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Supplemental Information

Heme Synthesis Inhibition Blocks Angiogenesis via Mitochondrial Dysfunction

Trupti Shetty, Kamakshi Sishtla, Bomina Park, Matthew J. Repass, and Timothy W. Corson

Supplemental Figures

A

Treatment	PPIX
	(pmol/mg)
DMSO	4.058
NMPP	97.244

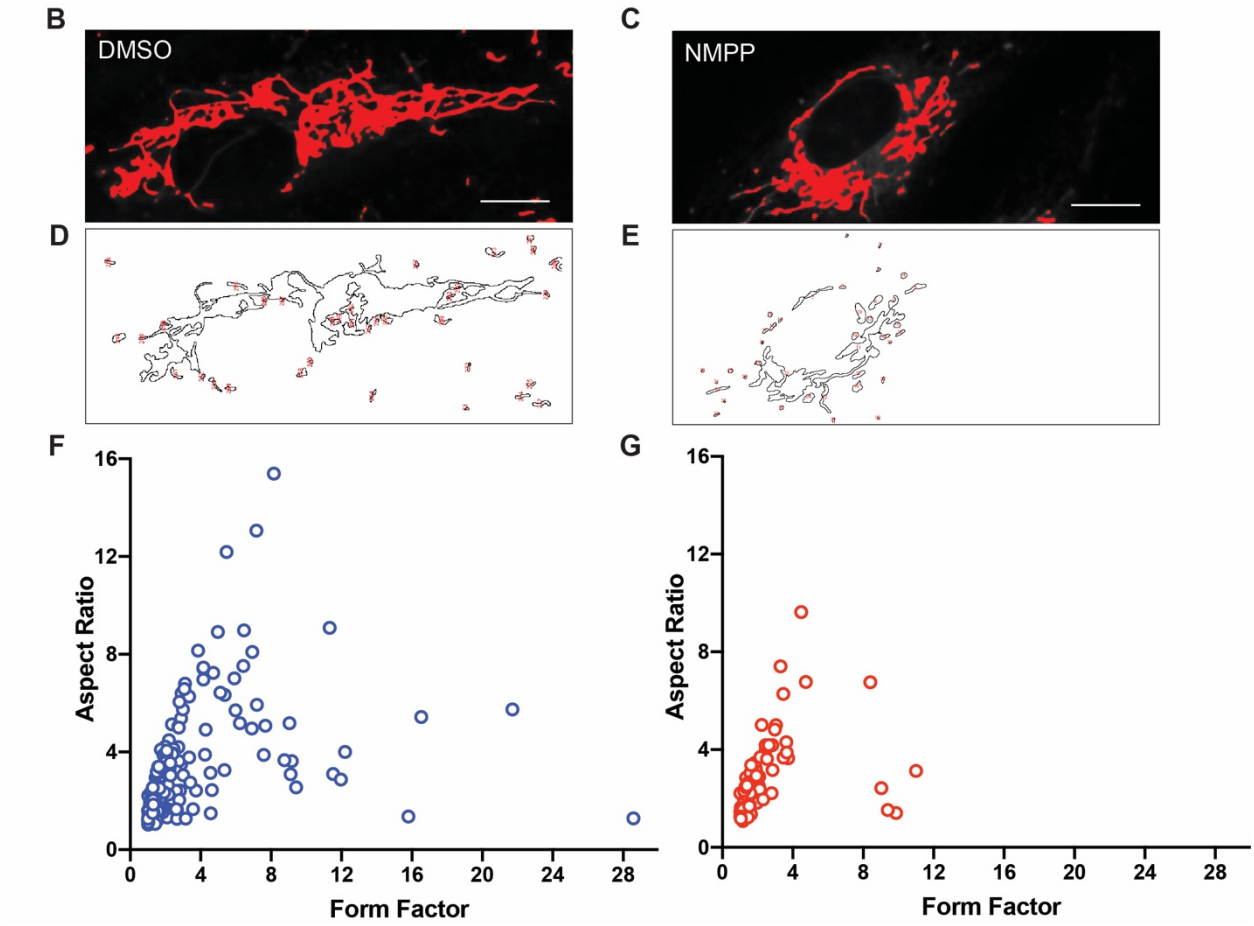

Figure S1. Mitochondrial morphometric analysis and PPIX levels in HRECs treated with NMPP, Related to Figure 1.
(A) PPIX levels quantified by ultra-performance liquid chromatography in HRECs treated with NMPP. Representative cells depicting particle analysis for measuring mitochondria. **(B, C)** Representative slice from Z-stack images showing threshold intensity set for DMSO and NMPP treated HRECs. **(D, E)** Representative slice from Z-stack images showing outlines of assessed mitochondria. **(F, G)** Correlation of form factor versus aspect ratio under DMSO and NMPP treatments for the representative cells shown above. Scale bars = 10 μm.

