratio and made the final concentration at 0.5mg/ml (MTT working solution). 100μl MTT working solution was added into each well (including 3-5 empty wells as negative control/blank reading) and incubated for 3-4 hours at 37°C until intracellular purple formazan crystals are visible under microscope. Afterwards, 100 μL of DMSO was added into each well (including negative control wells) and the plate were incubated in dark on an orbital shaker for 15-20 minutes at 37°C. The absorbance values were read at 570nm using an xMark™ Microplate Absorbance Spectrophotometer (Bio-rad, Cat# 1681150). For ARPE-19 cells, cells were seeded on a 96-well plate ($1x10^5$ cells/ well) in 100ul medium/well on the day before the assay, treated with PBS/PQ (0.5mM) for 2 hours and followed by 24-hour treatment of SR9009 or SR9011 (1μM or 10 μM). The MTT measurement procedure was then carried out as described above for primary mouse RPE cells.

Chromatin immunoprecipitation (ChIP)

Major procedure of the ChIP experiment was described previously (*13, 14*). ChIP assays were performed in 4 groups of ARPE-19 cells: cells infected with lentivirus constructed with 1) *shRev-erbα*, or 2) *shControl*; and cells treated with 3) REV-ERBα agonist SR9009, or 4) vehicle control solution, for 24 hours. Anti-REV-ERBα, anti-HDAC3 and anti-NCOR1 antibodies (details in **Supplementary Table S1**) were used for immunoprecipitation. The enrichment of de-crosslinked chromatin DNA fragments precipitated by antibodies were analyzed by qPCR using selected primers described in **Supplementary Table S2**.

Promoter cloning and dual-luciferase assay

Three human *NFE2L2(NRF2)* promoter regions containing the putative RORE/RevRE-binding motif (PuGGTCA) were amplified by PCR using Phusion High-Fidelity DNA polymerase (New England Biolabs, Cat#: M0530). Primers used for cloning were described in **Supplementary Table S2**. PCR products were then purified by gel extraction and cloned into the destination pGL3-Basic-IRES luciferase vector (*15*). pGL3-Basic-IRES was a gift from Joshua Mendell (Addgene plasmid # 64784; http://n2t.net/addgene:64784; RRID: Addgene_64784). All clones were verified by Sanger Sequencing conducted by Eton Bioscience (Boston, MA, USA) and confirmed by 100% sequence matching with the promoter region of *NFE2L2(NRF2)* promoter.

p3Flag-REV-ERBα over-expression of REV-ERBα was a gift from Dr. Laura Solt at the Scripps Research Institute. Promoter plasmids and the empty vectors were transfected into ARPE-19 cells. After 24 hours, cells were treated with either REV-ERBα agonist SR9009 or vehicle, or p3Flag-REV-ERBα over-expression of REV-ERBα or vehicle, for another 24 hours. A dual-luciferase reporter assay kit (Promega; E1910) was used to determine luciferase activity. Relative luciferase activities were calculated by normalizing firefly luciferase activity values to respective *Renilla* luciferase values.

Real-time qPCR

Isolated RPE cells were homogenized in 0.5 ml TRIzol (ThermoFisher Scientific, Cat#: 15596026). Total RNA was extracted using the PureLink RNA Mini Kit (Thermo Fisher Scientific; Cat#: 12183025) according to manufacturer specifications. RNA (1 µg) was reverse transcribed to 20 µl cDNA using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-rad, Cat# 1708840). Real-time qPCR was performed using 1 µl of cDNA and iTaq Universal SYBR Green Supermix (Bio-rad, Cat# 1725120) on a CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-rad, Cat# 1854095). PCR reactions were heated to 50°C for 2 min and held at 95°C for 10 min to activate the polymerase. Amplification was performed for 40 cycles each of 15 s, denaturing at 95°C, and 1 min of annealing at 60°C. Cycle threshold (Ct) values were analyzed by double delta CT method and normalized to either *Gapdh* or *18S*. PCR primers used in the experiments are described in **Supplementary Table S2**.

Western Blotting

Primary mouse RPE cells were isolated as described above and incubated in 100 µl RIPA buffer with protease inhibitors and protein extracted on ice for at least 30 min. Samples were centrifuged for 15 min at 15,000 *g* at 4°C and supernatants were collected for protein concentration determination using BCA Protein Assay kit (Thermo Fisher Scientific,Cat#: 23225). For Western blot analysis, 5 µg RPE protein was loaded on 4–12% NuPage Bis-Tris Protein gels (Invitrogen, Cat#: NP0321BOX) and electrophoretically transferred onto PVDF membrane (Bio-rad, Cat#: 1620177). Membranes were incubated for 1 hour at room temperature in blocking buffer [5% powdered milk in Tris-buffered saline with 0.1% Tween 20 (TBST)] then incubated overnight with various primary antibodies (**Supplementary Table 1**). Membranes were washed three times with TBST and incubated for 1 hour with secondary antibodies. Blots were developed using chemiluminescence (Thermo Fisher Scientific, Supersignal West Dura Cat#: 34075, or SuperSignal West Femto Cat#: 34095) on Azure 600 Imaging System (Azure Biosystems, Dublin, CA, USA). Densitometry analysis was performed using Image J software and normalized to β-Actin.

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Number of Supplemental Tables: 2

Number of Supplemental Figures: 12

Supplementary Table S1: Sources and application details of antibodies and conjugates

Antibody	Vender	Catalog No.	Application (Dilution)
Recombinant Anti-NR1D1 (REV-ERBα) antibody [EPR10376]	Abcam	ab17430	WB (1:1000), IHC (1:200)
Anti-REV-ERBα (E1Y6D) rabbit mAb	Cell Signaling	13418	ChIP (1:50)
β-Catenin antibody (E-5)	Santa Cruz	sc-7963	IHC (1:500)
Vitronectin	Novus	NBP2-20866	IHC (1:200)
Anti-C3 antibody [EPR19394]	Abcam	ab200999	WB (1:1000)
Anti-C3 antibody [11H9]	Abcam	ab11862	IHC (1:200)
Peanut Agglutinin (PNA), Rhodamine	Vector	RL-1072-5	IHC (1:1000)
Anti-8-Hydroxydeoxyguanosine (8-OHdG)	Millipore	AB5830	IHC (1:50)
Anti-NRF2 antibody	Abcam	ab92946	WB (1:1000)
SOD1/Cu-Zn SOD antibody	Novus	NBP2-24915	WB (1:1000)
SOD2 (D3X8F) XP® rabbit mAb	Cell Signaling	13141	WB (1:1000)
Catalase (D5N7V) rabbit mAb	Cell Signaling	14097	WB (1:1000)
Anti-HDAC3 (D2O1K) rabbit mAb	Cell Signaling	85057	ChIP (1:50)
Anti-ZO-1 antibody	Millipore	AB2272	IHC (1:500)
Alexa Fluor™ 488 Phalloidin	Invitrogen	A12379	IHC (1:500)
β-Actin (8H10D10) mouse mAb	Cell Signaling	3700	WB (1:2000)

