

SUPPLEMENTARY METHODS

Human tissue and cell lines. Primary cell cultures used included RPE cells (hRPE) isolated from human donor eyes (n=3; 15-93 years old; 1 female, 1 male, 1 gender unknown) collected by BioSight, the North Carolina Organ Donor and Eye Bank Inc., in which cells were cultured within 8 hours post-mortem, as previously described (1-3). 'Normal' donor eyes did not exhibit evidence of retinal/RPE changes upon post-mortem evaluation of the posterior pole under a dissecting microscope (Leica MZ6 Stereomicroscope, Leica Microsystems Inc. Buffalo Grove, IL, USA). Passages between 4 and 7 of the primary RPE cell cultures were used for the in vitro studies. Expression of RPE specific genes *BEST1* (Bestrophin 1), and *RPE65* (retinoid isomerohydrolase RPE65; Table S2), were confirmed in our cell lines prior to use (4). RF/6A cells, a spontaneously transformed choroidal endothelial cell line (CEC) derived from the eyes of a rhesus macaque fetus, passages 35–40, and ARPE19 cells, a spontaneously arising human RPE cell line derived from the eyes of a 19 year-old male donor, passages 21–28, were obtained from ATCC (Manassas, VA, USA) and used in preliminary studies. Final conditions of both ARPE19 and the primary RPE cell cultures were such that cells were post-confluent and demonstrated zonula occludens immunoreactivity (1-3, 5). Additionally, freshly isolated human donor RPE (n = 31; aged 18-94 years) were used for *LXR* expression studies (Table S1). For histological studies, donor eyes were retrieved from patients diagnosed with dry AMD through the Alabama Eye Bank and BioSight (n=9). Death to preservation time was less than 3 hours. Following enucleation, eyes were fixed by immersion in 4% paraformaldehyde. Approximately 16 hours later the anterior segments were removed, posterior poles were embedded in paraffin, and sectioned at 5 μ m.

RNA isolation and qPCR. Total RNA isolated from cultured cells, freshly isolated human RPE cells, and choroid, along with RNA quality assessment, cDNA reverse transcription, and qPCR, were

completed as previously described (1-3, 5). Donor demographics are listed in Table S1. qPCR was performed using the Bio-Rad CFX96 Realtime PCR Detection System (Bio-Rad). Melting curves for each pair of primers were inspected to confirm a single amplicon. The Ct values were normalized to the housekeeping gene, acidic ribosomal phosphoprotein P0, *36B4*. Gene expression fold changes were calculated using the $\Delta\Delta CT$ method. Primer sequences were selected from Primer Bank, <http://pga.mgh.harvard.edu/primerbank> (Table S2). The amplification products of *NR1H3*, *NR1H2*, *RXR α* , *RXR β* and *ACTB* primers obtained after qPCR were run on a 1% agarose gel and visualized with ethidium bromide.

siRNA transfection and transcriptional activity assay. siRNAs were used to knock down *NR1H3* and *NR1H2* expression *in vitro*. A control siRNA (siC) for a non-targeting sequence or 4 different *NR1H3* and *NR1H2* siRNAs (si*NR1H3*-1, si*NR1H3*-2, si*NR1H3*-3, si*NR1H3*-4, si*NR1H2*-1, si*NR1H2*-2, si*NR1H2*-3, si*NR1H2*-4; GE Dharmacon, Lafayette, CO, USA; Table S6) were transfected into hRPE or RF/6A cells using Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY, USA) and tested to account for knockdown efficiency (Figure S20). Briefly, complexes of siRNA (100 pmoles per 250,000 cells) and RNAiMAX in OPTI-MEM were added to six well cell culture plates according to the manufacturer's protocol. si*NR1H3*-1 and si*NR1H2*-3 for hRPE (Figure S20A), while si*NR1H3*-2 and si*NR1H2*-1 for RF/6A cells (Figure S20B) were found to be most effective at reducing LXR expression and used in subsequent experiments. Cells were used 24 hours post-transfection, in LXR activity assays. The transcriptional activity of *NR1H3* and *NR1H2* was measured using a luciferase-based reporter assay and *LXR* target gene expressions (Table S2) were quantified using qPCR. Briefly, 50,000 cells/well were seeded in 24-well plates in phenol red-free medium supplemented with 7.5% charcoal-stripped fetal bovine serum and cultured overnight. Lipofectin (Invitrogen)-mediated transfection was performed the following day, using plasmids

encoding the LXR-DR4–luciferase reporter, CMV- β -galactosidase and pBSII. The next day, cells were treated with LXR ligands (Table S3). Cells were lysed 24 hours later to measure luminescence. Luciferase (reporter) and β -galactosidase [chlorophenol red β -D-galactopyranoside (CPRG) as substrate; transfection normalization] activities were measured using a SpectraMax M5 spectrophotometer (Molecular Devices, San Jose, CA). Concomitantly, cells were treated with LXR ligands (Table S3) for RNA isolation and target gene expression studies. RNA was extracted at the indicated time points for quantitative real-time PCR (qPCR).

JC-1 assay. hRPE cells were plated in 96-well plates (20,000 cells/well). After 24 hours, cells were starved in DMEM/F12 1% FBS overnight. The cells were pre-treated with LXR ligands for 24 hours followed by treatment with oxidant injury-inducing agents and lipids (Table S3). Twenty four hours later, cells were stained with JC-1 dye (Sigma-Aldrich, Saint Louis, MO), which normally concentrates in the mitochondrial matrix forming red fluorescent aggregates, while dissipation of mitochondrial membrane potential prevents aggregate formation and leads to formation of green monomers, according to the manufacturer's protocol. The results were reported as the ratio of mitochondrial membrane permeable JC-1 monomers/J-aggregates as a measure of mitochondrial membrane potential gradient loss. For imaging, 15000 cells/chamber were plated on the 8-chamber culture slides (Falcon, Big Flats, NY). It was followed by starvation, pre-treatment with LXR ligands, treatment with oxidant-injury inducing agents and JC-1 staining as mentioned above. Cells were imaged using a Nikon C2si confocal microscope, visualized and processed using Nikon Elements software. Valinomycin was used as a positive control to dissipate mitochondrial membrane potential.

AdipoRed™ assay. hRPE cells were cultured, treated as outlined above, and stained with Adipored™ reagent (Lonza, Walkersville, MD) according to manufacturer's protocol. The results were reported as relative fluorescence (excitation: 485nm, emission: 572nm).

Cell viability. Cell viability was measured using CellTiter-Blue® (Promega, Madison, WI), according to the manufacturer's protocol. The results were reported as relative fluorescence (excitation: 560 nm, emission: 590 nm).

Animals. Male and female *Nr1h3/Nr1h2^{+/+}* (*WT*), *Nr1h3^{-/-}*, *Nr1h2^{-/-}*, *Nr1h3^{-/-}/Nr1h2^{-/-}* mice on the C57BL/6J background were maintained in a temperature (25 °C) and light controlled (12h light/12 h dark) environment and provided standard mouse chow ad libitum. Mice were backcrossed two times. Mice were aged to 10-14 months and screened for the confounding retinal degeneration 8 mutation and its absence was confirmed as previously described (1). The knockdown of *Nr1h3* was confirmed in RPE-choroid tissue by RT-PCR (Figure S7 and S19).

In vivo imaging. A Micron IV (Phoenix Research Laboratories Inc, Pleasanton, CA) retinal imaging microscope was used for in vivo imaging. Fundus images were obtained, following dilation, from anesthetized 10-14 month old *WT* (n=4), *Nr1h3^{-/-}* (n=5), *Nr1h2^{-/-}* (n=3), *Nr1h3^{-/-}/Nr1h2^{-/-}* (n=4) mice, as described previously (1, 6). The spectral domain optical coherence tomography (OCT) module of the Micron IV was used to image retinal layers guided from the bright field.

Electroretinography (ERG). ERGs were recorded using the Espion E² system (Diagnosys LLC) as described previously (1, 2). Briefly, 10-14 month old *WT* (n = 15), *Nr1h3^{-/-}* (n = 11), *Nr1h2^{-/-}* (n = 11), *Nr1h3^{-/-}/Nr1h2^{-/-}* (n = 2) mice were dark-adapted for four hours and anesthetized by an intraperitoneal injection of a ketamine/xylazine mixture (85/10 mg/kg). Pupils were dilated with 1% cyclopentolate-HCL and 2.5% phenylephrine, and the mouse body temperature was maintained at 37 °C using a water-based warming pad. ERG responses under dark-adapted ("scotopic") conditions

were evoked by a series of nine flashes ranging from 0.0001 cd·s/m² to 100 cd·s/m². For flashes up to 0.1 cd·s/m², responses of 10 trials were averaged. For 0.5 and 1 cd·s/m² flash responses, three trials were averaged. For brighter stimuli, responses to single flashes were recorded without averaging. Light-adapted (“photopic”) ERGs were evoked by a series of six flashes ranging from 0.2 cd·s/m² to 2,000 cd·s/m² whereas rod inputs were suppressed with a steady background illumination of 50 cd/m². Up to 10 trials were averaged for all flash responses. Analysis of a- and b-wave amplitudes was performed as described (1, 2). For c-waves, flash response (5 cd·s/m² to 100 cd·s/m²) were recorded for 7 seconds.

