Supplementary Information

Materials and Methods

Materials

Unless noted otherwise, reagents were obtained from Sigma-Aldrich (Saint Louis, MO) and tissue culture media and supplements were obtained from Gibco/ThermoFisher (Waltham, MA). Bafilomycin A1 (# 88899-55-2) was purchased from Toris (Minneapolis, MN). Salinosporamide A (Marizomib, # 437742-34-2) and Spautin-1 (#1262888-28-7) were purchased from Cayman Chemical (Ann Arbor, MI). 5-aminoimidazole-4-carboxamide riboside (AICAR, (# 72704) was purchased from StemCell Technologies (Cambridge, MA). Dorsomorphin was purchased from MP Biomedicals (#183060, Solon, OH). The human induced pluripotent (hiPSC) cell line, IMR90-4, was purchased from WiCell (Madison, WI).

Differentiation of RPE from human induced pluripotent cells (hiPSC)

The hiPSC was differentiated, as previously described.[1] Briefly, embryoid bodies were incubated in mTeSR-1 media (Stem cell technologies). For differentiation, mTeSR-1 was gradually replaced with NIM-KSR (DMEM/F12 nutrient medium, 1% N2 supplement, 1× nonessential amino acids (Gibco, #11140-050), 2 mM glutamax, 1.0 mM sodium pyruvate (MP Biomedicals, #199654), 2 μg/ml heparin, 10% knockout serum, 100 U/ml penicillin, and 100 μg/ml streptomycin), as follows: Day1, 1:3 (NIM-KSR:mTeSR-1); Day2, 1:1(NIM-KSR:mTeSR-1); and Day3-Day6, undiluted NIM-KSR. On Day7, the cell aggregates were seeded on 6-well plates (Corning) that were coated with 1% Matrigel (500 μl/well). From Day 7 to Day 21 the cell aggregates were kept in NIM-KSR media. After 21 days, when retinal cups appeared in the Matrigel cultures, the retinal cups were maintained in a serum free medium (DMEM-F12 (3:1), 2% B27, 1mM sodium pyruvate, 1× nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin). Media was changed 3 times a week. After 30-60 days, pigmented RPE cells appeared on the edge of the retinal cups. RPE cells were mechanically harvested, digested with 0.25% Trypsin-EDTA for 40 minutes, washed with 10% fetal bovine serum in DMEM high glucose medium, and plated onto 6-well plates that were coated with synthemax (5 $\mu g/cm^2$). RPE cells were maintained in KSR medium (10% knockout serum, 1% N2 supplement, 1× nonessential amino acids, 2 mM glutamax, 1 mM sodium pyruvate, 10 mM nicotinamide (Stem Cell Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin) supplemented with 10 μM rock inhibitor (Y27632 dihydrochloride, Enzo Life Sciences, Farmingdale, NY, #ALX-270-333-M005) until the cells became confluent when the rock inhibitor could be discontinued. The cultures were expanded by harvesting the cells with a 30 min incubation with trypsin (0.25% Trypsin-EDTA) and replating the cells on sythemax-coated T25 flasks (1 well/T25 flask). Cells were maintained in KSR with rock inhibitor until the cells were fully confluent. Cells were then frozen or, 5×10^5 cells were plated onto 12 mm Transwell inserts (Sigma-Aldrich, #3460) that were coated with synthemax (5 μg/filter). RPE cells were maintained in KSR media containing 10 μM rock inhibitor until cells were fully confluent. Cultures were then switched to SFM (DMEM, high glucose:F12 (3:1), 2% B27, 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamax, 100 U/ml penicillin, and 100 μg/ml streptomycin) for 60-90 days until the transepithelial electrical resistance (TER) reached a plateau. TER was measured with an EVOM resistance meter using EndOhm electrodes (World Precision Instruments, Sarasota, FL).[2]

Small Interfering RNA (siRNA)
The siRNAs for knockdown experiments, obtained from Dharmacon (Lafayette, CO), included On-target plus siRNA Reagents for humans: *CLDN19* (# LQ-016233-01-0005), *CLDN4* (# LQ-013612-00-0005), *SQSTM1*/P62 (# LQ-010230-00-0005), and a Dharmacon-recommended control siRNA, *PPIB* (Cyclophilin B, # D-001820-01-05). The *CLDN4* siRNA also served as a negative control, as claudin-4 is not expressed by RPE. The knockdowns were performed according to the manufacturer's protocol. Briefly, cultures of iPSC-RPE cells were fully differentiated on Transwell inserts and then transfected using 25nM siRNA (*CLDN19* or *CLDN4*) or 50nM siRNA (*SQSTM1* and *PPIB*) and DharmaFECT 4 transfection reagent (#T-2004-02). Cells were incubated with siRNA for 48 hours, then returned to SFM. Experiments were performed one-week post-transfection.