Supplementary Table S2: Primer sequences for RT-qPCR, ChIP, and cloning

Primer Name	Forward (5'-3')	Reverse (5'-3')
RT-qPCR		
Mouse		
Nr1d1 (Rev-erbα)	CTTCATCCTCCTCCTCCTTCTA	GTAATGTTGCTTGTGCCCTTG
Rpe65	GAAGTTGGATCTGAGCCTTTCT	CGGATGAATCTTCTGTGGTATGT
Nfe2l2	CCTTTGGAGGCAAGACATAGAT	GTTCCTTCTGGAGTTGCTCTT
Sod1	CTCAGGAGAGCATTCCATCATT	CTCCCAGCATTTCCAGTCTT
Sod2	AGCGTGACTTTGGGTCTTT	AGCGACCTTGCTCCTTATTG
Cat	CAGATGGAGAGGCAGTCTATTG	AAAGATCTCGGAGGCCATAATC
Clock	CCGACAAAGATCCCTACTGATAC	ACCAACTGACTGGGAGTTTATG
CD36	GCGACATGATTAATGGCACAG	GATCCGAACACAGCGTAGATAG
Ctsd	CTACCTGAACGTCACTCGAAAG	GATGTCCCTGTGTCCACAATAG
Itgav	AGAAGACGTTGGGCCTATTG	TGTAAGGCCACTGGAGATTTAG
MertK	CGACTTCTAGGCGTGTGTATAG	GTGTGGAGGTCTCCGTATTT
Gpx1	CTACACCGAGATGAACGATCTG	TCTTCATTCTTGCCATTCTCCT
Gpx4	CCGATATGCTGAGTGTGGTTTA	GGCTGCAAACTCCTTGATTTC
Hmox1	GTACACATCCAAGCCGAGAA	TGGTACAAGGAAGCCATCAC
Hmox2	GAATGCCTTGGACCTGAATTTG	CCTGGTCCAGTTCACTGAATATC
Human		
NR1D1 (REV-ERBα)	GTCGCTTCAAGAAGTGTCTCTC	GTTCATGGCACTCTGCATCT
RPE65	GGACTTGGCTTGAATCACTTTG	AAGATGGGTTCTGATGGGTATG
CLOCK	CCAGTGATGTCTCAAGCTACAA	CCGTTGTTCCAATTGGTCTTTC
NFE2L2	GTTGCCCACATTCCCAAATC	CGTAGCCGAAGAAACCTCAT
SOD1	TCGAGCAGAAGGAAAGTAATG	CCTGCTGTATTATCTCCAAACT
SOD2	TTCAATGGTGGTGGTCATATC	CCGTCAGCTTCTCCTTAAAC
CATALASE	CCCAGGAAGATCCTGACTAT	CTTGGTGAGATCGAATGGATTA

GPX1	CATCAGGAGAACGCCAAGAA	GCACTTCTCGAAGAGCATGA
GPX4	GCTGTGGAAGTGGATGAAGA	GTCGATGAGGAACTTGGTGAA
HMOX1	TCAGGCAGAGGGTGATAGAA	GCTCCTGCAACTCCTCAAA
HMOX2	GGGAAAGGAGACATGCGTAAA	CTTCCTCAGCACAGCCATAG
ChIP		
BMAL1 promoter	TTGGGCACAGCGATTGGTG	TAAACAGGCACCTCCGTCCC
NFE2L2 #1	GCCTTGGAGCAGGAGAAAT	AGAGCACAGGTGGAACATTAG
NFE2L2 #2	CCTTCTCCAACGAGAGTTCTTC	ACTAGACCCTGGATTCTGTCA
NFE2L2 #3	GTGTTTGCAGTTTATGGCTTCT	AAAGGTGCTGACTGGTTTCT
Cloning for Luciferase Assay		
NFE2L2 #1 Luc	CTAGCTAGCAGCTGGACCGAGAG CCAAAC	CCGGAATTCGCCAGGCACGAAAT AGGTGG
NFE2L2 #3 Luc	CTAGCTAGCCAGTTTATGGCTTC TTACC	CCGGAATTCTTCTCCGAGGAGAT ATAAA