Conventional transmission electron microscopy (cTEM). Enucleated eyes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) overnight at 4 °C (1, 2). The cornea and lens were removed and the posterior pole was washed in cacodylate buffer, incubated in osmium tetroxide for 1 h, dehydrated in a series of increasing ethanol concentrations, and embedded in Epon. The morphology of retina/RPE/choroid was examined by light microscopy in 1 µm thick sections in the nasal-temporal orientation at the optic nerve, counterstained with toluidine blue. The thickness of the retinal layers was measured using the Adobe Photoshop CC ruler tool at 100, 200, 300, 400 and 500 µm distances from the optic nerve head in both the nasal and temporal directions, and the results were graphed in a spider diagram (*WT* n = 2, *Nr1h3*^{-/-} n = 3). Ultrastructural analysis was performed by cTEM in a minimum of three sections from *WT* (n = 6), *Nr1h3*^{-/-} (n = 10), *Nr1h2*^{-/-} (n = 6), *Nr1h3*^{-/-}/*Nr1h2*^{-/-} (n = 4) (1, 2).

Quick freeze/deep etch (QFDE) TEM. Enucleated eyes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) overnight at 4 °C. Prior to QFDE tissue processing, eyes were dissected into sections approximately 1 mm x 1 mm in size following additional fixation until needed. Sections were mounted on laponite clay and “touch” frozen with a custom instrument on a liquid nitrogen

cooled copper block, processed using a custom modified Cressington CFE-40 freeze fracture system (Cressington, Watford, England, UK), and imaged by TEM (JEOL JEM1010; JEOL USA, Inc. Peabody, MA) as previously described (7).

Dark Adaptation ERG. Dark adaptation was performed as previously described (8, 9). Briefly, mice were dark adapted overnight and anesthetized with 86.9 mg/kg ketamine and 13.4 mg/kg xylazine under red light. Body temperature was maintained at 37°C by heating pad. Atropine sulfate 1% ophthalmic solution (Bausch & Lomb; Bridgewater, NJ) was used to dilate the pupils. Contact lens electrodes were placed bilaterally with appropriate reference and ground electrodes. AUTAS BigShot System (LKC Technologies Inc.; Gaithersburg, MD) was used to acquire scotopic a-waves. Eyes were exposed to a bright (150,000 lux) LED light for 15 seconds to photobleach an estimated 90% of the visual pigment. ERG response was monitored at fixed time points for up to 100 minutes post-bleach to monitor response recovery. Raw data was processed using MATLAB software (Math Works; Natick, MA). The sensitivity for each eye was calculated as the ratio of dim flash response amplitude and the corresponding flash intensity in the linear range of the intensity-response curve, and normalized to its dark adapted, pre-bleach level.

Blood collection and post-mortem tissue analyses. Mouse plasma was isolated and low density lipoprotein (LDL) and high density lipoprotein (HDL) levels were measured using a cholesterol assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's protocols. Eyes were enucleated and processed for histology, immunohistochemistry, lipofuscin assessment, conventional transmission electron microscopy, quick-freeze deep etch electron microscopy, and cytokine analyses as described below.

Cytokine analysis. Blood plasma was collected and the RPE/choroid complex was isolated from the posterior eyecups of *WT* and *Nr1h3^{-/-}* (n = 4) mice and homogenized in RIPA buffer, as previously

described (1, 6). Protein quantitation was performed using BCA assay and used to normalize the amount of protein loaded onto each Mouse Cytokine Array C3 membrane (RayBiotech, Norcross, GA). Each group had two technical and four biological replicates. The manufacturer's protocol was followed for processing the membranes. Images of the membranes were inverted to neutralize the effect of any differences in background, and dot intensity was quantified using ImageJ. Since each antibody on the membrane was blotted in duplicate, the mean intensity of the dots was calculated, averaged across the biological replicates and normalized to the vehicle.

RNA isolation and qPCR from mouse tissue. Total RNA was isolated from RPE-choroid and retina of 3-5-month-old *WT* and *Nr1h3*^{-/-} mice followed by RNA quality assessment, cDNA reverse transcription, and qPCR. The mouse primers are listed in Table S2.

Autofluorescence quantification. A Leica Spectral Laser Scanning Confocal Microscope was used to measure autofluorescence throughout the entire RPE layer of cryosections ($n = 2$ measurements in $n = 5$ eyes per genotype) as previously described (1, 2, 10). Lambda (λ) scans were performed using a 405 nm laser. Excitation and emission frequencies were measured with a 5 nm wide band through a spectral range from 422.5 nm to 722.5 nm using serial 30-image scans at ~ 10.3 nm intervals. Fluorescent intensities are represented as arbitrary units as defined by the confocal Leica software.

Histochemistry, immunohistochemistry of retinal cross-sections, and RPE/choroid flat mount analyses. The posterior poles of human eyes were paraffin embedded and sectioned for IHC. Mouse eyes were fixed in 4% paraformaldehyde and cryopreserved. Following removal of the anterior segment, specimens were sectioned from the superior cup through the optic nerve to the inferior cup in 10 μ m increments. Cryosections from the peripheral and central regions of the eye were stained with Oil Red O, as previously described, to visualize neutral lipids (11, 12), or probed with

antibodies (Table S4). Non-specific immunostaining in sections was blocked with normal serum (Jackson ImmunoResearch, West Grove, PA, USA) appropriate to the secondary antibody species. Secondary antibodies were conjugated to horseradish peroxidase (HRP) for immunohistochemistry or AlexaFluor 568 or 488 (Invitrogen) for immunofluorescence. Control slides containing sequential sections were probed with non-immune serum and buffer without primary antibody. RPE/choroid flat mounts were prepared from enucleated eyes as described (13), and stained with phalloidin–tetramethylrhodamine B isothiocyanate and F4/80 (Table S4) to visualize RPE cell borders and immune cells, respectively. Nuclei were stained with Hoechst 33258 (Invitrogen). Images were collected on a Nikon C2si confocal microscope, visualized and processed using Nikon Elements software to display maximum intensity projection. Images from each quadrant of the stained RPE–choroid flat mounts were stitched using Adobe Photoshop CC to obtain a complete image and ADGRE1⁺ cells were counted per flat mount (*WT*: n = 5, *Nr1h3*^{-/-}: n = 8).

ApoB100 mice and drug treatment. Mice which express apoB100 (apoB100 mice, genetic background: 50% C57BL/6 and 50% 129/Sv) (14) were used to test the LXR agonist, GW3965 (Duke Small Molecule Synthesis Facility), as a therapeutic agent. Three month old apoB100 mice (n = 7 per treatment group) were fed either low fat diet pellets [LFD; TD.150777; Purified Control Diet (10% Fat Kcal)] or GW3965 pellets [(LFD + GW); TD.150778; GW3965 Diet 120 mg/kg; 10/Fat)] manufactured by Envigo, for a period of 5 months, ad libitum. Mouse weights were monitored monthly. Fundus and OCT imaging was performed before and after the treatment period. After 5 months, mouse tissues (eyes and blood) were harvested and processed. Posterior poles were subjected to paraformaldehyde fixation, cryosectioned and stained with antibodies to GFAP, ADGRE1 and APOE (Table S4). Plasma LDL and HDL levels were measured using a cholesterol assay kit according to the manufacturer's protocols. Retinal cross sections stained with an antibody

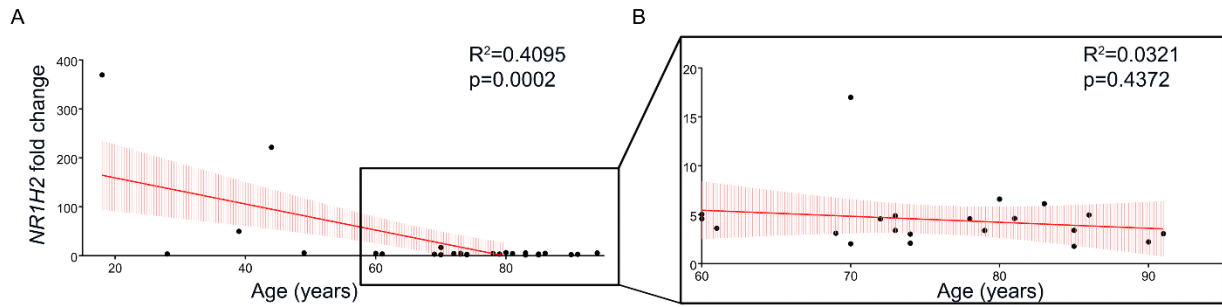
to F4/80 were evaluated and the number of immunopositive cells present in the sub-retinal space were counted (n = 9 sections per eye, n = 3 eyes per group) and plotted. APOE immunoreactivity in slides were quantified by measuring the length of the APOE⁺ Bruch's membrane (BrM) and plotting it as a ratio to the total length of the BrM in the section (n = 6 sections per eye, n = 3 eye per group).

References:

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SUPPLEMENTARY FIGURES

**Figure S1.**

***NR1H2* expression in human RPE cells decreases with age.** (A) Expression of *NR1H2* in RPE cells isolated post-mortem from donors (18 to 94 years old, fold change relative to ARPE19 cells, $n = 28$, Pearson R squared was calculated, $p = 0.0002$). (B) Expression of *NR1H2* in RPE cells isolated post-mortem from donors (60 to 94 years old, fold change relative to ARPE19 cells, $n = 23$, Pearson R squared was calculated, $p = 0.4372$).