Western blotting
Total protein was isolated in ice-cold lysis buffer (0.1% SDS and 50 mM Tris, pH 8.0, containing protease inhibitor cocktail (Sigma Aldrich, # 11697498001). Protein concentration was measured with a nanodrop spectrophotometry (Thermo Fisher Scientific). samples were resolved on a 4-15% Tris–Cl gradient gel (Bio-Rad, # 456-8086) and Mini-PROTEAN Electrophoresis system (Bio-Rad). The proteins were transferred to immunoblot-PVDF membranes (Bio-Rad, # 162-0177). The blots were incubated in blocking buffer for 2 hr at RT and then in primary antibodies overnight at 4°C. After washing, the blots were incubated at RT with secondary antibodies that were conjugated with horseradish peroxidase (HRP). Antibodies are described in Supplementary Table 1. β-actin was used as the loading control. Blots were developed using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo fisher scientific, #34096) The fluorescent signal was acquired with a ChemiDoc MP imager and analyzed with Image Lab software (Bio-Rad).

Immunofluorescence and Confocal Microscopy
Cultures were fixed in PBS containing 2% paraformaldehyde, 1mM $CaCl_2$, and 1 mM $MgCl_2$ for 5 mins at RT, permeabilized with 0.1% Triton X-100 (Bio-Rad) in PBS buffer, and blocked with 5% normal donkey serum (EMD Millipore, S30-100ML) for 2 hours at RT. The cultures were incubated with primary antibodies (Supplementary Table 1) overnight at 4°C, followed by the appropriate secondary antibodies. Alexa Fluor 647 phalloidin (Invitrogen, # A22287) was used to label F-actin. DAPI (4′,6-diamidino-2-phenylindole) was used to label the nucleus. Cultures were mounted with Fluoromount-G (Southern Biotech, # 00-4958-02). Confocal images were acquired using a Zeiss LSM 510 EXCITER microscope (ZEISS, USA). Zen software (ZEISS) was used to analysis data. Images were prepared by optimizing the window settings for the test samples and using those settings for the controls.

Phagocytosis assays
Porcine POS were isolated using the protocol established by Mao and Finnemann.[3] RPE was incubated with POS, 0.5 ml of 2×10^7/particles/ml (~10 particles/cell) in SFM for 2 hr at 37°C in a humidified CO_2 incubator. Cultures were washed with 0.5 mM EDTA in 1× PBS to remove bound and unbound POS. Cells were incubated for 0, 6, 24, 48, and 96 hr upon which cultures were washed 3× with 1×PBS. Cell lysates were prepared using ice-cold lysis buffer (0.1% SDS, and 50 mM Tris, pH 8.0 containing protease inhibitor cocktail (#11697498001, Sigma Aldrich) and prepared for western blotting. Samples were blotted with anti-rhodopsin antibodies and the intensity of the bands were normalized to actin. Because rhodopsin forms multimers, all of the multimers were integrated when quantifying the blots.