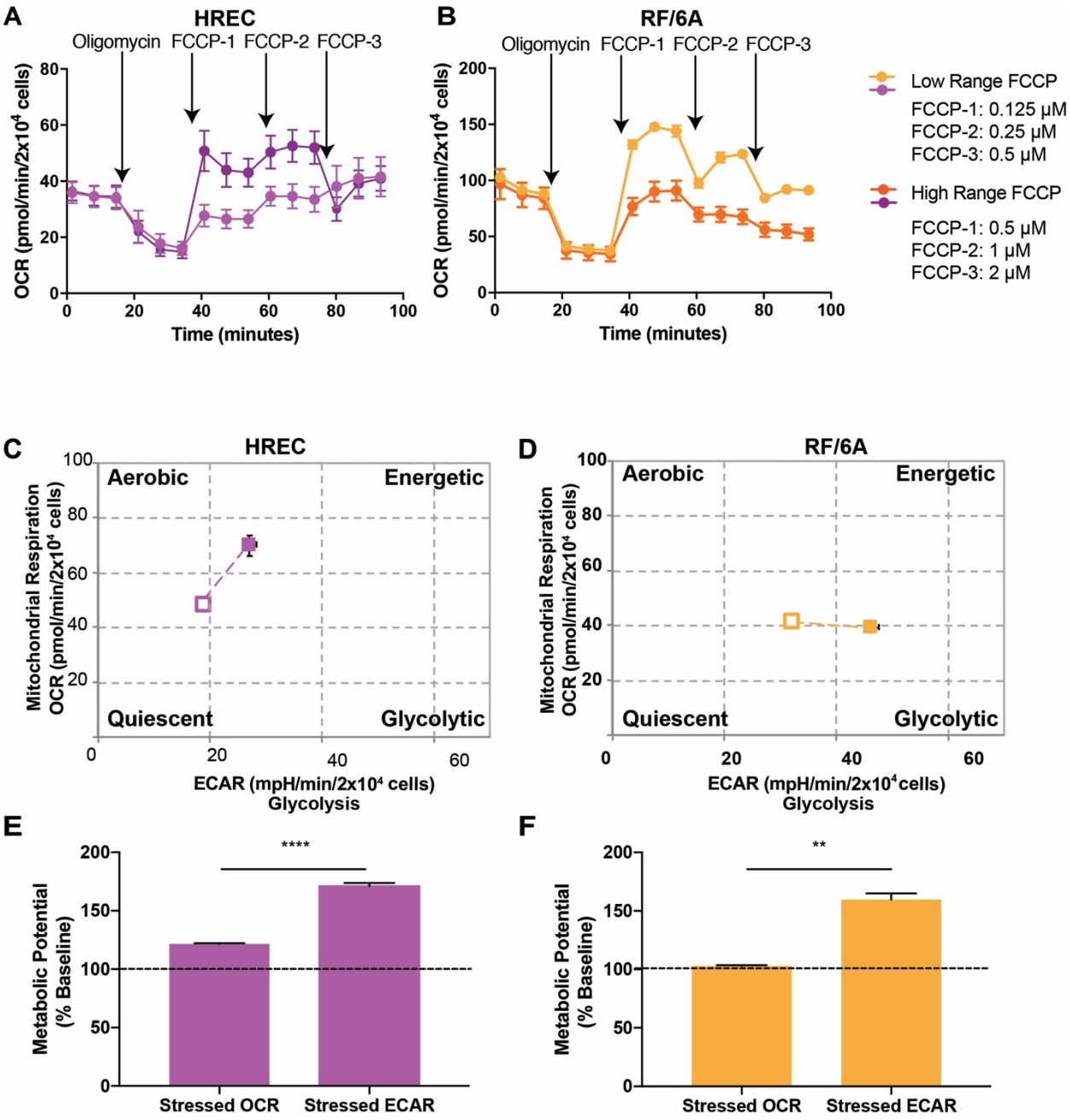

Figure S2. FCCP titration and cell energy phenotype of HRECs and RF/6A cells, Related to Transparent Methods and Figure 4.

Optimization of uncoupler FCCP concentration that produces maximal respiration in ocular endothelial cells. Oxygen consumption rate (OCR) traces of (**A**) HRECs and (**B**) RF/6A cells. 2×10^4 cells seeded. Mean ± SEM, n=3. Cellular energetic phenotype profiles under metabolic stress of (**C**) HRECs and (**D**) RF/6A cells. Open box indicates baseline phenotype, filled box indicates stressed phenotype. Representative plots of three independent experiments. Some error bars are too small to be seen. (**E, F**) % stressed OCR and ECAR produced over 100% baseline OCR and ECAR. Mean ± SEM, n=3; **p<0.01, ****p<0.0001, two-tailed unpaired Student's t-test.