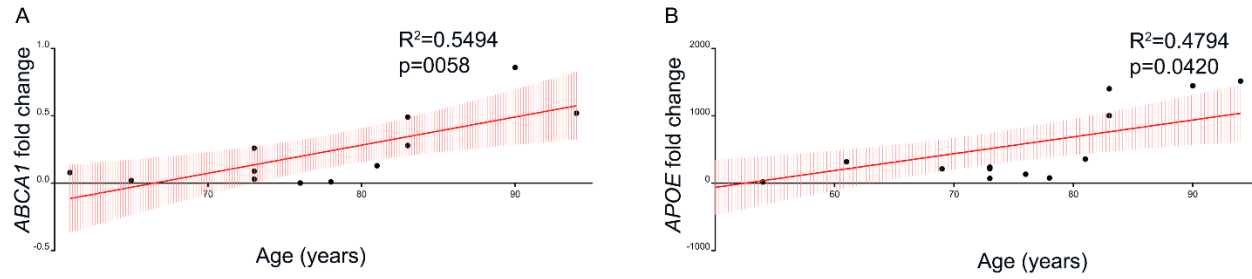


Figure S2.

***ABCA1* and *APOE* expression in human RPE cells displays a positive correlation with age.**

(A) Expression of *ABCA1* in RPE cells isolated post-mortem from donors (61 to 94 year old, fold change relative to ARPE19, $n = 12$, Pearson R squared was calculated, $p = 0.0058$). (B) Expression of *APOE* in RPE cells isolated post-mortem from donors (47 to 94 year old, fold change relative to ARPE19, $n = 15$, Pearson R squared was calculated, $p = 0.0420$).

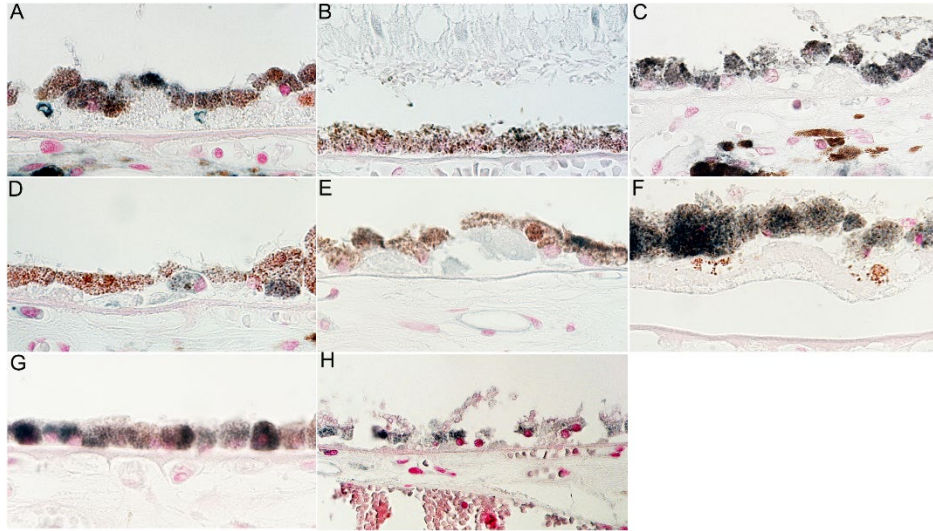


Figure S3.

NR1H3 staining in AMD donor eyes. Multiple examples of NR1H3 (12HCLC) staining (dark grey) pattern in RPE of AMD donor sections (A-H).

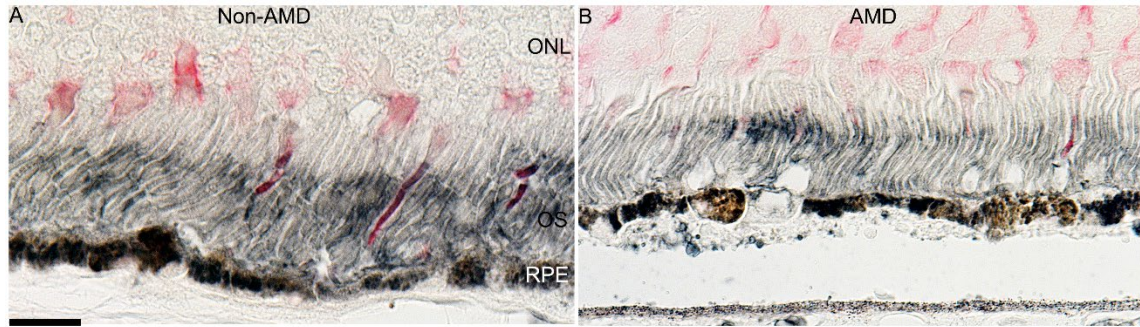


Figure S4.

NR1H3 staining in RPE and cone segments in AMD donor eyes. NR1H3 (K8607) (dark grey) and cone (R/G Opsin) (red) staining in retina/RPE/choroid complex of age-matched non-AMD (A) and AMD (B) donor sections. (Scale bar = 25 μ m) (RPE: retinal pigment epithelium, OS: outer segments, ONL: outer nuclear layer). Representative images are shown. Total number of eyes probed with antibodies ($n = 2 - 3$ from age-matched non-effected and AMD donors, respectively).

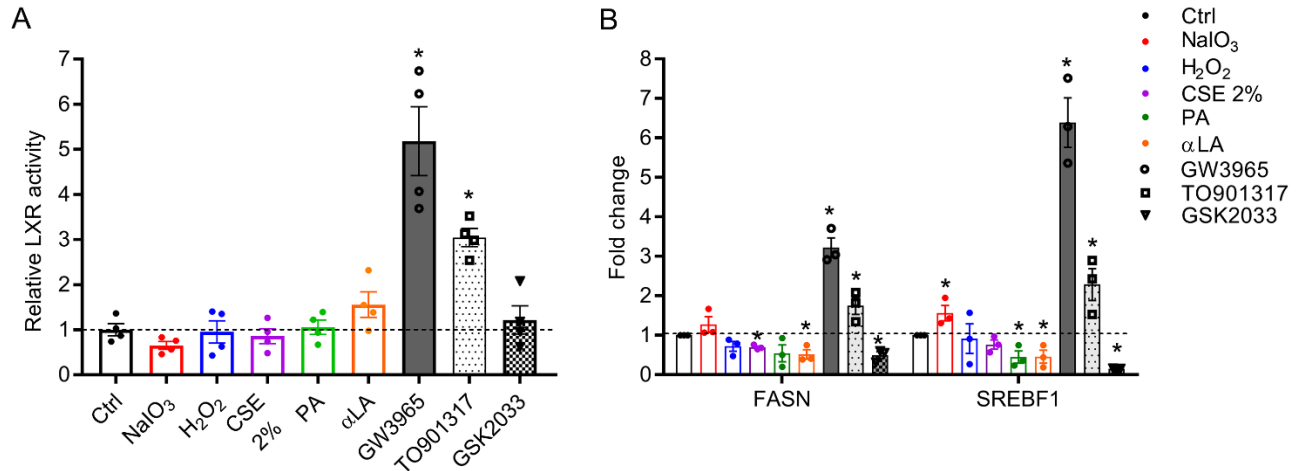


Figure S5.

Effect of oxidant injury and lipid on LXR activation. Human primary RPE cells from a 93 year old donor were treated with oxidant injury agents, lipids, LXR agonists and an antagonist. (A) LXR activation was measured by luciferase assay. DMSO and ethanol were used as vehicle controls. (B) Expression of *FASN* and *SREBF1* (LXR target genes) was measured. NaIO₃: sodium iodate, H₂O₂: hydrogen peroxide, CSE: cigarette smoke extract, PA: Palmitic acid, αLA: alpha Linolenic acid. n = 3, Mean + SEM, *: p < 0.05 relative to vehicle control, 1-way ANOVA and Dunnett's multiple comparisons test.

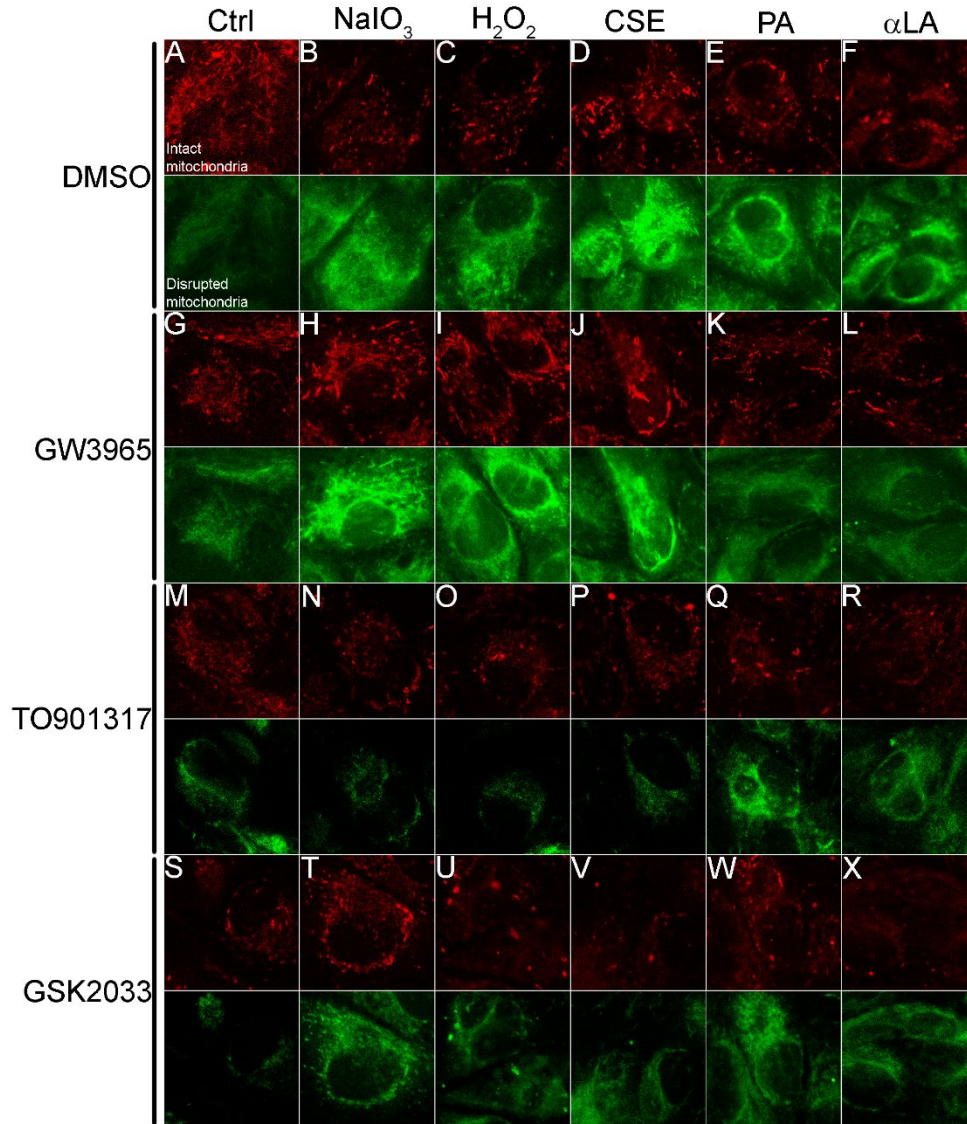


Figure S6.