Alternatively, phagocytosis was analyzed using a fluorescence activated cell sorter. Bovine rod POS, labeled with fluorescein isothiocyanate (FITC, Sigma-Aldrich) were a gift of J. Gong (Yale Univ. Dept. of Ophthalmology) and prepared as follows: Bovine rod POS were purchased from InVision BioResources (Seattle, WA), and labeled with FITC according to the manufacturer's protocol. Briefly, isolated POS membranes were pelleted in Hank's balanced salt solution, and re-suspended in 0.1 M sodium bicarbonate buffer, pH 9–9.5. FITC (2 mg/ml) was solubilized in dimethyl sulfoxide and added to the POS for a final concentration of 50 ng/ml. After a 2 hr incubation at room temperature with gentle shaking, the FITC-labeled POS were washed twice, pelleted in a micro-centrifuge tube (4 min at 4,500×*g*), and re-suspended in SFM at a concentration of 0.4 μg/μl. The FITC-ROS were counted under a fluorescent microscope and 25×10^6 particles fed to RPE cultured on Transwell filters (~25 POS particles/RPE cell). The cultures were incubated for 2 hr at 37°C in a humidified CO_2 incubator. Cultures were then rinsed with growth medium to remove excess FITC-POS and the incubation continued for 3 more hr. The cells harvested with 0.25% Trypsin-EDTA and washed with 1× PBS containing 2% fetal bovine serum. The samples were analyzed with a LSRII flow cytometer at Yale Flow Cytometry (Yale University School of Medicine, New Haven, CT). The data acquired with BD FACSDIVA software (BD Biosciences).

Pharmacology experiments

For all experiments, 500 μl of the final dilution of the following drugs were added to the apical medium chamber of the Transwell inserts, incubated in a humidified, 37°C incubator for 24 hrs, and assayed for phagocytosis. Western blotting for AMPK, pAMPK, and rhodopsin was performed following a 2 hr incubation with POS and 6 hr incubation without POS, as described above. Bafilomycin A1was dissolved in DMSO to form a 100 mM stock solution and diluted 1:1000 to form a 100 μM working solution in SFM. Marizomib was dissolved in DMSO to form a 100 μM stock solution and diluted 1:1000 to form a 100 nM working solution in SFM. AICAR was dissolved in DMSO to form a 75 mM stock solution and diluted 1:18.75 to form a 4 mM working solution in SFM. Dorsomorphin was dissolved in DMSO to form a 10 mM stock solution and diluted 1:500 to form a 20 nM working solution in SFM.

Quantitative Real-Time, Reverse-Transcriptase Polymerase Chain Reaction (qRT^2-PCR)

Total RNA was extracted using the RNeasy mini kit (Qiagen). cDNA was reverse transcribed according to the manufacturer's instructions (iScript cDNA Synthesis Kit, BioRad). Expression of mRNA was assessed using iTaq SYBR Green Supermix (BioRad) and RNA primers that were synthesized at the Keck Center (Yale University). The primers are listed in Supplementary Table 2. Three independent experiments were performed. *GAPDH* was used as a control to normalize the data. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.[4]

Enzyme Assays

Catalase (# 707002) and Superoxide Dismutase (# 706002,) assay kits were purchased from Cayman Chemical (Ann Arbor, MI). Cells were scraped from the Transwell inserts in PBS and centrifuged. The cell pellet was resuspended and sonicated in 500 μl/filter of cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, 70 mM sucrose, and protease inhibitor cocktail. Samples were centrifuged at 1,500×*g* for five min at 4°C. Supernatants (10 ul) were assayed according to the manufacturer's instructions. Three independent experiments were performed.

Statistics

Statistical analyses that were performed using Prism8 software (GraphPad, San Diego, CA) included: Student's t-test for two-way comparisons, Student's one-way t-test to compare the deviation of ratios from 1.0, and Pearson's correlation co-efficient. The number of independent experiments is indicated in the figure legends. p-values < 0.05 were considered significant.

References

1. Singh D, Wang SB, Xia T, et al. A biodegradable scaffold enhances differentiation of embryonic stem cells into a thick sheet of retinal cells. *Biomaterials* 2018;154:158-168.
2. Peng S, Gan G, Rao VS, Adelman RA, Rizzolo LJ. Effects of proinflammatory cytokines on the claudin-19 rich tight junctions of human retinal pigment epithelium. *Invest Ophthalmol Vis Sci* 2012;53:5016-5028.
3. Mao Y, Finnemann SC. Analysis of Photoreceptor Outer Segment Phagocytosis by RPE Cells in Culture. *Methods Mol Biol* 2013;935:285-295.
4. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402-408.