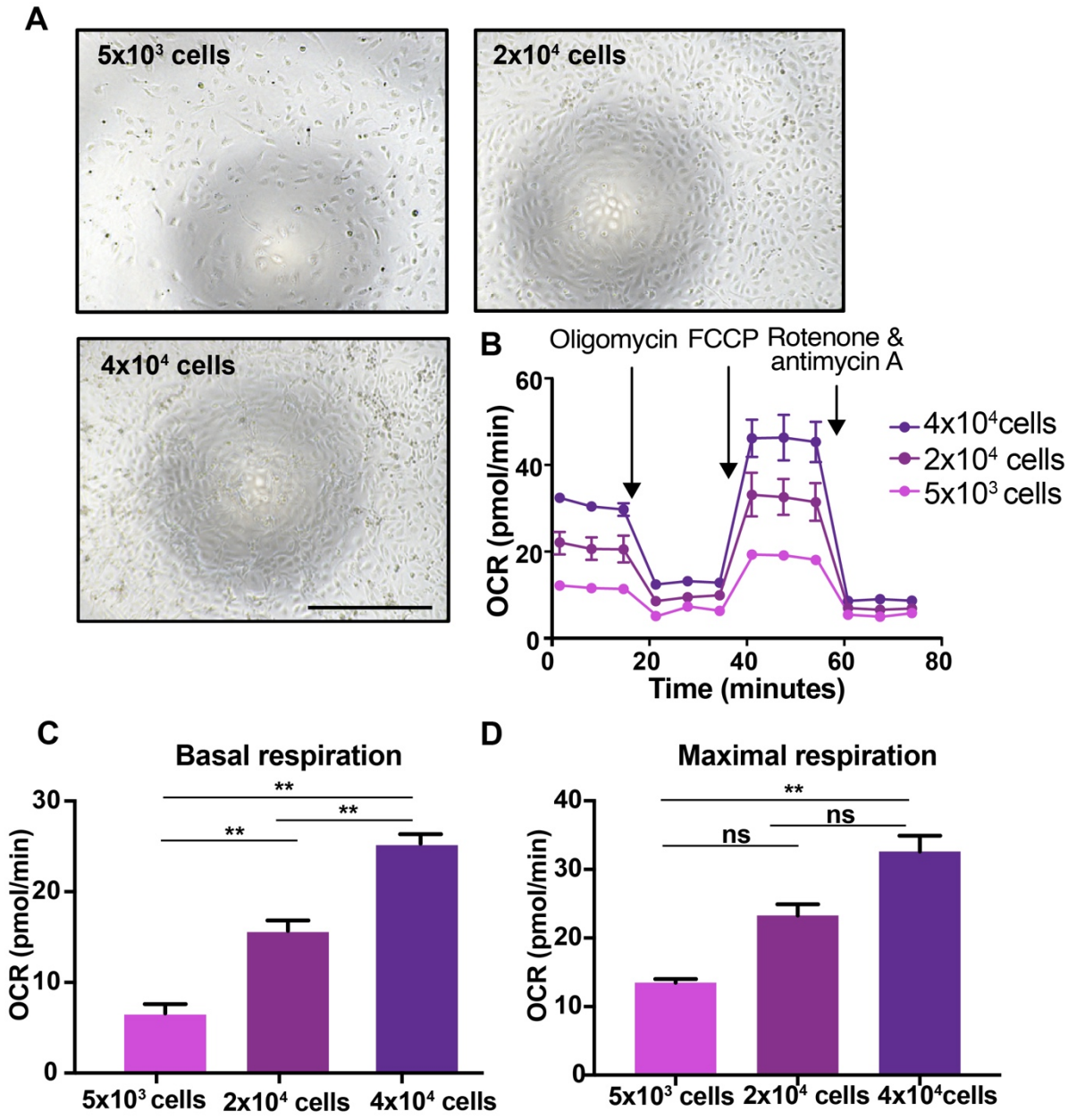

Figure S3. Optimizing cell seeding density, Related to Transparent Methods and Figure 4.
(A) Representative images from three independent experiments of HRECs. Scale bar = 500 μm. **(B)** Related OCR traces, **(C)** basal respiration, and **(D)** maximal respiration. Mean ± SEM, n=3; ns, not significant, **p<0.01, ANOVA with Tukey's post-hoc tests.