LXR activation differentially ameliorates oxidant injury and lipid overload in a ligand-dependent manner. Human RPE cells were pretreated with LXR agonists and antagonists followed by oxidant injury and/or lipid overload and the effect of LXR activation on mitochondrial membrane potential was measured by exposure to the JC-1 dye, in which intact mitochondria are stained red, whereas the disrupted mitochondria are stained green. NaIO₃: sodium iodate, H₂O₂:

hydrogen peroxide, CSE: cigarette smoke extract, PA: Palmitic acid, α LA: alpha Linolenic acid.

Representative images from a total of 3 experiments shown.

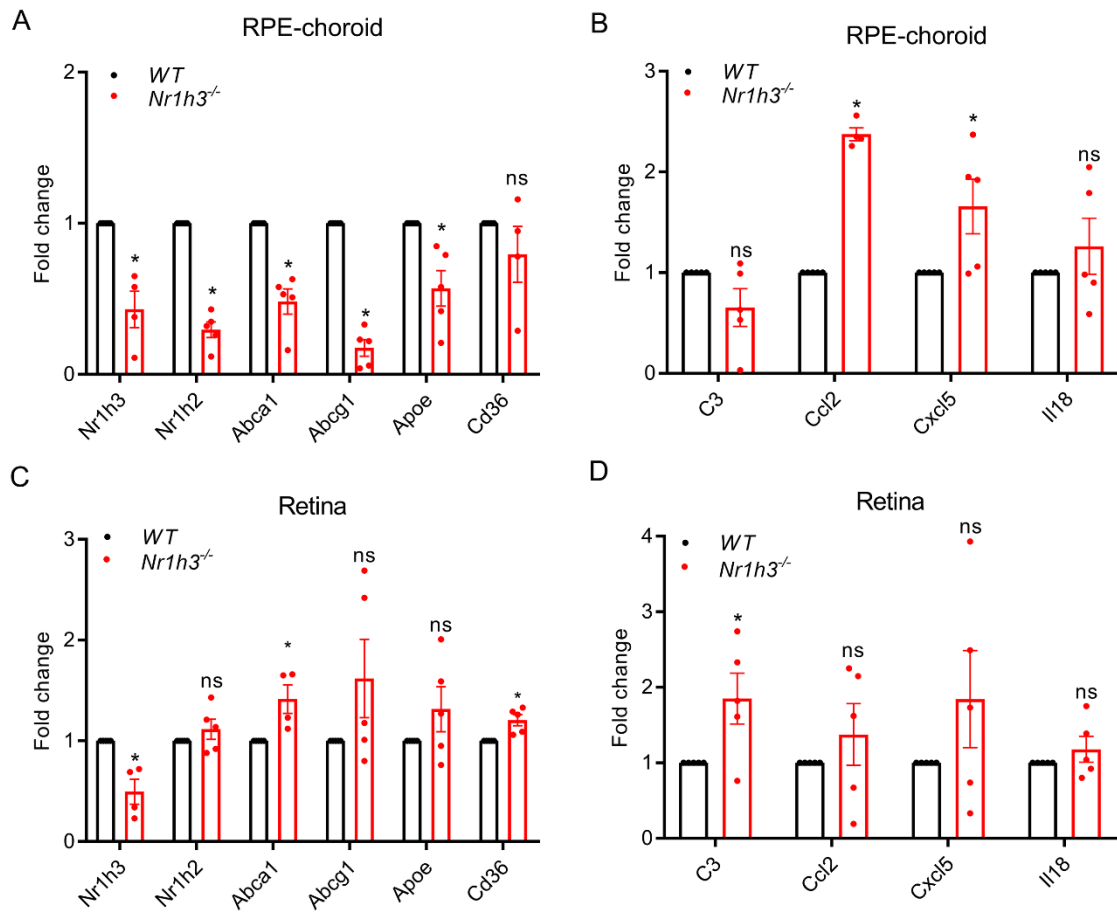


Figure S7.

Expression of *Nr1h3* and target genes is downregulated in 10 to 14 month old *Nr1h3*^{-/-} mice.

mRNA expression of *Nr1h3*, *Nr1h2*, *Abca1*, *Apoe* and *Cd36* in RPE-choroid (A) and retina (C) of 10 to 12 month old *WT* and *Nr1h3*^{-/-} mice (n = 5, Mean ± SEM, *: p < 0.05, Multiple t-tests).

mRNA expression of *C3*, *Ccl2*, *Cxcl5* and *Il18* in RPE-choroid (B) and retina (D) of 10 to 12 month old *WT* and *Nr1h3*^{-/-} mice (n = 5, Mean ± SEM, *: p < 0.05, Multiple t-tests).

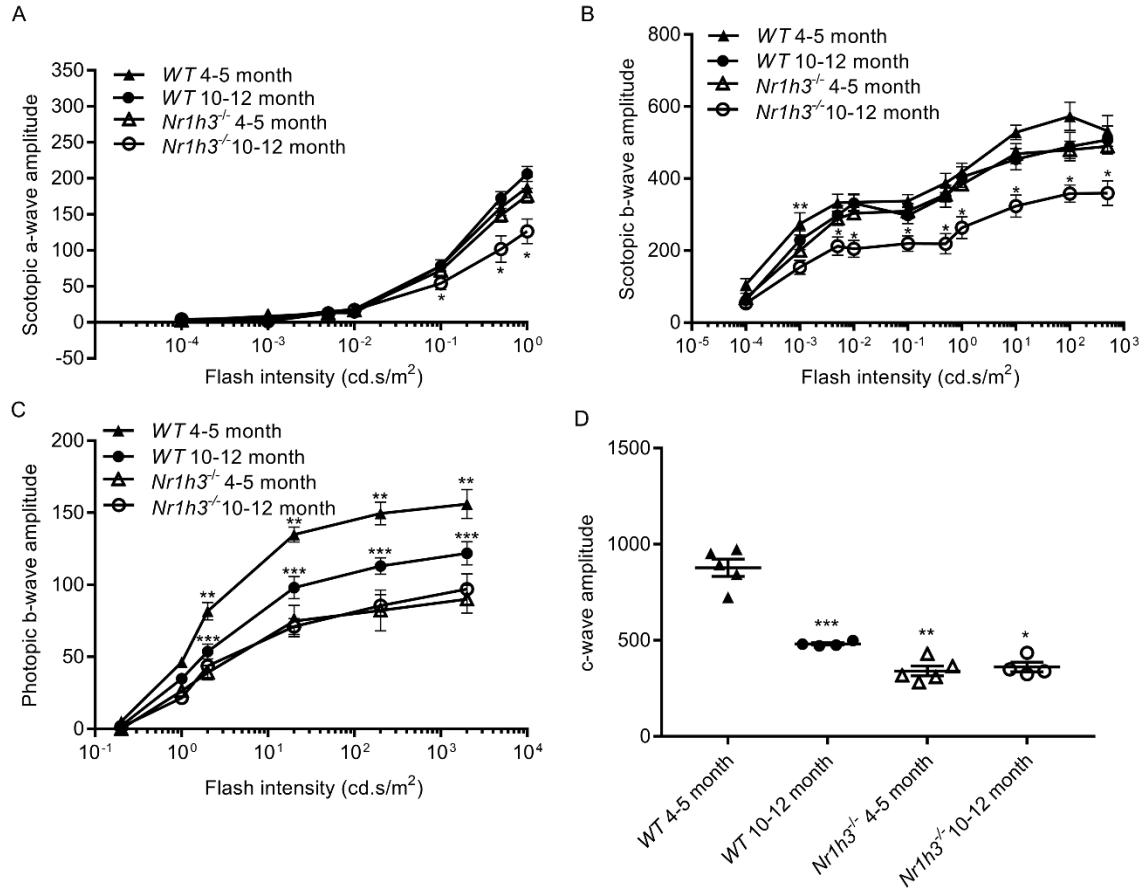


Figure S8.