Supplementary Table 1: Antibodies

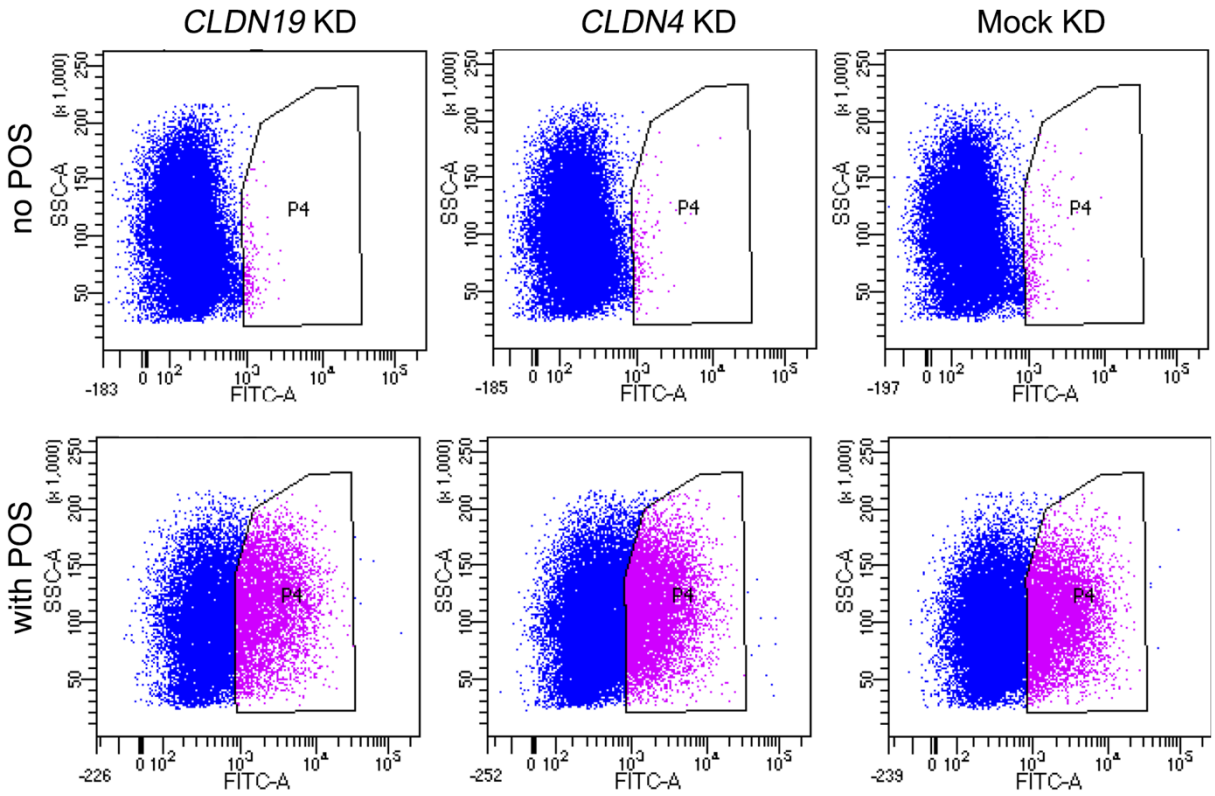
Antigen	Host	[a]Procedure/ Dilution[a]	Conjugate	Catalog number	Supplier
β-Actin (BA3R)	Mouse Monoclonal	IB 1:5000	None	MA5-15739	Thermo Fisher Scientific
ADAM9	Rabbit Polyclonal	IB 1:500	None	AB2959	Millipore
AMPKα (D5A2)	Rabbit Monoclonal	IB 1:1000	None	5831	Cell Signaling
Phospho-AMPK (D4D6D)	Rabbit Monoclonal	IB 1:1000	None	50081	Cell Signaling
Claudin-19 (C-5)	Mouse Monoclonal	IF 1:200 IB 1:1000	None	sc-365967	Santa Cruz
LC3B (D11)	Rabbit Monoclonal	IB 1:1000	None	3868	Cell Signaling
PI3 Kinase class III (D9A5)	Rabbit Monoclonal	IB 1:1000	None	4263	Cell Signaling
Raptor (24C12)	Rabbit Monoclonal	IB 1:1000	None	2280	Cell Signaling
Phospho-raptor	Rabbit Polyclonal	IB 1:1000	None	2083	Cell Signaling
Rhodopsin (4D2)	Mouse Monoclonal	IB 1:1000	None	MABN15	Millipore
SQSTM1/P62	Rabbit Polyclonal	IB 1:1000	None	PA5-34781	Thermo Fisher Scientific
Tyrosinase (JA52-11)	Rabbit Monoclonal	IB 1:500	None	NBP2-67232	Novus Biologicals
Ubiquitin (Ubi-1)	Mouse Monoclonal	IB 1:1000	None	MAB1510	Millipore
ULK1 (D8H5)	Rabbit Monoclonal	IB 1:1000	None	8054	Cell Signaling
Phospho-ULK1 (D1H4)	Rabbit Monoclonal	IB 1:1000	None	5869	Cell Signaling
ZO-1	Rabbit Polyclonal	IF 1:250	None	18-7430	Thermo Fisher Scientific
Rabbit IgG	Goat	IB 1:10000	Horseradish Peroxidase	7074P2	Cell Signaling
Mouse IgG	Horse	IB 1:10000	Horseradish Peroxidase	7076S	Cell Signaling
Rabbit IgG	Donkey	IF 1:250	Cy2	711-225-152	Jackson ImmunoResearch
Mouse IgG	Donkey	IF 1:250	Cy3	715-165-151	Jackson ImmunoResearch