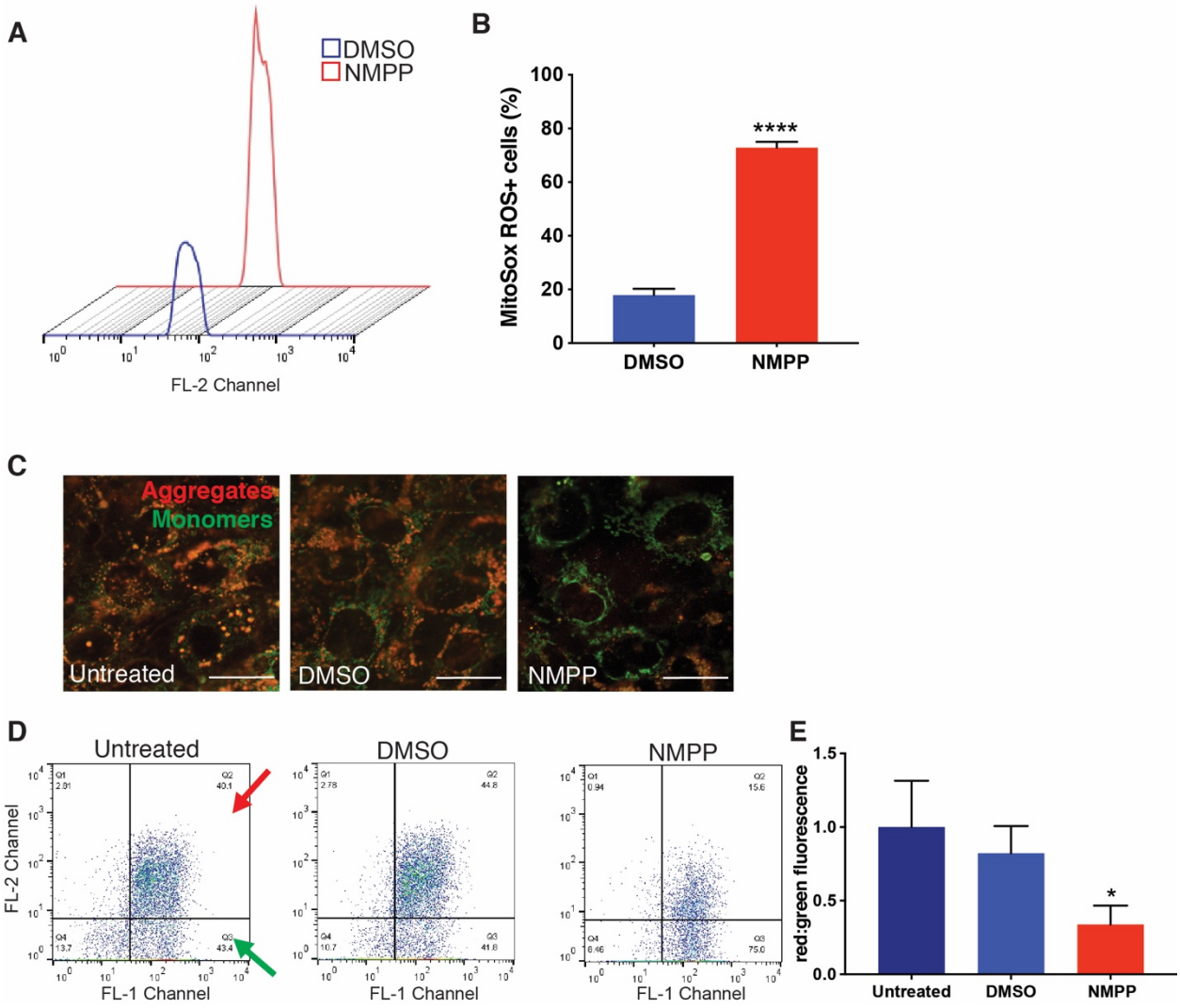

Figure S4. FECH blockade caused mitochondrial defects in RF/6A cells, Related to Figures 1 and 2. **(A)** RF/6A cells labeled with mitoSox ROS were assessed using flow cytometry. Representative fluorescence peaks. **(B)** Quantification of cells positive for red fluorescence. ****p<0.0001 vs. DMSO, unpaired Student's t-test**. (C)** RF/6A cells stained with JC-1 dye showing green monomers and red aggregates under NMPP treatment. **(D)** Representative dot plots of FL1 versus FL2 channel measuring red and green fluorescence using flow cytometry after NMPP treatment. Red and green arrows indicate quadrants expressing FL1-red and FL2-green fluorescent cells. **(E)** Quantification of red:green fluorescence from flow experiment. *p<0.05 vs. untreated, one-way ANOVA with Tukey's post hoc tests. Bar graphs indicate mean ± SEM, n=3; Scale bars = 20 μm.

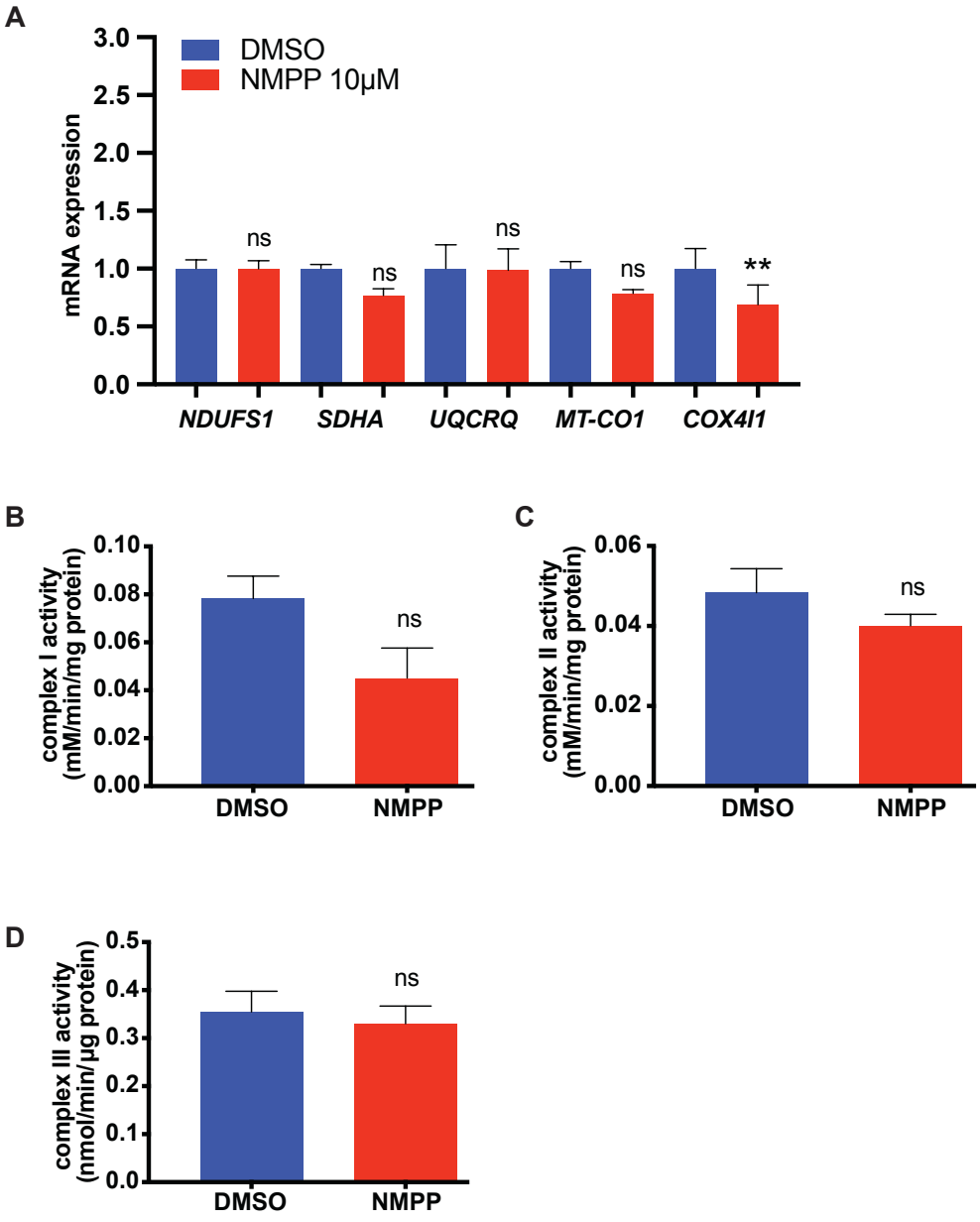

Figure S5. Loss of heme decreases transcription of nuclear encoded CcO but has no effect on activities of complexes I-III, Related to Figure 3.
(A) mRNA quantification of genes encoding components of ETC complexes I-IV **(B-D)** HRECs treated with NMPP were assessed for activities of **(B)** complex I, **(C)** complex II and **(D)** complex III. Bar graphs indicate mean ± SEM, n=3, ns = not significant, **p<0.01, unpaired Student's t-test.