***Nr1h3*^{-/-} mice display age-dependent decline in visual function.** Averaged ERG responses from 4 to 5 month old (n = 6) and 10 to 12 month old (n = 7) dark-adapted *WT* and *Nr1h3*^{-/-} mice. Plots of (A) scotopic a-wave amplitudes, (B) scotopic b-wave amplitudes, and (C) photopic a-wave amplitudes as a function of flash intensity are shown (Mean ± SEM, Multiple t-tests, *: *Nr1h3*^{-/-} young versus old, $p < 0.01$; **: *WT* young versus *Nr1h3*^{-/-} young, $p < 0.01$; ***: *WT* young versus old, $p < 0.01$). (D) Plot of c-wave amplitudes. (Mean ± SEM, 1-way ANOVA, Dunnett's multiple comparisons test, *: *Nr1h3*^{-/-} young versus old, $p < 0.01$; **: *WT* young versus *Nr1h3*^{-/-} young, $p < 0.01$; ***: *WT* young versus old, $p < 0.01$).

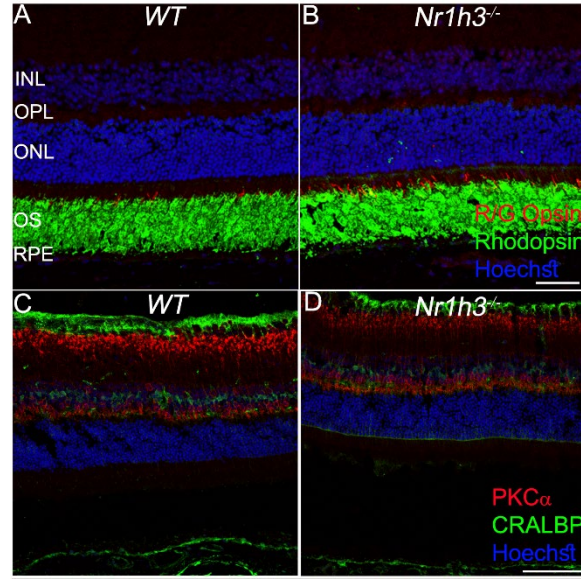


Figure S9.

Retinal markers in mice with genetic ablation of *LXRα*. Rhodopsin (green) and R/G Opsin (red) staining in 10 to 14 month old (A) *WT* and (B) *Nr1h3*^{-/-} mice. Representative images are shown (n = 3 per group; Scale bar = 50 μM). CRALBP (green) and PKCα (red) staining in 10 to 14 month old (C) *WT* and (D) *Nr1h3*^{-/-} mice. Representative images are shown (n = 3 per group; Scale bar = 20 μM).

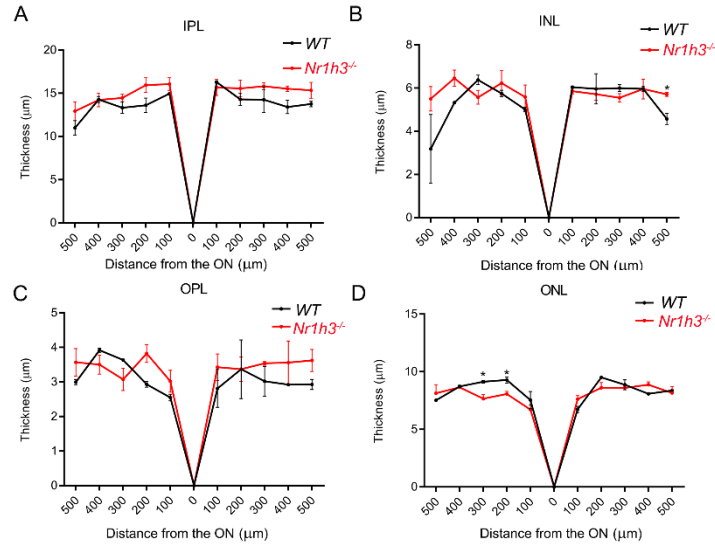


Figure S10.

Thickness of retinal layers in mice with genetic ablation of *Nr1h3*. Spider diagrams depicting the thickness of (A) inner plexiform layer (IPL), (B) inner nuclear layer (INL), (C) outer plexiform layer (OPL) and (D) outer nuclear layer (ONL) in retinal sections from 10 to 14 month old *WT* (n = 2) and *Nr1h3*^{-/-} (n = 3) mice (Mean ± SEM, *, p < 0.05, Multiple t tests, one per row).

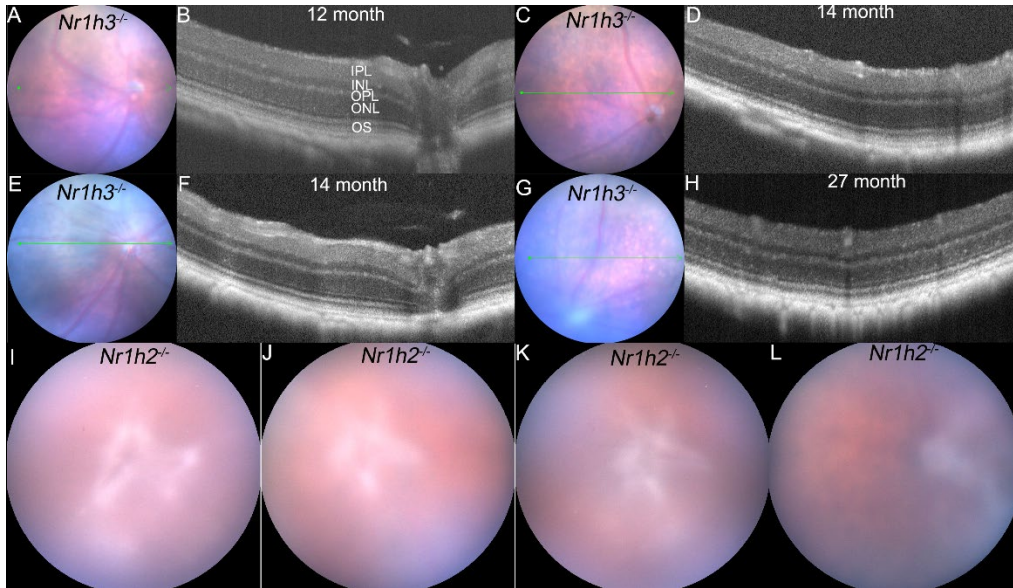


Figure S11.

In vivo images from aged *Nr1h3*^{-/-} and *Nr1h2*^{-/-} mice. Fundus and OCT images from (A, B) 12 month old, (C, D, E, F) 14 month old and (G, H) 27 month old *Nr1h3*^{-/-} mice. (I, J, K, L) Fundus images from *Nr1h2*^{-/-} mice, 12 to 14 month old, demonstrating stellate cataracts. IPL: inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer and OS: outer segments.

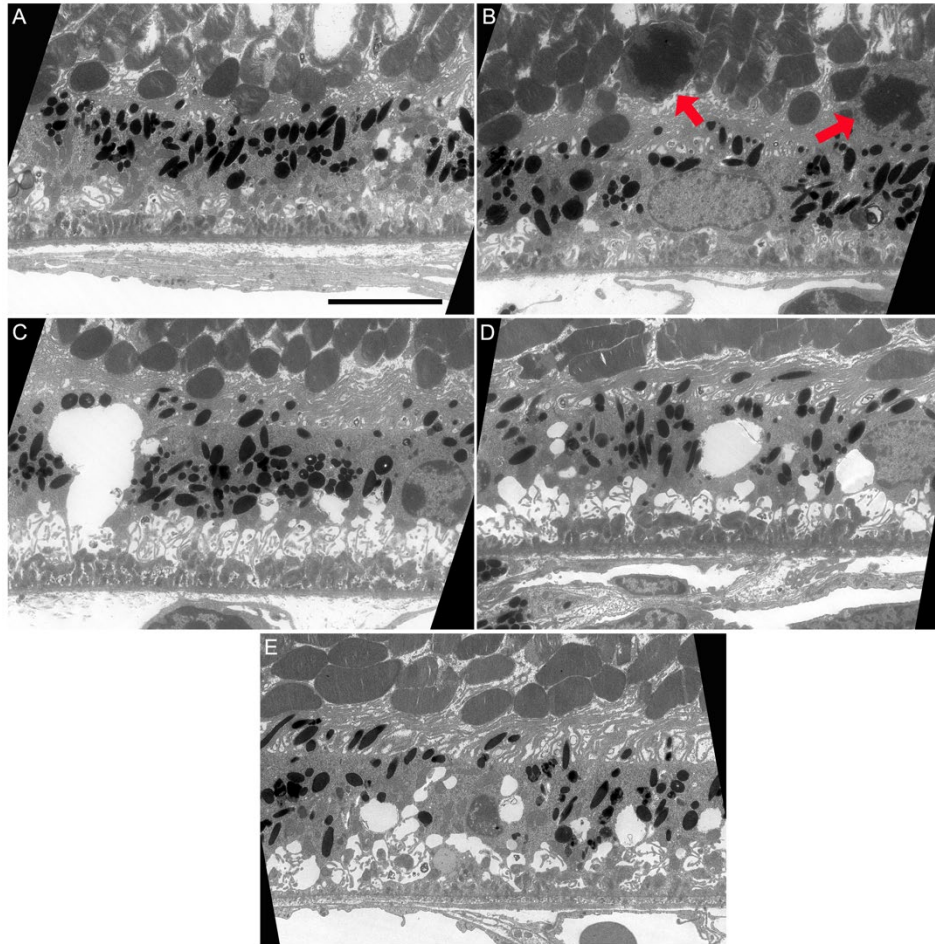


Figure S12.

cTEM images of *Nr1h3*^{-/-} mice. Representative electron micrographs showing the outer retina of 10 to 14 month old *Nr1h3*^{-/-} mice. (A, B, C, D, E). Continuous sub-RPE deposits are evident. (B) Two sub-retinal cells are also shown (red arrows). Scale bar = 5 microns.