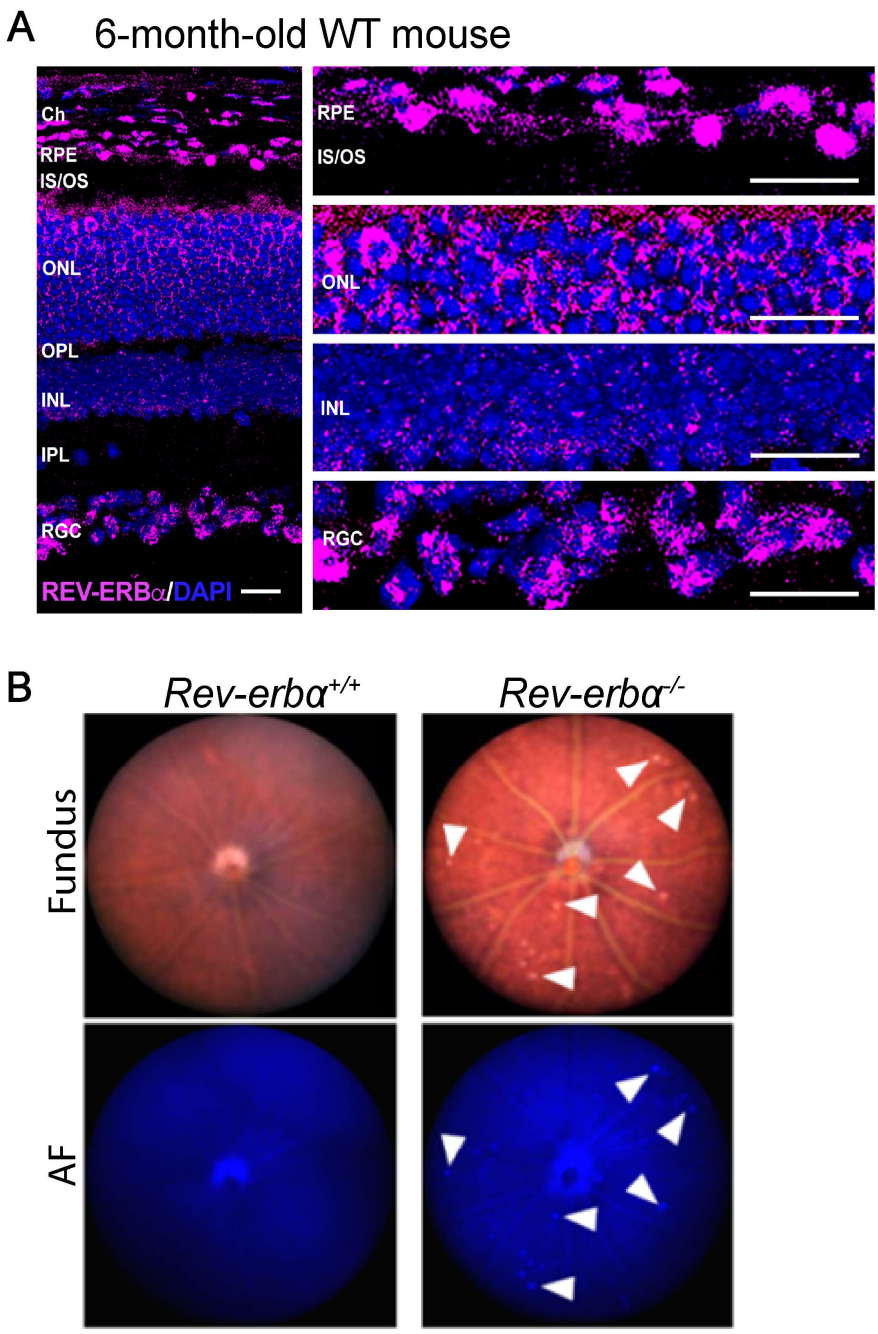

Figure S1. Localization of REV-ERBα in retinal neurons and fundus imaging of *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ retinas. (A) Immunohistochemistry of REV-ERBα antibody staining in retinal cross sections showing REV-ERBα (magenta) in different layers colocalized with DAPI (blue, nuclei marker). Ch, choroid; INL, inner nuclear layer, IPL, inner plexiform layer; IS/OS, inner segments and outer segments of photoreceptors; ONL, outer nuclear layer; RGC, retinal ganglion cell. Scale bars, 20 μm. (B) *Rev-erbα*$^{-/-}$ retinas show whitish-yellow deposits (arrowheads) in fundus imaging, with hyper-autofluorescence (AF) at 5 months old.

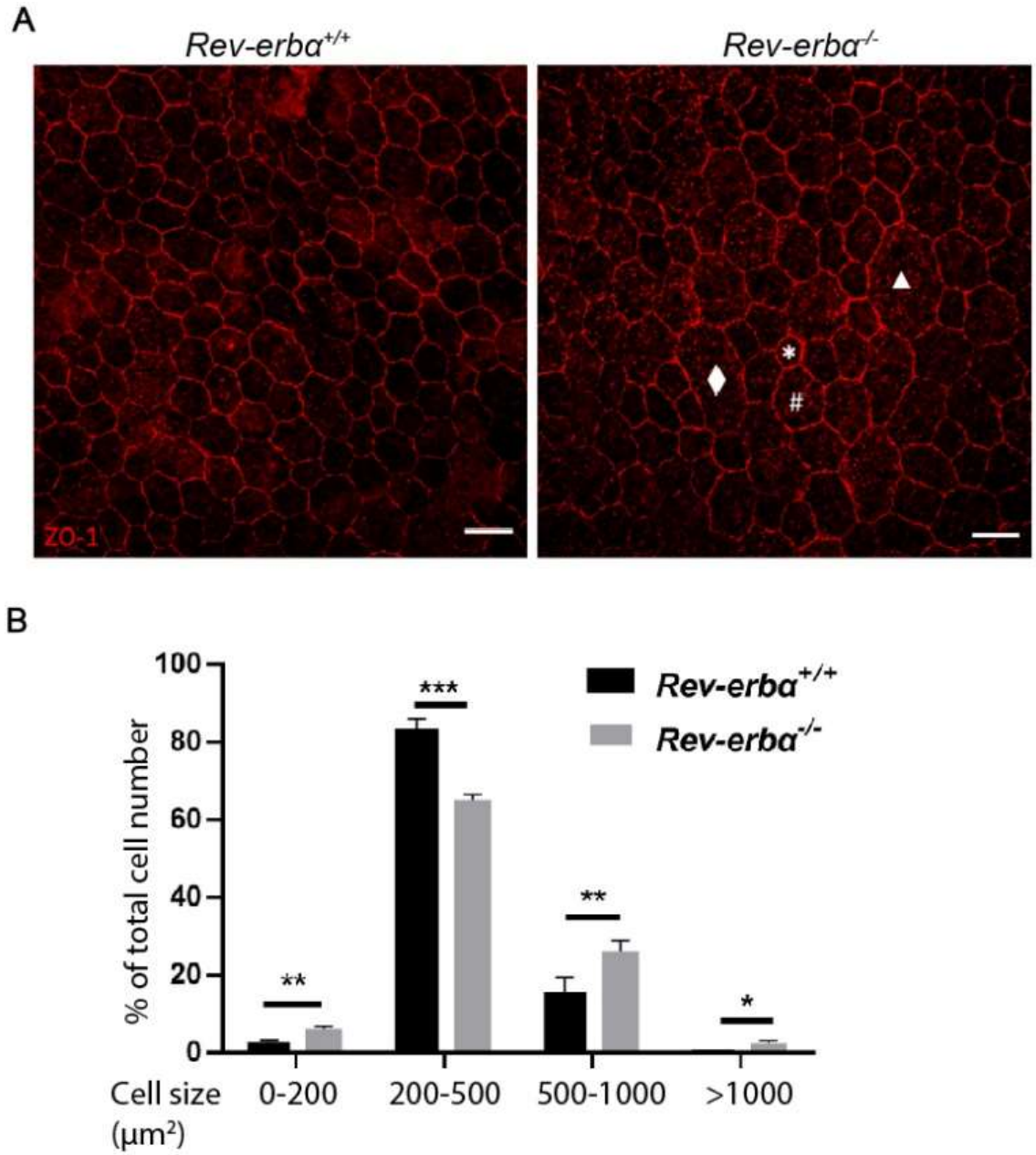

Figure S2. Decreased number of RPE cell size in aged *Rev-erbα*$^{-/-}$ RPE/choroid flat mounts. (A) Representative images of 12-month-old *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ RPE/choroid complex flat mounts immunostained with ZO-1 (Red). The asterisk (*) indicates a representative abnormally shrunk RPE cell with the size of <200μm^2; the pound (#) indicates a normal sized RPE cell with the size of 200-500μm^2; white diamond and triangle indicate abnormally enlarged RPE cells with the size of 500-1000 μm^2 and 1000> μm^2 respectively. Scale bar, 20μm. (B) Quantification of RPE cell number in four categories of RPE cell size. Error bars indicate SD. *: P<0.05, **: P<0.01, ***: P<0.001.