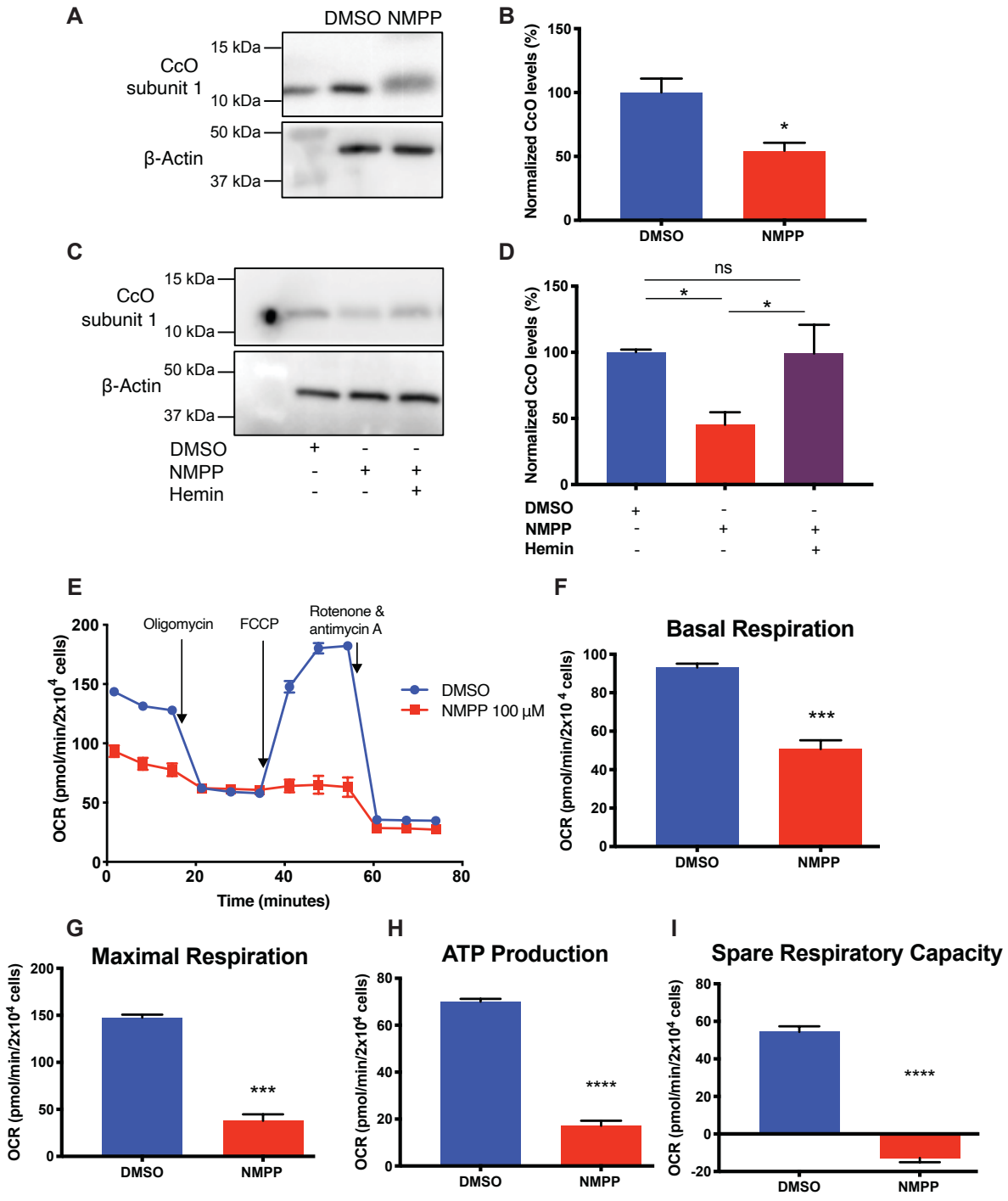

Figure S6. FECH inhibition decreases mitochondrial respiration and CcO expression, which is rescued by hemin in RF/6A cells, Related to Figures 3 and 4.
(A) RF/6A cells treated with NMPP were blotted for nuclear-encoded CcO subunit 1 (COX4I1) with **(B)** quantification as shown. **(C, D)** RF/6A cells treated with 100 µM NMPP show rescue of CcO subunit 1 protein levels after hemin supplementation. **(E)** Representative OCR kinetic traces for RF/6As under NMPP chemical inhibition. **(F)** Basal respiration, **(G)** maximal respiration, **(H)** OCR-linked ATP production, and **(I)** spare respiratory capacity were calculated based on OCR curves for the respective treatment group. Bar graphs indicate mean ± SEM, n=3; *p<0.05, ***p<0.001, ****p<0.0001, **(D)** one-way ANOVA with Tukey's post hoc tests; (**B, F-I**) unpaired Student's t-test.