[a]IF, Immunofluorescence; IB, Immunoblot

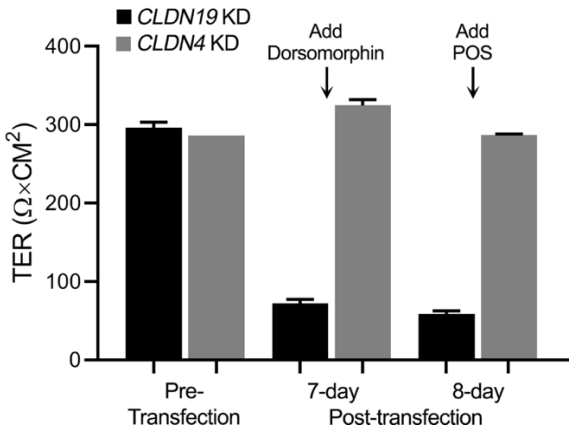
Supplementary Table 2: Primers designed using http://primer3.ut.ee/

Gene		Sequence (5'→3')
Beclin-1	Forward	CTGGTAGAAGATAAAACCCGGTG
	Reverse	AGGTAGAGCGTGGACTATCCG
LC3	Forward	ACATGAGCGAGTTGGTCAAG
	Reverse	GTTCACCAGCAGGAAGAAGG
BCL-2	Forward	GCCTTCTTTGAGTTCGGTGG
	Reverse	TACTCAGTCATCCACAGGGC
P62	Forward	TGCCCAGACTACGACTTGTG
	Reverse	GAGAAGCCCTCAGACAGGTG
GAPDH	Forward	TCACCAGGGCTGCTTTTAAC
	Reverse	GCAAAGCTTCCCGTTCTCAG
MTOR	Forward	TCCGAGAGATGAGTCAAGAGG
	Reverse	CACCTTCCACTCCTATGAGGC
ATG5	Forward	TCGAGATGTGTGGTTTGGACG
	Reverse	ATGGTTCTGCTTCCCTTTCAG
UVRAG	Forward	GCACCCTAGCCAAGAACAAG
	Reverse	GGGTAGGAGAGTGCCATTG
ATG12	Forward	TGCAGCTTCCTACTTCAATTGC
	Reverse	TTTCTTGGTGTCGCCAGCAG
ITGB1	Forward	TTCAAGGGCAAACGTGTGAG
	Reverse	GAAGGCTCTGCACTGAACAC
ULK1	Forward	GTGGGCAAGTTCGAGTTCTC
	Reverse	CCTCCAAATCGTGCTTCTCG
MT1F	Forward	ACCTCCTGCAAGAAGAGCTG
	Reverse	CTTCTCTGACGCCCCTTTG
MT1H	Forward	TCCTGCAAGTGCAAAAAGTG
	Reverse	CTTCTCTGACGCCCCTTTG
MT1B	Forward	TCCTGCAAGTGCAAAGAGTG
	Reverse	TGATGAGCCTTTGCAGACAC
MT1G	Forward	TCCTGCAAGTGCAAAGAGTG
	Reverse	CAGCTGCACTTCTCCGATG
MT1X	Forward	ACCTCCTGCAAGAAGAGCTG
	Reverse	CTTGTCTGACGTCCCTTTGC
MT1A	Forward	TCCTGCAAATGCAAAGAGTG
	Reverse	CAGCTGCACTTCTCTGATGC
MT1E	Forward	CACAAACCCCAACTGTACCC
	Reverse	CTCAGAACCCAGACCCAGAG
MT2A	Forward	ATGGATCCCAACTGCTCC
	Reverse	CAGCTTTTCTTGCAGGAGG