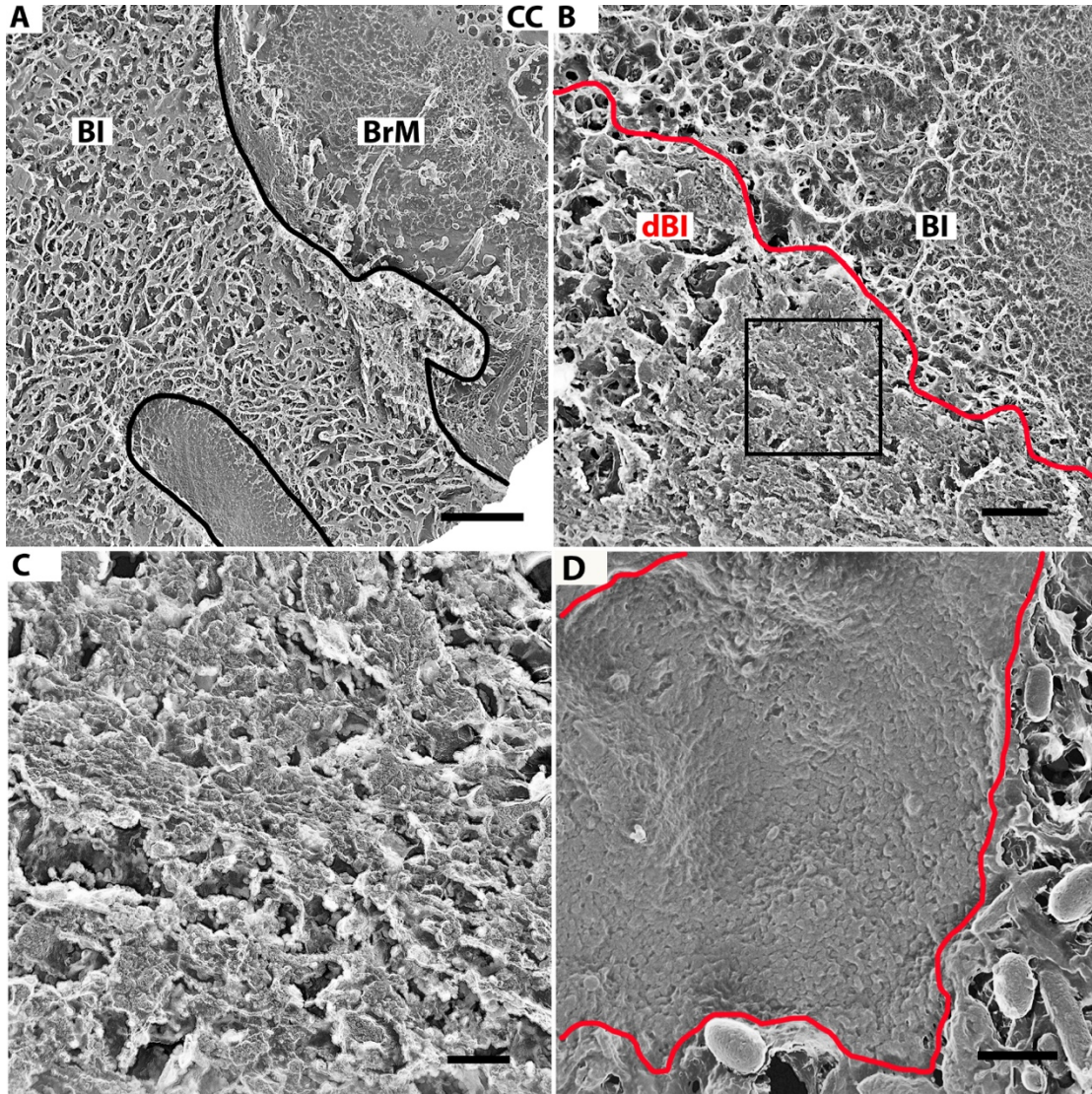


Figure S13.

RPE basal infoldings are disrupted and lipid droplets are present in *Nr1h3*^{-/-} mouse eyes. A detailed view of insets shown in Figure 6. Panels A, B and C represent insets H, J and K, respectively. (A, B) Scale bar = 1 μ m; (C) Scale bar = 250 nm. (D) Representative image of a cut through Bruch's membrane from a *Nr1h3*^{-/-} mouse in a region with an abundance of spherical deposits; red line delineates 'deposit' region (n = 4; Scale bar = 1 μ m).

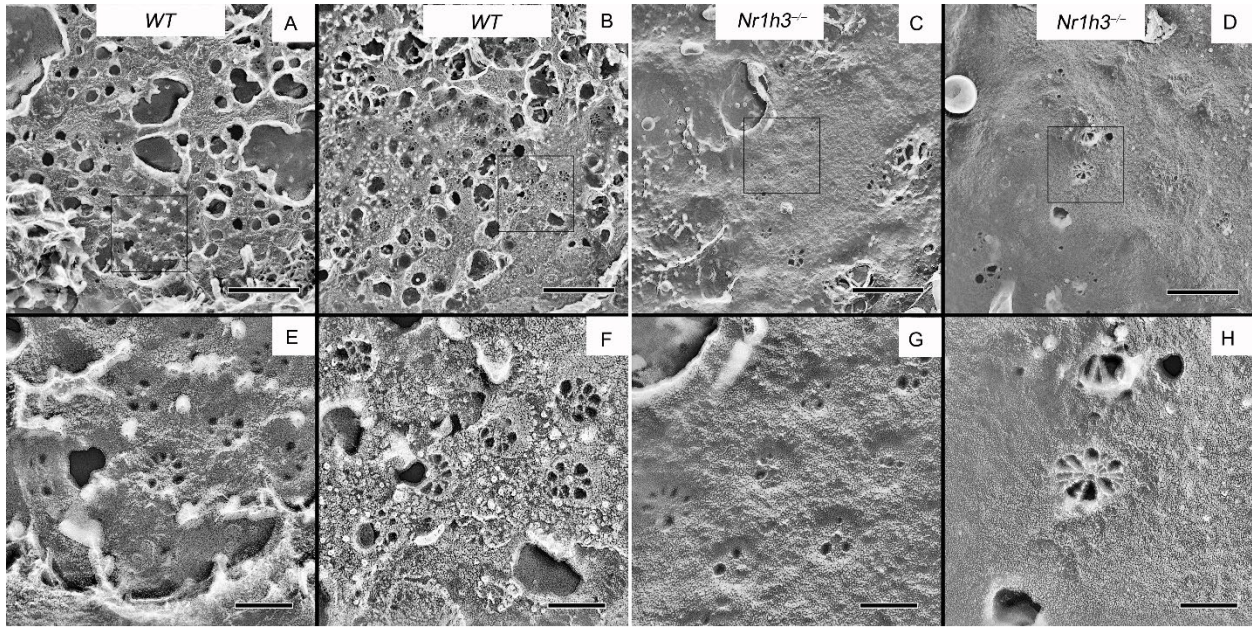


Figure S14.

Fenestration density and structure in *WT* mice and *Nr1h3*^{-/-} mouse eyes. Panels A, B, C, and D show a lower magnification of a region of the choriocapillary (CC) surface with fenestrations and panels E, F, G and H display a higher magnification of the square region of their above respective image. In *WT* mice (A, B, E and F), CCs are typically seen as circular pores or “wagon wheel” structures. In *Nr1h3*^{-/-} mice (C, D, G and H), some of the wagon wheel shapes of the fenestrations are disrupted as evident by fewer number of pores or “spokes”. Representative images are shown (n = 4; Scale bar: A, B, C, D = 500 nm; E, F, G, H = 100 nm).

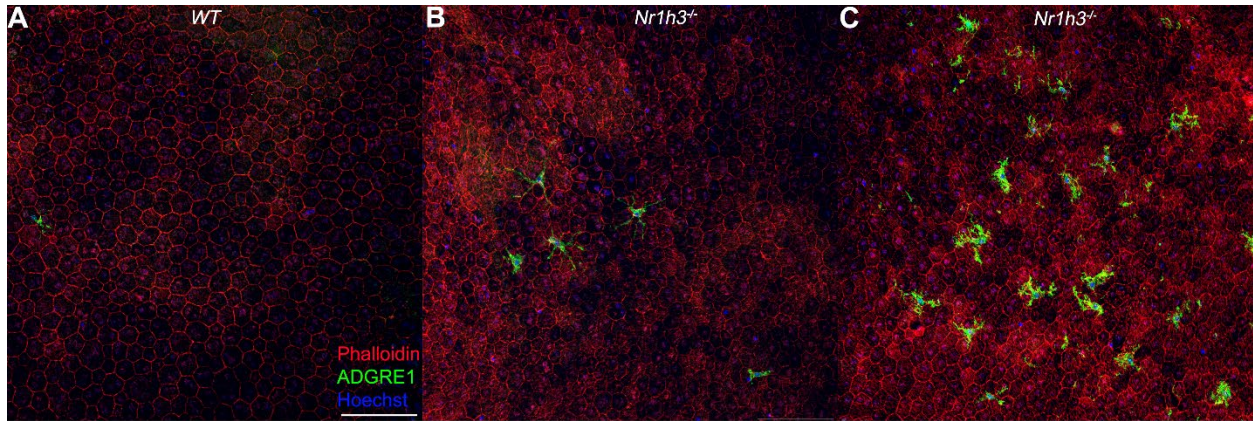


Figure S15.