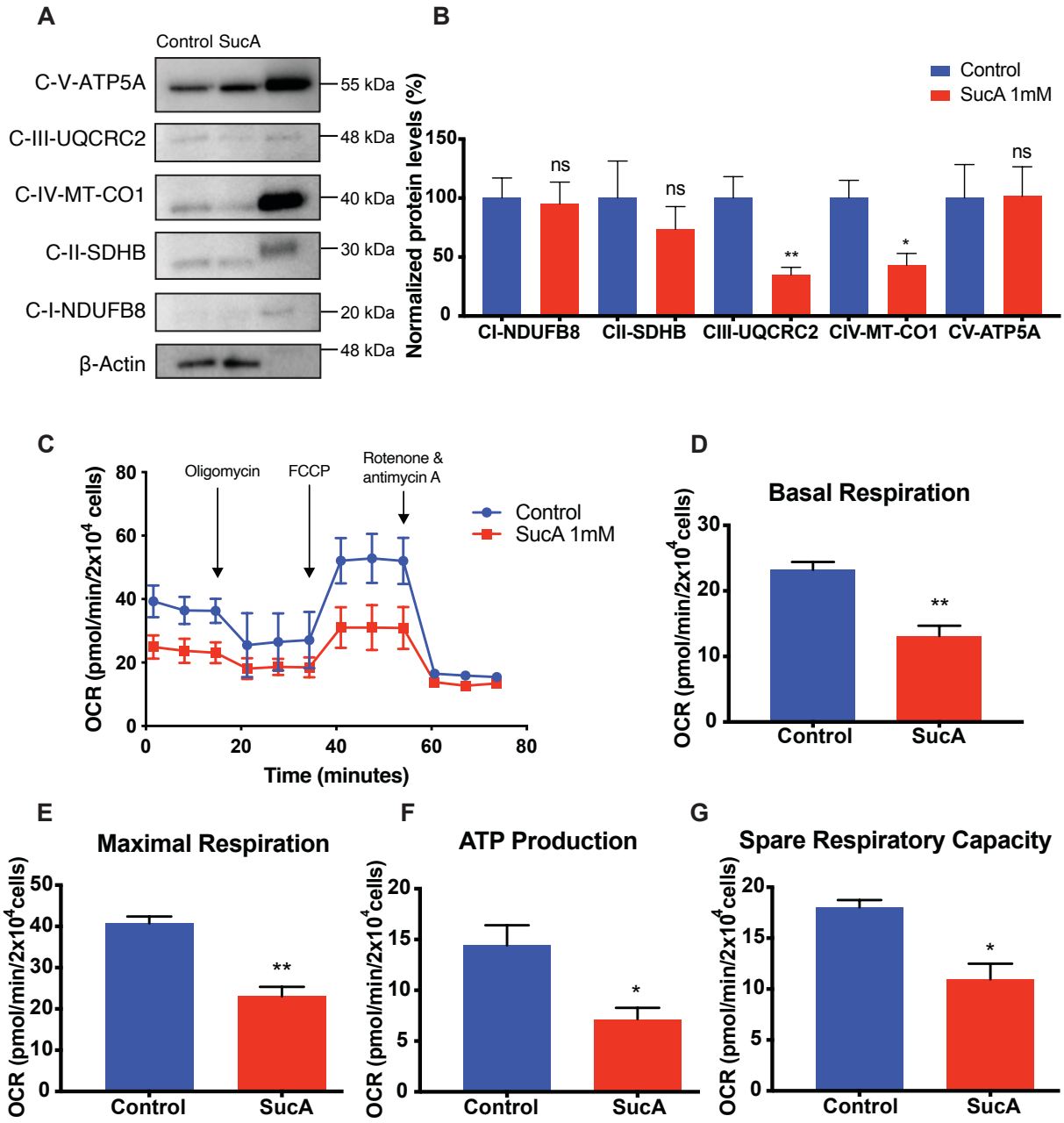

Figure S7. Loss of heme through ALAD blockade reduces mitochondrial respiration, Related to Figures 3 and 4.

(A) HRECs treated with succinylacetone were immunoblotted for Complexes I-V as indicated. **(B)** Quantification of the blots was graphed as shown relative to control. **(C)** OCR kinetic traces for HRECs under succinylacetone treatment. **(D)** Basal respiration, **(E)** maximal respiration, **(F)** OCR-linked ATP production, and **(G)** spare respiratory capacity were calculated based on OCR curves for the respective treatment group. Representative OCR curve of three individual experiments. Bar graphs indicate mean ± SEM, n=3; *p<0.05, **p<0.01, unpaired Student's t-test.

Transparent Methods

Experimental Model and Subject Details

Cell Culture

Human primary retinal microvascular endothelial cells (HRECs) and Attachment Factor were purchased from Cell Systems (Kirkland, WA, USA). Female HRECs were grown in endothelial growth medium (EGM-2) and used between passages 4 and 8. EGM-2 was prepared by combining components of an EGM-2 "bullet kit" (Cat no. CC-4176) and endothelial basal medium (EBM, Lonza, Walkersville, MD, USA; Cat No. CC-3156). As primary cells, these were not subject to authentication. Rhesus macaque choroidal endothelial (RF/6A) cells were obtained from ATCC (Manassas, VA, USA) and grown in Eagle's Minimum Essential Medium (EMEM, ATCC Cat No. 30-2003) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories, Etobicoke, ON, Canada; Cat No. A15-751). Since short tandem repeat profiles are not available for Rhesus, these cells were not authenticated after receipt from the supplier, and sex was not reported. All cells were grown at 37°C, 5% CO_2, 100% humidity and tested for mycoplasma contamination regularly. *N*-methyl protoporphyrin (NMPP) was purchased from Frontier Scientific (Logan, Utah, USA), prepared in DMSO, and applied to cells at 1 μM or 10 μM (as indicated) for 48 hours. FECH siRNA (SASI_Hs01_00052189 and SASI_Hs01_00052190) was purchased from Sigma and MISSION® siRNA Universal from Sigma was used as a negative siRNA control. Succinylacetone (Sigma-Aldrich, Saint Louis, MO, USA) was prepared in sterile water and used at 1 mM final well concentration for 24 hours and water was used as vehicle control.

Animals

Animal studies were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee, and were consistent with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. C57BL/6J wild-type, healthy female mice, 8 weeks of age were purchased from Jackson Laboratories and group housed under standard conditions. NMPP at 10 μM final vitreous concentration was injected intravitreally into naïve mice under ketamine/xylazine anesthesia as described (Sulaiman et al., 2016), and 24 hrs post-injection, animals were euthanized, and retinas were immediately isolated from the animals and processed for the energetics experiment.

