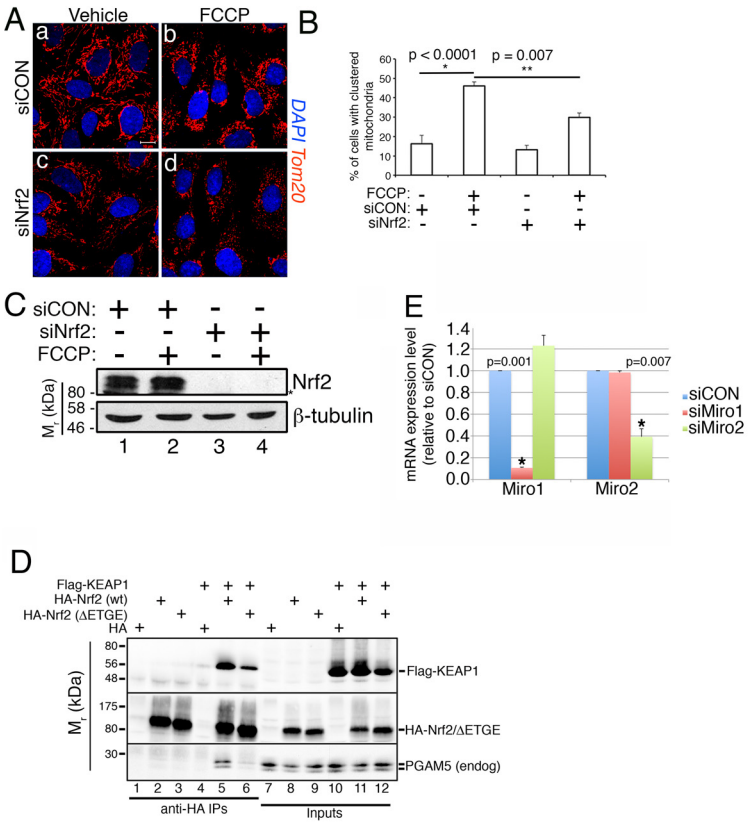
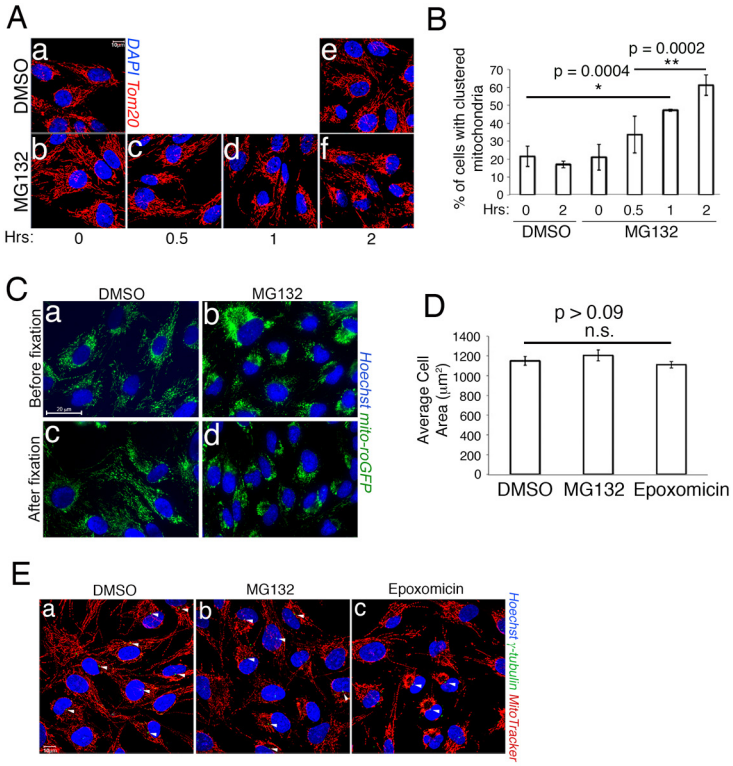
SUPPLEMENTAL FIGURES