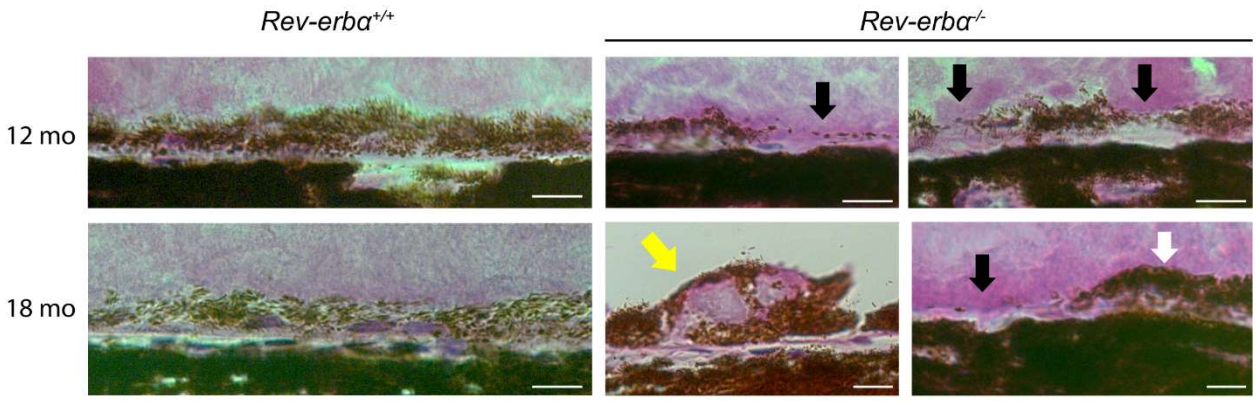

Figure S3. Histopathology of RPE degeneration and sub-retinal deposits in *Rev-erbα*$^{-/-}$ eyes. Left panels: representative images indicate normal sub-retinal layers from of H&E stained eye cross-sections from 12- and 18-month-old *Rev-erbα*$^{+/+}$ mice. Right panels: representative degenerative RPE pathologies *Rev-erbα*$^{-/-}$ mice. Black arrows indicate hypopigmented and/or discontinued RPE layers at 12, and 18 months old, yellow arrow indicates drusen-like sub-retinal deposit, and white arrow indicates hyperpigmentation in 18-month-old *Rev-erbα*$^{-/-}$ mice. Scale bar: 10 μm.

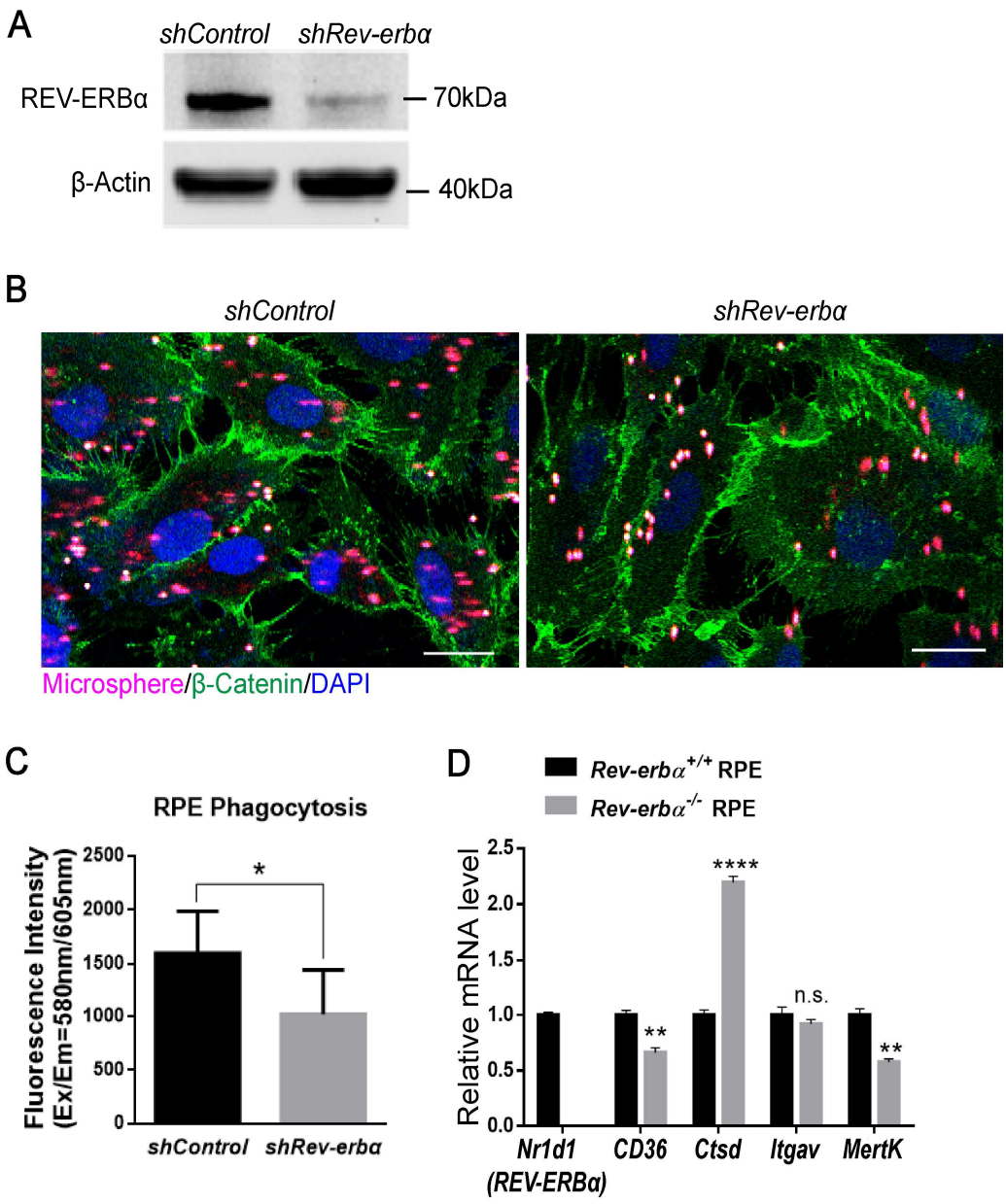

Figure S4. Inhibition of REV-ERBα reduced ARPE-19 cell phagocytic activity and altered phagocytosis-related gene expression. (A) Confirmation of REV-ERBα knockdown by *shRev-erbα* in ARPE-19 cells by Western blotting. (B) Confocal microscopy demonstrated lower levels of phagocytosis of microspheres by *shRev-erbα*-treated ARPE-19 cells compared with *shControl*-treated cells. Green: β-Catenin, Blue: DAPI, Red: fluorescent conjugated polystyrene microspheres (1 μm diameter). Scale bar: 20μm. (C) Fluorescence intensity of ingested microspheres indicative of phagocytosis was quantified using a microplate reader at 580 nm excitation and 605 nm emission. n=6/group. (D) RPE were isolated from age-matched (4 months old) *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mouse eyes, and mRNA expression were analyzed by real time qPCR. n=3/group; error bars indicate SD; *: P<0.05, **: P <0.01; **** P<0.0001. n.s.: not significant.