Method Details

Protoporphyrin IX quantification

Protoporphyrin IX (PPIX) analysis was performed at the Iron and Heme Core facility at the University of Utah. Cells were washed with PBS, pelleted, and stored frozen at -80°C. The cells were suspended in 50 mM potassium phosphate pH 7.4 and homogenized by sonication. 200 μL extraction solvent (EtOAc:HOAc, 4:1) was slowly added to 50 μL concentration-adjusted sample and shaken. The mixture was centrifuged at 16000x*g* for 0.5 min and the supernatant was collected. About 10 μL of the supernatant solution above was injected into a Waters Acquity ultra performance liquid chromatography (UPLC) system with an Acquity UPLC BEH C18, 1.7 μm, 2.1 x 100 mm column. PPIX was detected at 404 nm excitation and 630 nm emission. Solvent A was 0.2% aqueous formic acid while Solvent B was 0.2% formic acid in methanol. The flow rate was kept at 0.40 mL per minute and the column maintained at 60˚C for the total run time of 7 min. The following successive linear gradient settings for run time in minutes versus Solvent A were as follows: 0.0, 80%; 2.5, 1%; 4.5, 1%; 5.0, 80%.

Mitochondrial morphology

Cells were plated on 35 mm coverslip bottom dishes. HRECs were stained using MitoTracker Green (Thermo Fisher, Cat no M7514) at 200 nM for 10 minutes in the dark at 37°C. Imaging was performed immediately following staining using an LSM700 confocal microscope (Zeiss, Thornwood, NY, USA) under a 63× oil immersion lens and acquired *Z*-stacked images were analyzed using ImageJ software (Trudeau et al., 2010). Briefly, individual cells were selected and particle analysis was performed to determine form factor ($\text{perimeter}^2/4\pi$*area) and aspect ratio (length of major and minor axes). Mitochondria of 12 cells per condition were analyzed and the mean per cell was considered for further statistical tests.

Mitochondrial membrane potential assessment

Membrane potential ($\Delta\Psi_m$) was measured with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbo cyanine iodide (JC-1) (Santa Cruz, Santa Cruz, CA, USA) dye (Perelman et al., 2012). Cells were stained with JC-1 dye at 5 µg/mL final concentration for 10 minutes in the dark at 37°C. Cells were washed with 1× HBSS and prepared for flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA) in Fluorobrite DMEM. JC-1 dye after accumulation in mitochondria forms red aggregates and green monomers that emit fluorescence at 590 nm and 510 nm respectively. For live imaging, cells grown in coverslip bottom 35mm dishes were stained and imaged using the Zeiss confocal microscope under a 63× oil immersion objective.

Mitochondrial ROS measurement

Cells were labeled with MitoSox ROS (Thermo Fisher Scientific) dye at 5 µM final concentration for 10 minutes in the dark at 37°C using phenol red-free Fluorobrite DMEM. Cells were washed and stained with Hoechst 33342 stain for 10 minutes at room temperature. For flow cytometry, cells were labeled with dye at 1 µM final concentration, incubated in the dark at 37°C and immediately loaded for ROS fluorescence detection in the FL-2 channel using flow cytometry (Kauffman et al., 2016).

mRNA quantification using qPCR

RNA was isolated using Trizol reagent and 1 µg RNA was used for cDNA synthesis, made using iScript cDNA synthesis kit from Bio Rad (Cat no. 1708897, Hercules, CA, USA). TaqMan probes for *MFN2* (Hs00208382_m1)*, DNM1L* (Hs01552605_m1)*, OPA1* (Hs01047013_m1)*, FECH* (Hs01555261_m1), *NDUFS1* (Hs00192297_m1), *SDHA* (Hs00188166_m1), *UQCRQ* (Hs00429571_g1), *MT-CO1* (Hs02596864_g1), and *COX4I1* (Hs00971639_m1) were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). qPCR was performed using Fast Advanced Master Mix, TaqMan probes, and a ViiA7 thermal cycler (Applied Biosystems). Gene expression was determined using $\Delta\Delta C_t$ method and *HPRT* (Hs02800695_m1) as housekeeping control and normalized to individual sample controls.

Immunoblotting

Cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA, Cat no. R0278) and processed for protein quantification followed by SDS-PAGE. For ETC proteins, total OXPHOS Rodent WB Antibody Cocktail (Abcam, ab110413, Cambridge, MA, USA) at 1:250 dilution was used. CcO nuclear-encoded subunit 1 (COX4I1) antibody was purchased from Thermo Fisher Scientific (Cat no. PA5-19471, Pittsburgh, PA, USA) and used at 1:1000 dilution. β-actin antibody was purchased from Sigma (Cat no. A5441) and used at 1:2000 dilution. FECH antibody was purchased from LifeSpan BioSciences (Seattle, WA, USA, Cat no LS C409953) and used at 1:500 dilution prepared in 5% BSA solution. Chemiluminescent reagent ECL Prime was purchased from GE Healthcare (Buckinghamshire, UK, Cat no. RPN2232).

Complexes I-IV activity assays

For complex I activity, HRECs treated with NMPP (10 µM) or DMSO were harvested and processed using a kit (Abcam, Cambridge, MA, USA, Cat no. ab109721) according to manufacturer's instructions. Briefly, 250 µg of protein lysates were loaded into plates pre-coated with immobilized complex I and the oxidation of NADH to NAD+ was measured as the increase in absorbance at 450 nm, read on a Synergy H1 plate reader (BioTek, Winooski, VT, USA). Complex II enzyme activity was measured using an assay kit (Abcam, Cambridge, MA, USA, Cat no. ab109908) following manufacturer's instructions. Cell lysates from HRECs treated with either DMSO or NMPP (10 µM) were processed and 250 µg protein was loaded into plates coated with anti-complex II antibody. The activity of complex II was determined by measuring the production of ubiquinol coupled to the dye 2,6- dichlorophenolindophenol as a reduction in absorbance at 600 nm. For complex III activity, cell lysates from NMPP (10 µM) or DMSO treated HRECs were processed using complex II + III enzyme assay kit (Abcam, Cambridge, MA, USA, Cat no. ab109905) according to manufacturer's instructions. Complex III activity was determined as a gain in absorbance from the reaction of oxidized cytochrome *c* to the reduced form at 550 nm. ELISA was performed on cell lysates treated using Complex IV ELISA kit (Abcam, Cambridge, MA, USA, Cat no. ab109909) following manufacturer's instructions. Briefly, 10 µg protein was loaded into plates coated with CcO antibody and oxidation of reduced cytochrome *c* by the immunocaptured CcO from the sample was measured, using

absorbance at 550 nm. Kinetic reads were obtained and slopes directly correlating to enzyme activity were determined. Total amount of CcO was also assessed on the same samples.