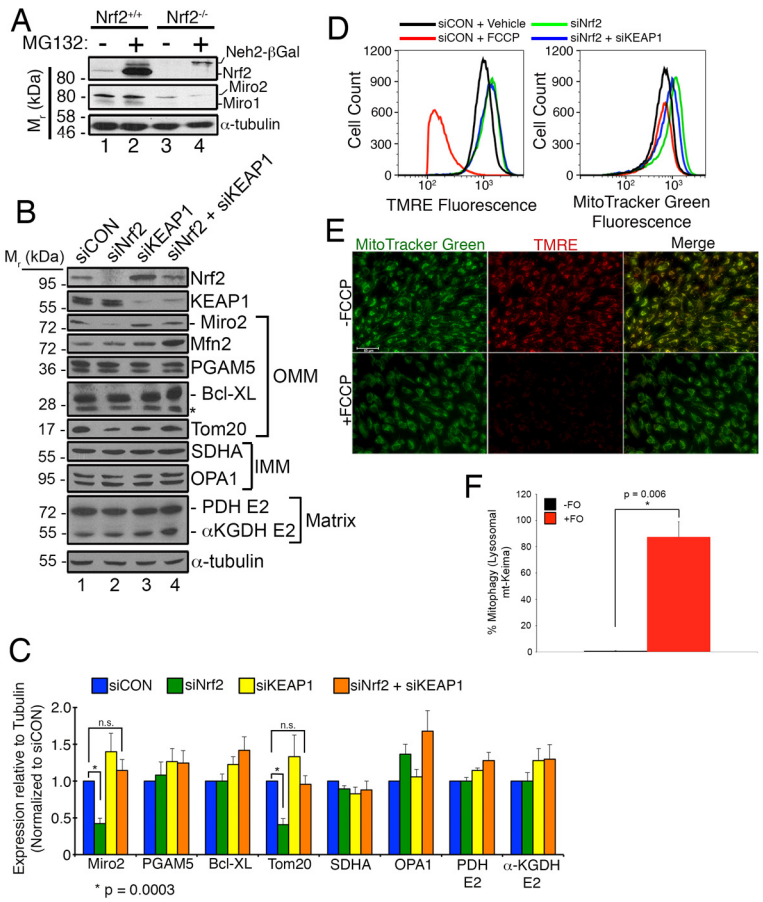
Supplemental Figure 1

Supplemental Figure 1. (A) RPE-1 cells transfected with siCON or siNrf2 and treated with 5 mM FCCP for 4 hrs. Mitochondria labeled with a-Tom20 (red) and nuclei with DAPI (blue). Size bar is 10 μm. **(B)** Graph of mitochondrial clustering frequency from (B). Data compiled from 3 independent experiments. Error bars reflect +/- S.D. **(C)** Representative western blot demonstrating Nrf2 knockdown efficiency. Asterisk denotes non-specific band recognized by a-Nrf2 antibody. The migration of molecular weight markers is indicated on the left. **(D)** HEK293T cells were transfected with the indicated plasmids and 2 days later lysed for anti-HA immunoprecipitations. HA-tagged Nrf2 co-precipitated Flag-KEAP1 and endogenous PGAM5 (lane 5). ETGE is a 4-residue motif within the Neh2 domain of Nrf2 that mediates binding to KEAP1. The DETGE Nrf2 mutant is deficient in KEAP1 binding and and fails to co-precipitate endogenous PGAM5 (lane 6). The migration of molecular weight markers is indicated to the left of each blot. **(E)** Graph of qPCR data demonstrating that siRNAs targeting Miro1 and Miro2 selectively reduce mRNA levels of each intended target. mRNA expression levels for each sample are compared to siCON. Statistical significance in (B) was determined by two-way ANOVA with Tukey's post-hoc correction and in (E) by one-way ANOVA.



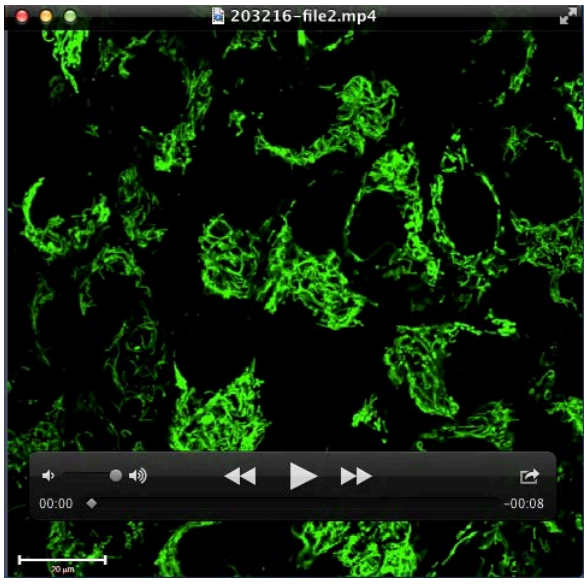
Supplemental Figure 2

Supplemental Figure 2. (A) RPE-1 cells were treated with DMSO or 10 mM MG132 for the indicated times. Mitochondria labeled with a-Tom20 (red) and nuclei with DAPI (blue). **(B)** Graph of mitochondrial clustering frequency from (A). Data compiled from 3 independent experiments. Error bars reflect +/- S.D. **(C)** mito-roGFP-expressing RPE-1 cells were treated with DMSO or 10 mM MG132 for 2 hrs. Epifluorescent microscopy was performed before (panels a and b) and after (panels c and d) fixation in 3.7% formaldehyde. **(D)** Graph of cell area in RPE-1 cells treated with DMSO, 10 mM MG132, or 1 mM epoxomicin for 2 hrs. 20X phase images were captured and free-form ROIs were drawn around cell borders. Cell area was measured using OpenLab™ software. Graph depicts data compiled from 3 independent experiments. Error bars reflect +/- S.E.M. **(E)** RPE-1 cells were treated with DMSO, 10 mM MG132, or 1 mM epoxomicin for 2 hrs and then fixed with ice-cold methanol and permeabilized with 0.02% Triton X-100. Centrosomes were visualized with anti-g-tubulin (green, examples marked with white arrowheads), mitochondria with MitoTracker (red), and nuclei with Hoechst 33342 (blue). Size bars in (A), (C) and (E) are 10 µm. Statistical significance in (B) and (D) was determined by two-way and one-way ANOVA, respectively, with Tukey's post-hoc corrections.