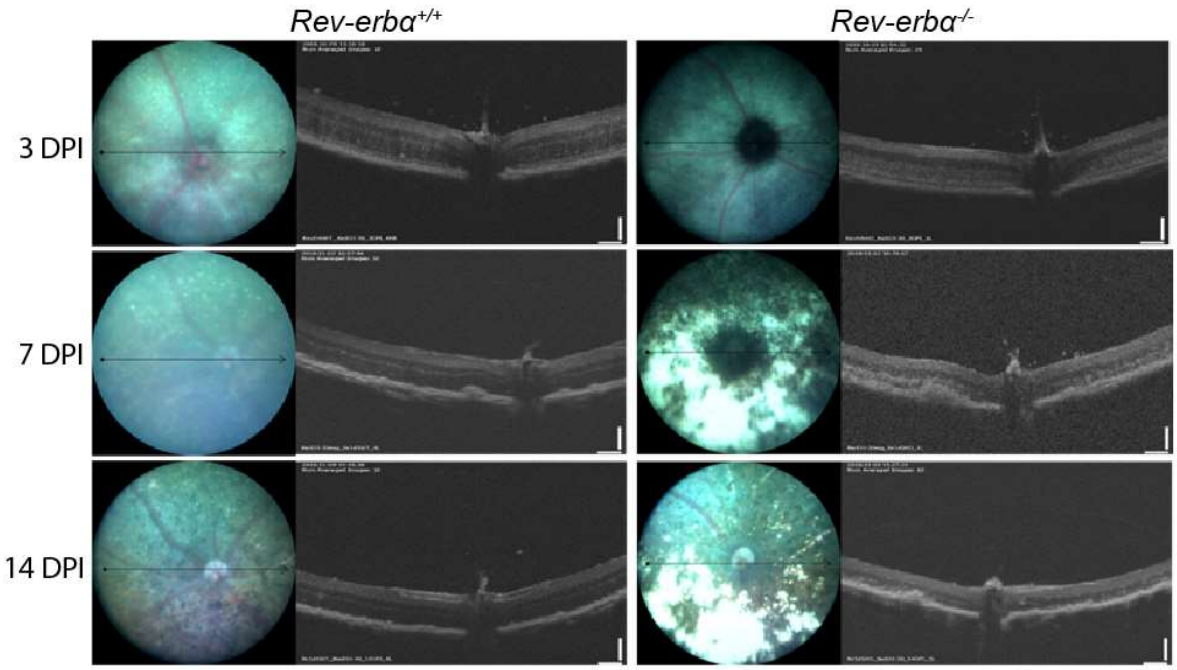

Figure S5. Representative fundus and OCT images from $NaIO_3$-treated *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice. Fundus and OCT imaging were performed on 3, 7, and 14 days post-$NaIO_3$ injection (DPI). Scale bars: 100 μm.

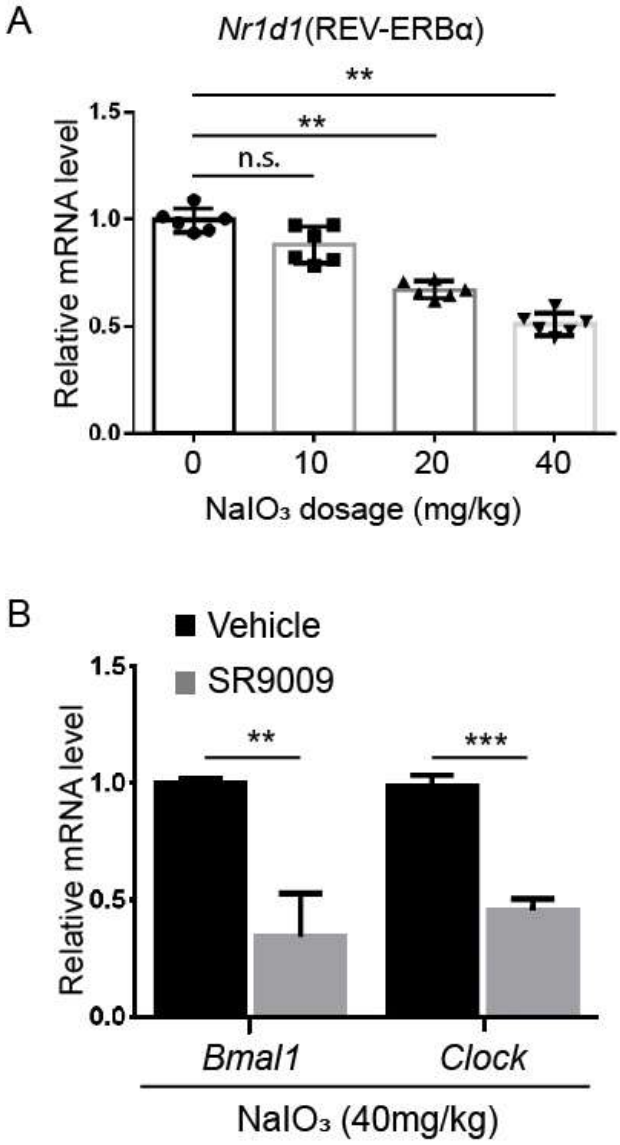

Figure S6. REV-ERBα expression was decreased under oxidative stress, but its activity was boosted by SR9009 treatment in mouse RPE. (A) Real-time qPCR results showing that mRNA expression of *Nr1d1*(REV-ERBα) in WT mouse RPE was decreased along increasing $NaIO_3$ concentration of treatment dose. qPCR results were normalized to *18S* and then normalized again to the expression level in no $NaIO_3$ treatment (0 mg/kg) group. n=6/group. (B) Real-time qPCR results showing that the expression of two known REV-ERBα target genes *Bmal1* and *Clock* was repressed in mouse RPE cells upon SR9009 treatment. qPCR results were normalized to *18S* and then normalized again to the expression levels in vehicle treated group. n=3/group. Error bars indicate SD; **: P <0.01; *** P<0.001. n.s.: not significant.