Sub-retinal ADGRE1⁺ cells in *Nr1h3*^{-/-} mice. Representative images of high magnification images of RPE-choroid flatmounts from 10 to 14 month old (A) *WT* (n = 5) and (B, C) *Nr1h3*^{-/-} (n = 8) mice showing RPE cells stained with phalloidin (red) and immune cells stained with ADGRE1 (green). Scale bar = 100 μM.

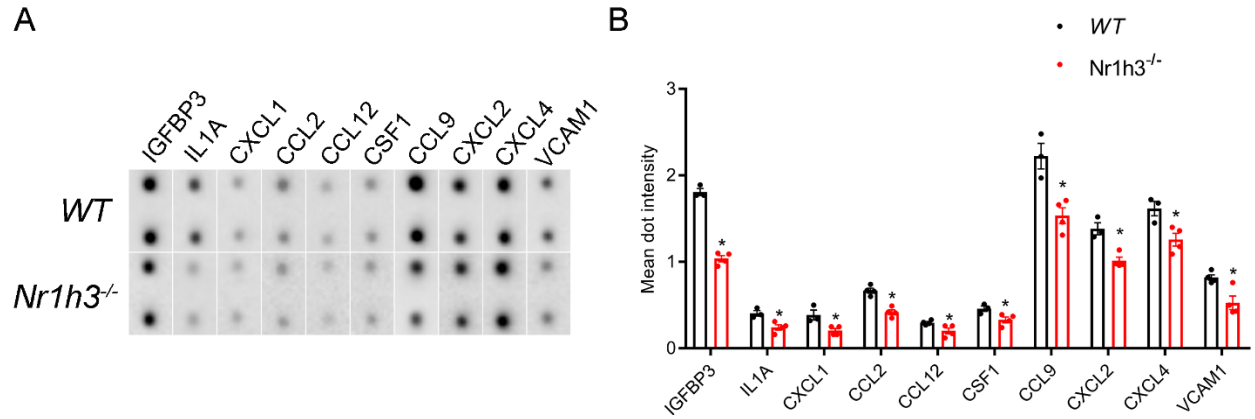


Figure S16.

Nr1h3^{-/-} mice exhibited a significant downregulation of cytokines in the systemic circulation.

Protein isolated from plasma of 10 to 14 month old *WT* and *Nr1h3*^{-/-} mice were blotted on a Mouse Cytokine Array C3 (62 proteins). (A) Representative images of significantly regulated cytokines are shown. (B) Quantification of staining intensity of significantly regulated cytokines (n = 4 per group, Mean + SEM, *: p < 0.05, Multiple t-tests).

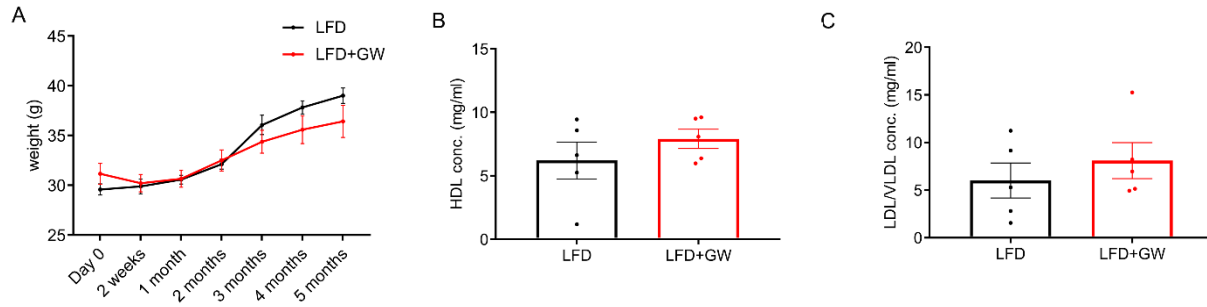


Figure S17.

GW3965 diet has no effect on weights; and plasma HDL and LDL/VLDL levels in *apoB100* mice. (A) Weights of 8 to 9 month old *apoB100* mice on LFD (n = 6) and LFD + GW (n = 7) diet (Mean $\pm$ SEM). Plasma (B) HDL and (C) LDL/VLDL levels in 8 to 9 month old *apoB100* mice on LFD (n = 5) and LFD + GW (n = 5) diet (Mean $\pm$ SEM).

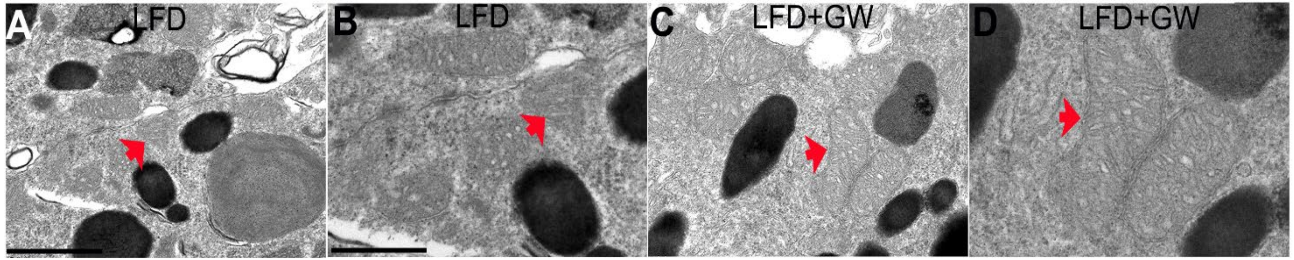


Figure S18.

Effect of the GW3965 diet on mitochondrial morphology in *apoB100* mice. Representative electron micrographs showing the integrity of the mitochondria in RPE cells from LFD (A, B) (n = 2) and LFD + GW (n = 3) groups (C, D). (A, C) Scale bar = 500 nm, (B, D) Scale bar = 250 nm.

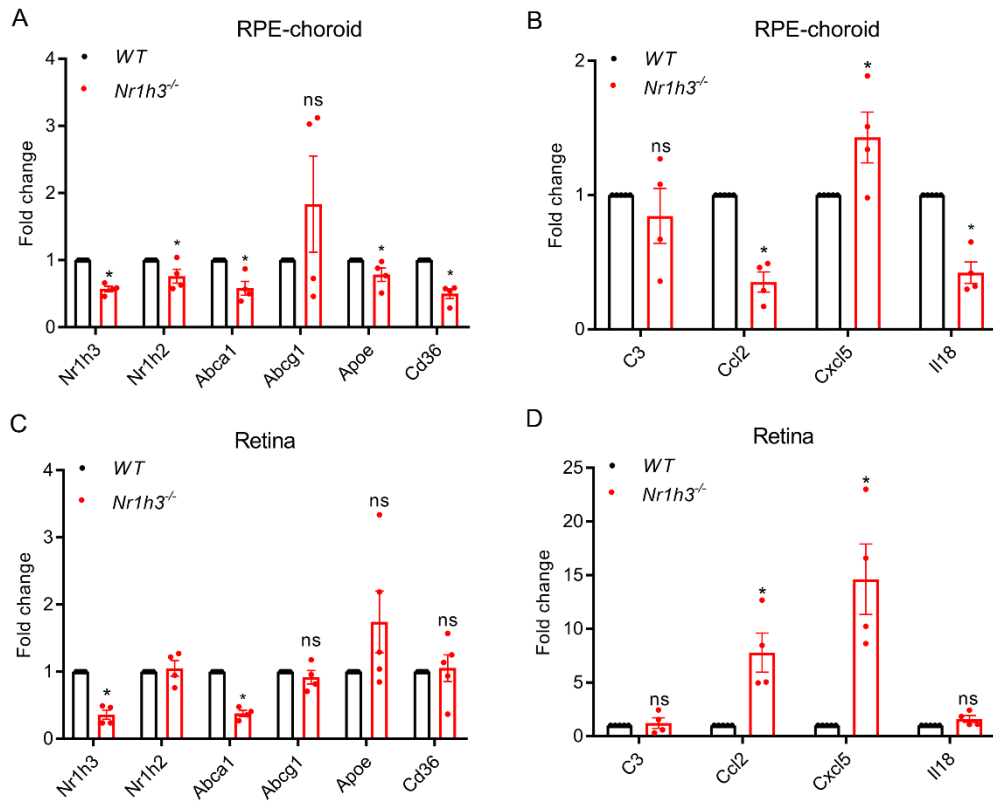


Figure S19.

Expression of *Nr1h3* and its target genes are downregulated in RPE of young *Nr1h3*^{-/-} mice.

mRNA expression of *Nr1h3*, *Nr1h2*, *Abca1*, *Apoe* and *Cd36* in RPE-choroid (A) and retina (C) of 3 to 5 month old *WT* and *Nr1h3*^{-/-} mice (n = 4, Mean ± SEM, *: p < 0.05, Multiple t-tests). mRNA expression of *C3*, *Ccl2*, *Cxcl5* and *Il18* in RPE-choroid (B) and retina (D) of 3 to 5 month old *WT* and *Nr1h3*^{-/-} mice (n = 4, Mean ± SEM, *: p < 0.05, Multiple t-tests).