Hemin rescue
For hemin rescue experiments, hemin (Sigma-Aldrich, Cat no H9039) 10 μM (final well concentration) was freshly prepared in DMSO. Cells pretreated with NMPP were supplemented with hemin in EBM2 containing 0.2% FBS for 6 hrs, followed by harvesting and processing for immunoblotting or ELISA.

FCCP titration, cell density determination, and cell energy phenotype
For determining optimal FCCP concentration, two ranges of FCCP were used (Figure S2A, B). Low range concentrations of 0.125, 0.25 and 0.5 μM and high range concentrations of 0.5, 1 and 2 μM were used for titration. We found 1 μM and 0.125 μM suitable for HRECs and RF/6A cells respectively. Similarly, for identification of appropriate cell seeding density to determine OCR and ECAR, cells at 5×10^3, 2×10^4 and 4×10^4 cells per well were plated overnight, and photographed using an EVOS fl digital microscope, followed by Seahorse XF analyses described below.

For assessment of cellular energy phenotype, cells (2×10^4 per well) were grown, as indicated, overnight followed by assessment of metabolic phenotype using the Seahorse XFp Cell Energy Phenotype test kit (Cat. No. 103275-100). Stressor mix was prepared by combining 1 μM oligomycin and 0.125 or 1 μM FCCP. Cells were incubated in base medium containing glutamine, sodium pyruvate and glucose for 1 hour in a room air incubator prior to measurements. Five cycles of readings were taken after the injection of stressor mix on the cells and kinetic traces indicating OCR and ECAR were produced in tandem.

Mitochondrial energetics
For measuring mitochondrial function, oxygen consumption rate (OCR) was measured using the Seahorse XFp Cell Mito Stress test kit (Cat No. 103010-100) (Figure S3) (Dranka et al., 2011) at the Indiana University School of Medicine Angio BioCore. Cells (2×10^4 per well) treated with NMPP (10 μM) or succinyl acetone (1 mM) were seeded overnight in XFp miniplates. Sensor cartridges were hydrated overnight in XF calibrant in a room air incubator. Next day, XF Base assay medium was prepared by addition of 5.5 mM glucose and 0.61 mM sodium pyruvate. For HRECs, 3.2 mM L-glutamine was used, while for RF/6A cells 1.6 mM concentration was used as per their respective routine culture medium compositions. Unbuffered base medium was filter sterilized followed by pH adjustment to 7.4 using 0.2 N NaOH solution. Cells were rinsed with base medium twice to remove overnight culture medium and this was replaced with base assay medium. Cells were incubated for a minimum of 1 hour in a room air incubator before proceeding with the assay. Mitochondrial inhibitors at final well concentrations of 1 μM oligomycin, 0.125 μM (for RF/6A cells) or 1 μM FCCP (for HRECs) and 0.5 μM rotenone/antimycin A were prepared using freshly buffered base medium. Mitochondrial inhibitors were then loaded into the hydrated sensor cartridges. After incubating cells in a room air incubator, XFp miniplates and the sensor cartridges were loaded into the instrument and OCR readings were determined using the XFp analyzer. The assay program was set up to measure three cycles of oligomycin followed by FCCP and a final injection of rotenone/antimycin A injected at indicated times. OCR in response to serial injections of oligomycin, FCCP and rotenone/antimycin mixture were recorded real-time in a kinetic assay.

For retinal ex vivo OCR measurements, protocols were adapted as previously described (Joyal et al., 2016; Kooragayala et al., 2015). Briefly, eyes were enucleated and retina was isolated from the posterior cup. Retinal punches (1 mm diameter) were dissected from an area adjacent to optic nerve to minimize variability in retinal thickness. Retinal punches were incubated in Seahorse XF DMEM Medium pH 7.4 containing 5 mM HEPES supplemented with 12 mM glucose and 2 mM L-glutamine for 1 hour in a room air incubator at 37°C. For OCR measurement, 0.5 μM FCCP and 0.5 μM rotenone/antimycin A were injected and OCR readings were determined.

Glycolytic function
Glycolytic parameters were measured using the Seahorse XF Glycolysis Stress test kit (Cat No. 103020-100). Cells (2×10^4 per well) were grown overnight followed by glucose starvation in assay medium containing sodium pyruvate and glutamine for 1 hour in a room air incubator. Compounds modulating

glycolysis were prepared using the same assay medium. Glucose at a final well concentration of 10 mM, oligomycin at 1μM and 2-deoxyglucose at 50 mM were loaded onto ports of the hydrated sensor cartridge followed by measurement of extracellular acidification rate (ECAR) using the XFp analyzer.

Quantification and Statistical Analysis

All Seahorse kinetic traces were analyzed using Wave 2.4 software (Agilent) and GraphPad Prism v. 8.0. FCS files for flow cytometry were analyzed using FlowJo v10. Comparisons between groups were performed with either unpaired, two-tailed Student's t-test or one-way ANOVA with Tukey's post-hoc tests as indicated. p values < 0.05 were considered statistically significant. Mean±SEM shown for all graphs unless indicated otherwise; n is listed in figure legends.

Supplemental References

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