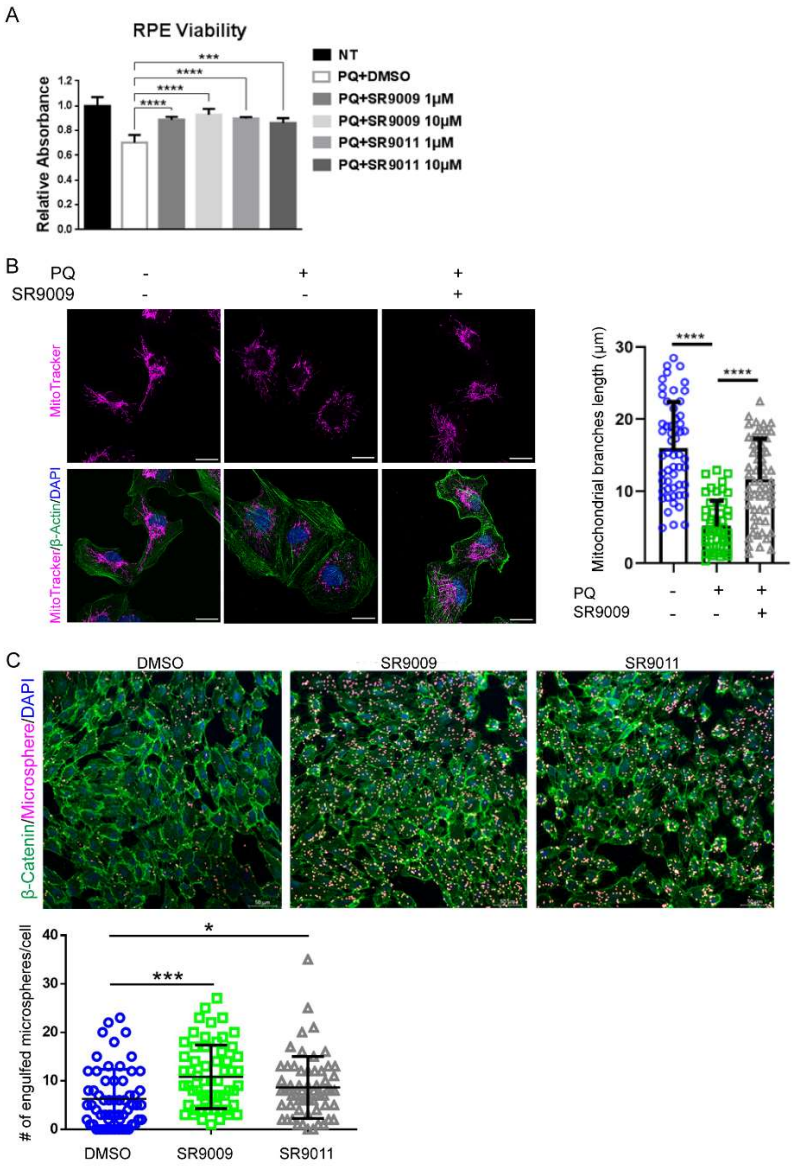

Figure S7. REV-ERBα agonists improved RPE health and function *in vitro*. (A) ARPE-19 cells were treated with PQ (0.5 mM, 2 hours) and followed by treatment with SR9011 or SR9009 (1 or 10μM, 24 hours). Cell viability was analyzed by MTT assay, n=6/group. (B) Mitochondrial morphology of ARPE-19 cells was visualized by Mitotracker staining (magenta) together with immunostaining of β-actin (green) and DAPI (blue). Cells were treated with PBS/PQ (0.5 mM, 2 hours) followed by DMSO/SR9009 treatment (10 μM, 24 hours). Average length of mitochondrial branches was quantified from 50-60 cells of each group. Scale bar, 20 μm. (C) ARPE-19 cells were treated with SR9009, SR9011 or DMSO control (10 μM each treatment, 24 hours), followed by challenge with microspheres (diameter of 1μm, at a density of 10 microspheres/cell) for 6 hours to evaluate their phagocytic activity. Microsphere, magenta; β-Catenin, green; DAPI, blue. Scale bar, 50μm. The number of engulfed microspheres per cell after REV-ERBα agonist treatment was quantified from 40-50 cells of each group. Error bars indicate SD; * P<0.05; *** P<0.001; **** P<0.0001.

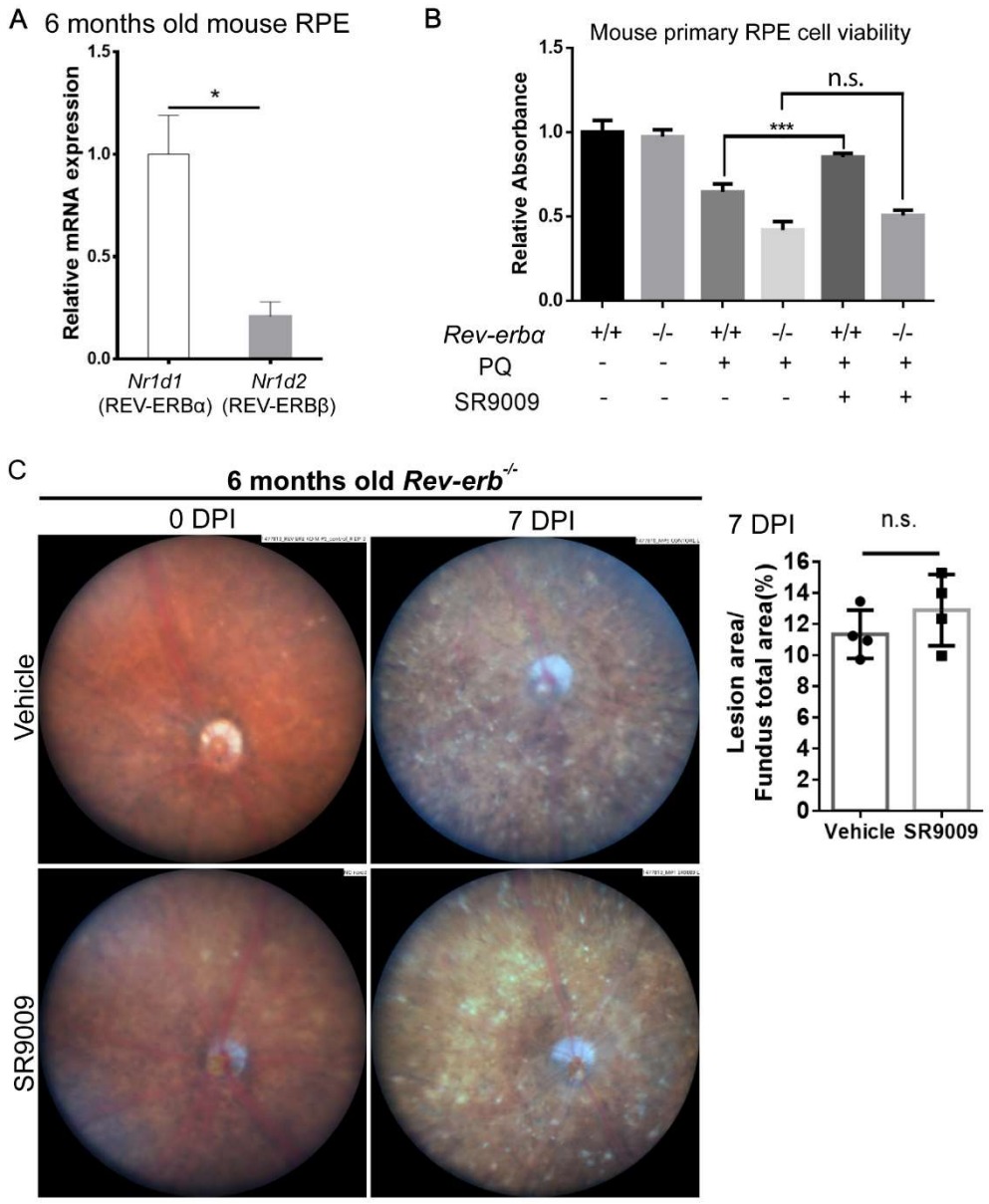

Figure S8. REV-ERB agonist SR9009 specifically target REV-ERBα in mouse RPE cells. (A) Primary mouse RPE was isolated from 6-month-old WT mice for detecting mRNA expression of *Nr1d1* (REV-ERBα) and *Nr1d2* (REV-ERBβ). Real time qPCR results were normalized to *18S* and then normalized again to the expression level of *Nr1d1*(REV-ERBα). n=3/group. (B) Primary RPE cells isolated from 8-week-old *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice were treated with either PBS or paraquat (PQ, 0.5 mM, 4 hours) and followed by treatment with vehicle/SR9009 (10 μM, 24 hours). Cell viability was analyzed by MTT assay, n=6/group. (C) Representative fundus images (0 day post 40mg/kg $NaIO_3$ injection, 0 DPI and 7 days post 40mg/kg $NaIO_3$ injection, 7 DPI) and quantification (7DPI) of lesion area from 6-month-old *Rev-erbα*$^{-/-}$ mice injected with either Vehicle or REV-ERB agonist SR9009 (100mg/kg, b.i.d.). n=4 eyes/group. Error bars indicate SD; *: P <0.05; *** P<0.001. n.s.: not significant.