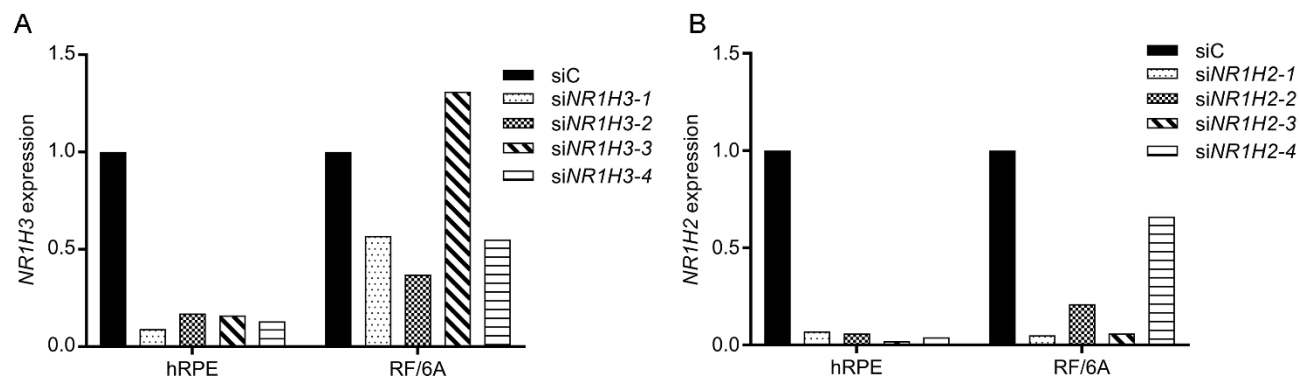


Figure S20.

Screening of siRNAs to target *NR1H3* and *NR1H2*. (A) Screening of four *NR1H3* targeting siRNAs in hRPE and RF/6A cells. (B) Screening of four *NR1H2* targeting siRNAs in hRPE and RF/6A cells.

SUPPLEMENTARY MATERIAL

Table S1. Demographics of donors.

Donor#	Age	Gender	Cause of Death	Recovery time
Non-AMD 1	84	Female	Breast Cancer	4 hr 45 min
Non-AMD 2	Not known	Not known	Not known	Not known
1	18	F	Pulmonary embolism	4 hr
2	28	M	Metastatic cancer	4 hr 15 min
3	39	M	Congestive heart failure	5 hr 15 min
4	44	F	Cardiovascular disease	5 hr 45 min
5	47	F	Cardiovascular disease	5 hr 5 min
6	49	M	Not known	4 hr 45 min
7	60	M	Cardiovascular disease	4 hr 16 min
8	60	F	Colon/Gastric cancer	6 hr
9	61	F	Cancer	6 hr 18 min
10	69	M	Respiratory failure	5 hr
11	70	M	Cardiovascular disease	5 hr 40 min
12	70	M	Cardiovascular disease	6 hr 29 min
13	72	M	Cerebrovascular accident	6 hr 28 min
14	73	F	Respiratory failure	5 hr
15	73	F	Not known	Not known
16	74	M	Subdural hematoma	4 hr
17	74	F	Cardiovascular disease	6 hr 23 min
18	76	M	Congestive heart failure	6 hr 50 min
19	78	F	Cardiovascular disease	3 hr 20 min
20	79	M	Cardiovascular disease	4 hr 40 min
21	80	F	Leukemia	3 hr 18 min
22	81	F	Cardiovascular disease	3 hr
23	83	M	Cardiovascular disease	5 hr 25 min
24	83	M	Cardiovascular disease	5 hr
25	85	M	Colon/Gastric Cancer	4 hr
26	85	F	Cardiopulmonary arrest	4 hr
27	86	M	Not known	6 hr
28	90	M	Gastric Cancer	4 hr
29	90	M	Cancer	4 hr 10 min
30	91	F	Emphysema	3 hr
31	94	M	Congestive heart failure	3 hr 10 min

Table S2. List of primers and primer sequences.

Gene	Primers	Sequence 5'→3' Human
18S	Reverse	ACCGCAGCTAGGAATAATGGA
	Forward	GCCTCAGTTCCGAAAACCA
36B4	Forward	GGACATGTTGCTGGCCAATAA
	Reverse	GGGCCCCGAGACCAGTGTT
ACTB	Forward	CTTCCTTCCTGGGCATGGAGTC
	Reverse	GGGTACATGGTGGTGCCGC
ABCA1	Forward	GGAGAGCATGTCCAATGTTCC
	Reverse	CGTCCTGTTCAATTCGTGCAA
APOE	Forward	GTTGCTGGTCACATTCCTGG
	Reverse	GCAGGTAATCCCAAAAGCGAC
BEST1	Forward	AACTGAGCCTACCACACAACA
	Reverse	CGGATTTCGACCTCCAAGCC
FASN	Forward	GTCCACCGACCTCCCGTTA
	Reverse	CCTCATGGTCTAGGTGCTTGT
NR1H3	Forward	CCCTTCAGAACCCACAGAGATC
	Reverse	GCTCGTTCCCCAGCATTTT
NR1H2	Forward	CGCTAAGCAAGTGCCTGGTT
	Reverse	CAGCCAGATGCAATCAATGCC
RPE65	Forward	CCTGCTGGTGGTTACAAGAAA
	Reverse	CCTGCCTGTTACATGAGCTGT
RXRA	Forward	GAGCCCAAGACCGAGACCTA
	Reverse	AGCTGTTTGTCGGCTGCTT
RXRB	Forward	AGCCCCCAGATTAACTCAACA
	Reverse	GATTGCACATAGCCGTTTGC
SREBF1	Forward	TCACCAGGACAGAAGGGAAG
	Reverse	CTCTGGCACCTTTTGCTAGG
Abca1	Forward	AAAACCGCAGACATCCTTCAG
	Reverse	CATACCGAAACTCGTTCACCC
Abcg1	Forward	CTTTCCTACTCTGTACCCGAGG
	Reverse	CGGGGCATTCCATTGATAAGG
ApoE	Forward	GACCCAGCAAATACGCCT
	Reverse	CATGTCTTCCACTATTGGCTC
C3	Forward	CCAGCTCCCCATTAGCTCTG
	Reverse	GCACTTGCCCTCTTTAGGAAGTC
Ccl2	Forward	TTAAAAACCTGGATCGGAACCAA
	Reverse	GCATTAGCTTCAGATTTACGGGT
Cd36	Forward	AGATGACGTGGCAAAGAACAG
	Reverse	CCTTGGCTAGATAACGAACTCTG
Cxcl5	Forward	TGCGTTGTGTTTGCTTAACCG
	Reverse	CTTCCACCGTAGGGCACTG
Il18	Forward	GACTCTTGCGTCAACTTCAAGG
	Reverse	CAGGCTGTCTTTTGTCAACGA

<i>Nr1h3</i>	Forward	CTCAATGCCTGATGTTTCTCCT
	Reverse	TCCAACCCTATCCCTAAAGCAA
<i>Nr1h2</i>	Forward	CGACTCCAGGACAAGAAGC
	Reverse	TCAAGAAGACACCACCAAGG

Table S3. List of treatments, concentrations, and sources.

Drugs	Concentration	Source	Catalog#
GW3695	1 μ M	Sigma-Aldrich	G6295
TO901317	10 μ M	Sigma-Aldrich	T2320
GSK2033	10 μ M	Sigma-Aldrich	SML1617
NaIO₃	100 μ M	Sigma-Aldrich	S4007
H₂O₂	100 μ M	Sigma-Aldrich	H1009
Cigarette smoke extract (CSE)	2%	Duke	
Palmitic acid (PA)	20 μ M	MP Biomedicals	0210090510
Alpha-linolenic acid (αLA)	20 μ M	Acros Organics	AC302825000

Table S4. Antibodies/stains used for immunofluorescence and histology.

Antibody/Stain	Source	Dilution	Catalog#
Apolipoprotein E (APOE)	Millipore	1:1000	AB947
CRALBP	Thermo Fisher	1:2500	MA1-813
EO6	Avanti Polar Lipids	1:100	330001S
ADGRE1	Bio-Rad	1:500	MCA497CA
GFAP	Dako	1:100	Z0334
Hoechst 33258 pentahydrate	Invitrogen	2µg/ml	H3569
NR1H3 12HCLC	Invitrogen	1:100	712441
NR1H3 K8607	Perseus Proteomics	1:200	PP-K8607
NR1H2 K8917	R&D systems	1:200	PP-K8917-00
Oil red o	Sigma-Aldrich	0.7%	O0625
Phalloidin tetramethylrhodamine B isothiocyanate	Sigma-Aldrich	1:500	P1951
PKCα	Abcam	1:200	ab4124
Red-green opsin	Millipore	1:1000	AB5405
Rhodopsin	Abcam	1:1000	ab5417

Table S5. List of reagents, kits and source.

Reagent/Kit	Source	Catalog#
Adipored™	Lonza	PT-7009
CellTiter-Blue®	Promega	G8080
Cholesterol assay kit	Abcam	ab65390
CPRG substrate	Sigma-Aldrich	59767
Ethidium bromide	Sigma-Aldrich	E1510
GW3965 diet	Envigo	TD.150778
JC-1	Sigma-Aldrich	T4069
LFD diet	Envigo	TD.150777
Lipofectamine™	Invitrogen	18324012
Beetle luciferin, potassium salt	Promega	E1603
Mouse cytokine array C3	RayBiotech	AAM-CYT-3-8

Table S6. List of siRNAs and sources.

siRNA	Company	Catalog#
siC	Qiagen	1027281
<i>siNR1H3 -1</i>	Dharmacon	J-003413-06
<i>siNR1H3 -2</i>	Dharmacon	J-003413-07
<i>siNR1H3 -3</i>	Dharmacon	J-003413-08
<i>siNR1H3 -4</i>	Dharmacon	J-003413-09
<i>siNR1H2 -1</i>	Dharmacon	J-003412-07
<i>siNR1H2 -2</i>	Dharmacon	J-003412-08
<i>siNR1H2 -3</i>	Dharmacon	J-003412-10
<i>siNR1H2 -4</i>	Dharmacon	J-003412-22