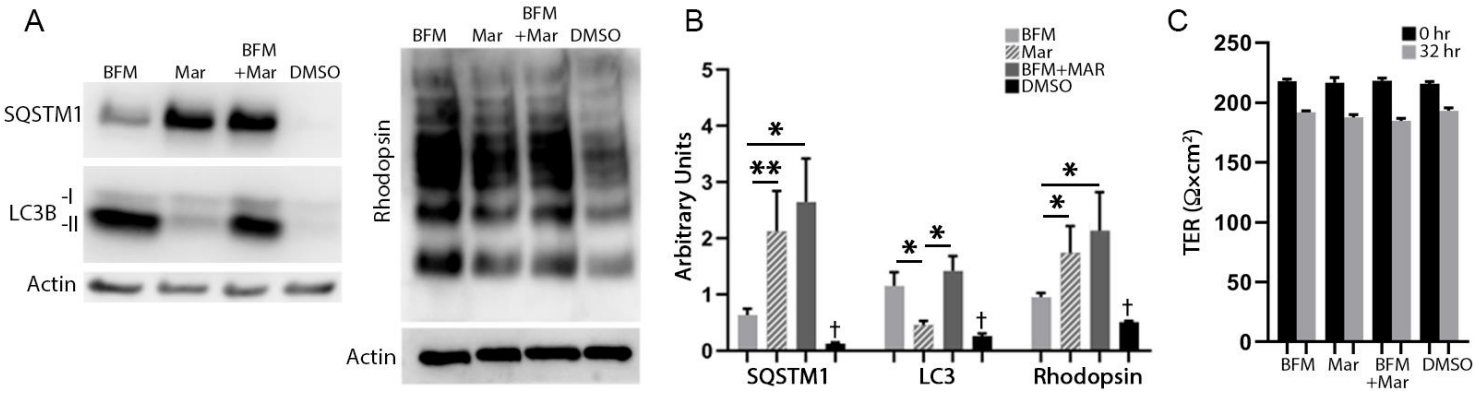
Supplementary Figures



Supplementary Fig. S1. Knockdown of CLDN19 had no effect on the binding and internalization of FITC-labeled POS. Cultures were transfected with siRNA as indicated above the panels. A representative of three independent experiments is shown. The P4 fraction contains FITC-positive cells. The background was very low, and no significant difference was observed among the three conditions.



Supplementary Fig. S2. Dorsomorphin does not affect the functionality of RPE. Seven days post-second transfection, cultures were treated with dorsomorphin for 24 hr. Cultures were then incubated with POS for 2 hr followed by an EDTA wash to remove non-phagocytized POS. TER was measured 6 hr later, before harvesting the cells for analysis, as reported in Fig. 5B,C&D. (n = 3)



Supplementary Fig. S3. Inhibition of proteasomes retarded the degradation of rhodopsin and increased the expression of SQSTM1. Cultures were incubated for 24 hr in DMSO or DMSO plus bafilomycin A1 (BFM), marizomib (Mar), or BFM + Mar. POS were added for 2 hr, cultures washed with EDTA to remove bound and unbound POS, and incubated for 6 more hr. A) Cell lysates were analyzed by western blotting. B) Quantification of the western blots. C) TER at the start and end of the experiment. As expected, the lysosomal inhibitor, bafilomycin, increased the steady-state levels of LC3 and SQSTM1. Although marizomib, a specific inhibitor of proteasomes, would be expected to increase the steady-state levels of SQSTM1, the small increase in LC3B and the decreased degradation of rhodopsin was novel. There was no apparent synergy between bafilomycin and marizomib. The TER of all the cultures decreased slightly ($p < 0.02$), but there was no statistical difference between the test samples and the DMSO control. Error bars, SE; †all comparisons with DMSO, $p < 0.05$; * $p < 0.05$; ** $p < 0.01$ (n=4-9)