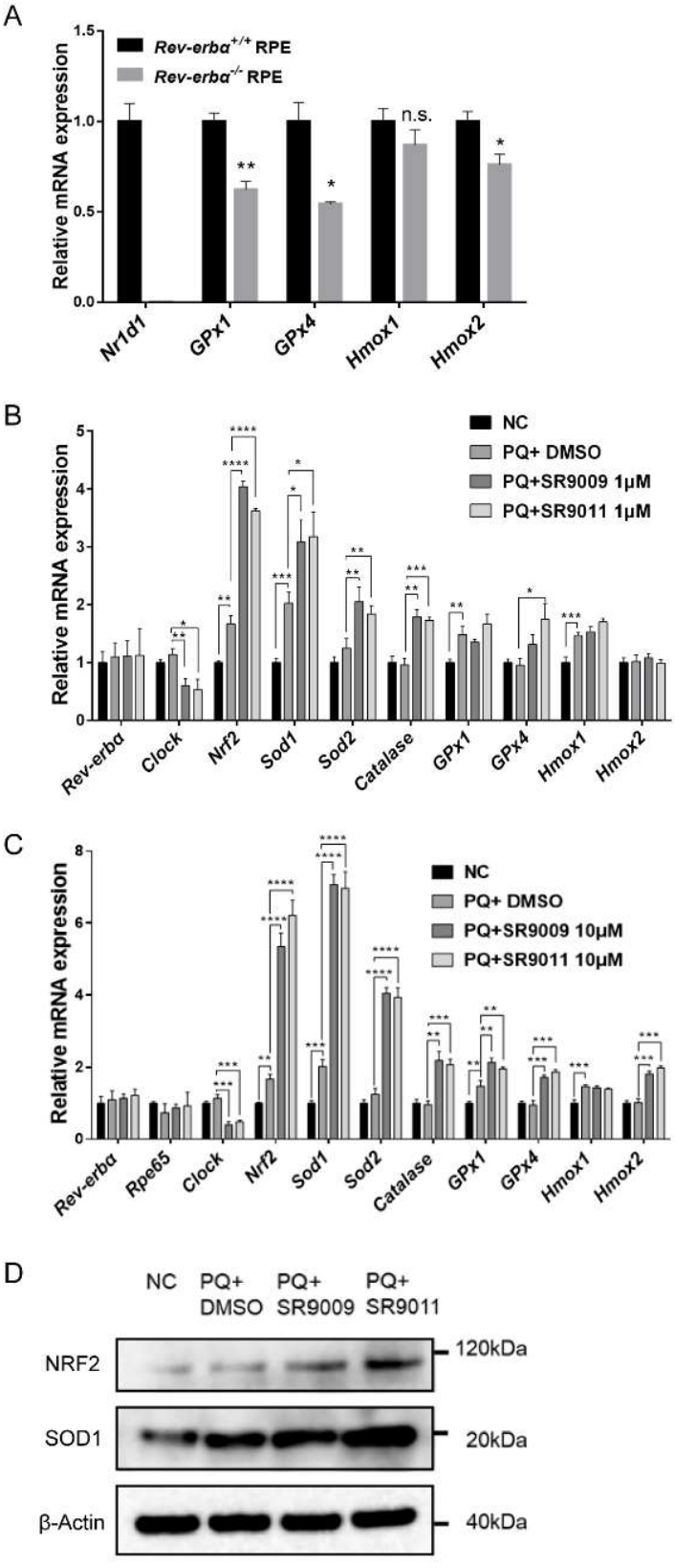

Figure S9. REV-ERBα regulates the expression of NRF2 downstream target genes in RPE cells. (A) mRNA expression of *Nr1d1* (REV-ERBα) and downstream target antioxidant genes of NRF2 (*GPx1*, *GPx4*, *Hmox1* and *Hmox2*) in *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ RPE isolated from 12-month-old mice. Real time qPCR results were normalized to *18S* and then normalized again to the expression levels in *Rev-erbα*$^{+/+}$ RPE cells. n=6/group. (B&C) ARPE-19 cells were treated with PQ (0.5 mM, 2 hours) followed by treatment with either SR9011, SR9009 or control DMSO (B: 1 μM; C:10 μM; both 24 hours). mRNA expression of oxidation-related genes was analyzed by RT-qPCR. n=3/group. (D) Protein levels of NRF2 and SOD1 were analyzed by Western blot in ARPE-19 cells after treatment with PQ (0.5 mM, 2 hours), followed by treatment with SR9009, SR9011 or DMSO control (10 μM, 24 hours). Error bars indicate SD; *: P <0.05, ** P<0.01; *** P<0.001; ****P <0.0001; n.s.: not significant.

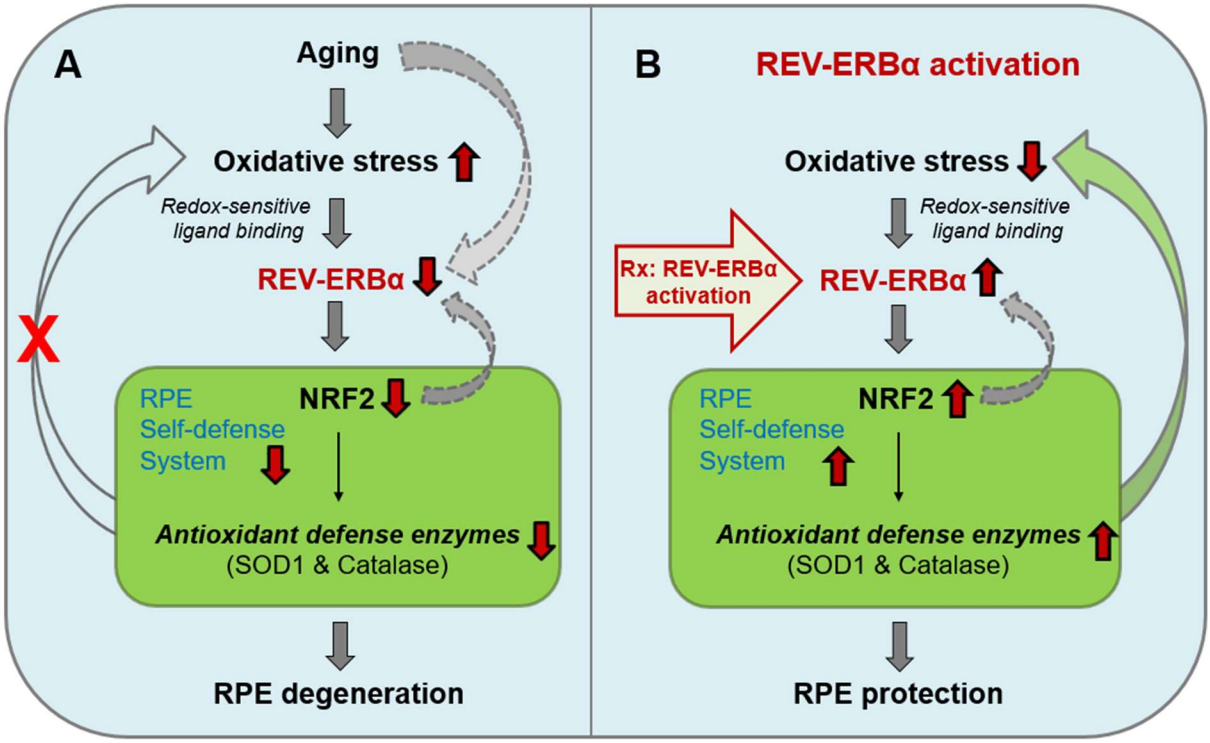

Figure S10. A proposed scheme of REV-ERBα protecting RPE against degeneration through enhancing NRF2-dependent antioxidant self-defense system. (A) REV-ERBα is a redox-sensitive ligand-dependent receptor and transcriptional factor. Aging (and resultant oxidative stress and inflammation) leads to decline of REV-ERBα in RPE. REV-ERBα deficiency causes decreased expression of NRF2, a key regulator of RPE antioxidant enzymes (SOD1 & catalase) and self-defense mechanism, thereby leading to oxidative stress damage, and exacerbated age-dependent RPE degeneration. (B) Activation of REV-ERBα represents a novel approach to enhance NRF2-mediated antioxidant enzymes and defense system and thereby protecting against RPE damage.

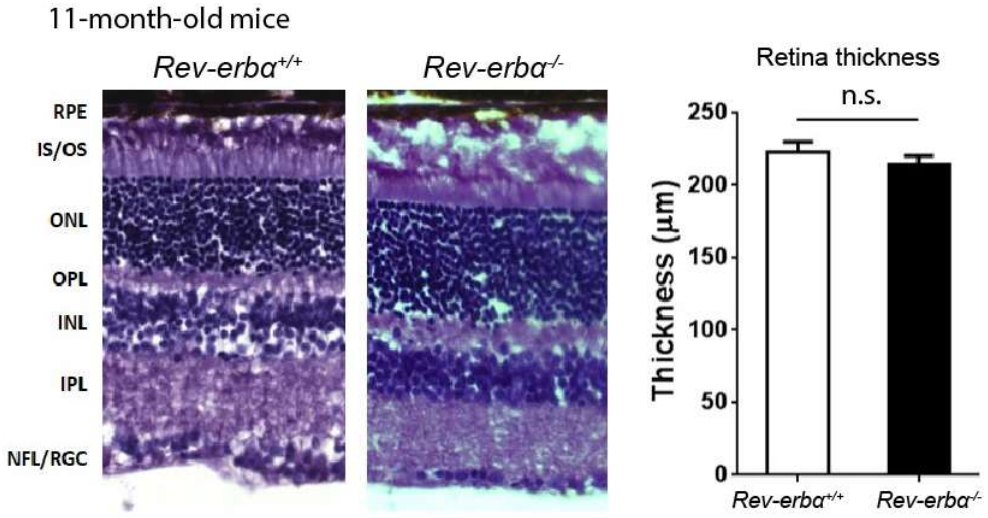

Figure S11. Retina thickness of *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ eyes remains comparable. Representative images of H&E staining of cross-sections from 11-month-old *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ retina. Retina thickness was quantified from 3 eyes in each group. Error bars indicate SD; n.s.: not significant.

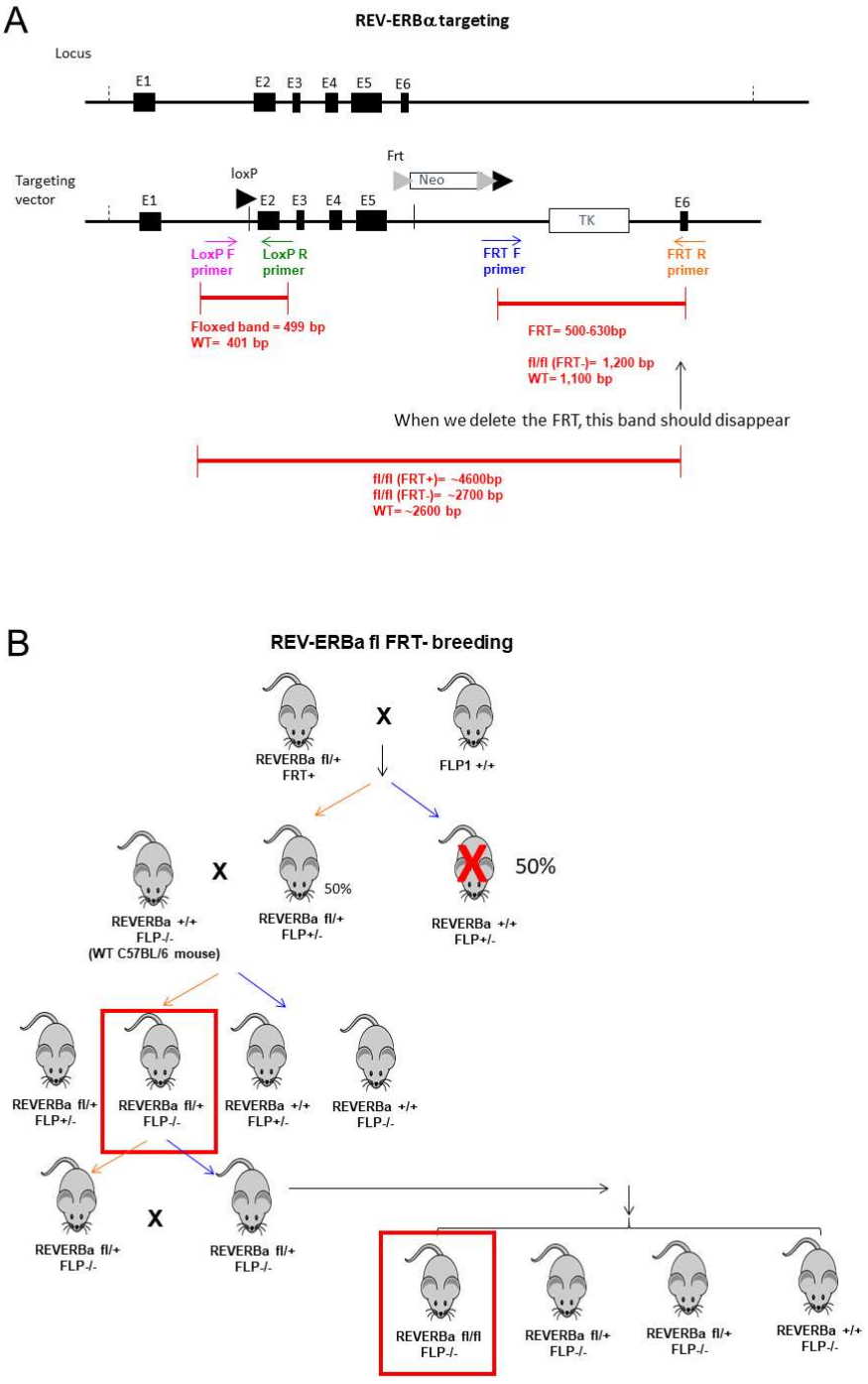

Figure S12. Strategy of *Rev-erbα* targeting and breeding to generate *Rev-erbα*$^{flox/flox}$ mice. (A) *Rev-erbα* gene locus (top) and targeting replacement vector used for electroporation and map of the genomic locus containing the *Rev-erbα*(*Nr1d1*) gene, loxP-FRT trap from exon 2 to 5, and *TK* gene (bottom). (B) Breeding scheme used to produce *Rev-erbα*$^{flox/flox}$ mice.