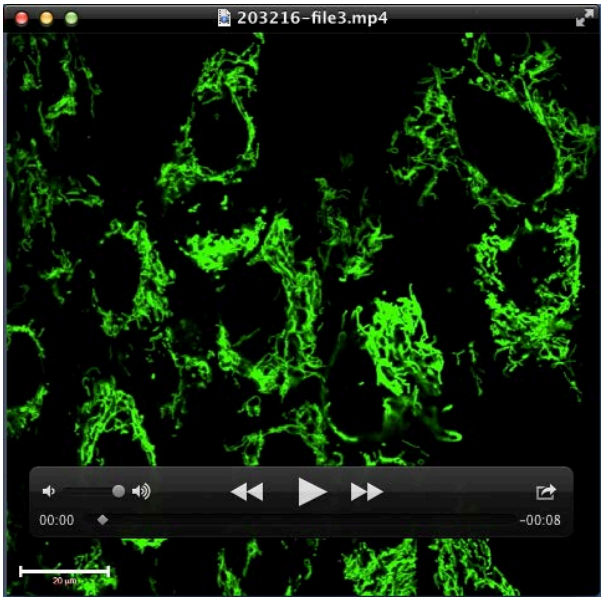


Supplementary Figure 3

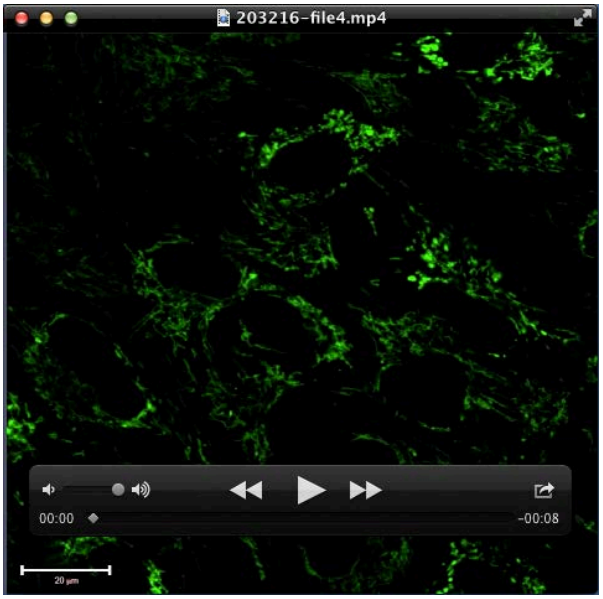
Supplemental Figure 3 **(A)** Western blots showing that Nrf2 knockout ($Nrf2^{-/-}$) MEFs have reduced Miro proteins and MG132 treatment stabilizes residual Neh2-bGal. The migration of molecular weight markers is indicated to the left of each blot. Strain-matched wild type control MEFs ($Nrf2^{+/+}$) shown for comparison. **(B)** Representative western blots showing levels of mitochondrial proteins (OMM – outer mitochondrial membrane; IMM – inner mitochondrial membrane) in cells transfected with the indicated siRNAs. PDH E2 and a-KGDH E2 were both detected with an antibody against lipoic acid. **(C)** Graph showing densitometric quantification of each mitochondrial protein in (B) relative to tubulin loading control. All data are normalized to siCON, which was set at a value of 1. Graph represents data compiled from 6 independent experiments. **(D)** Representative FACS traces for measuring TMRE and MTG uptake in cells treated with the indicated siRNAs or with FCCP as in Figs. 8F-8G. **(E)** Photomicrographs of control and depolarized mitochondria. TMRE uptake by mitochondria does not occur in cells treated with 5 mM FCCP. Scale bar is 65 mm. **(F)** Mitophagic flux in RPE-1 cells expressing mt-mKeima and YFP-parkin. Cells were treated with vehicle (-FO, black bar) or a combination of 5 mM FCCP and 5 mM Oligomycin A (+FO, red bar) for 4 hrs prior to being harvested for FACS. Graph represents % of cells containing lysosomal mt-mKeima and was compiled from 3 independent experiments. Statistical significance in (C) and (F) were determined by one-way ANOVA and paired, two-tailed t-test, respectively. Error bars represent +/- S.E.M.



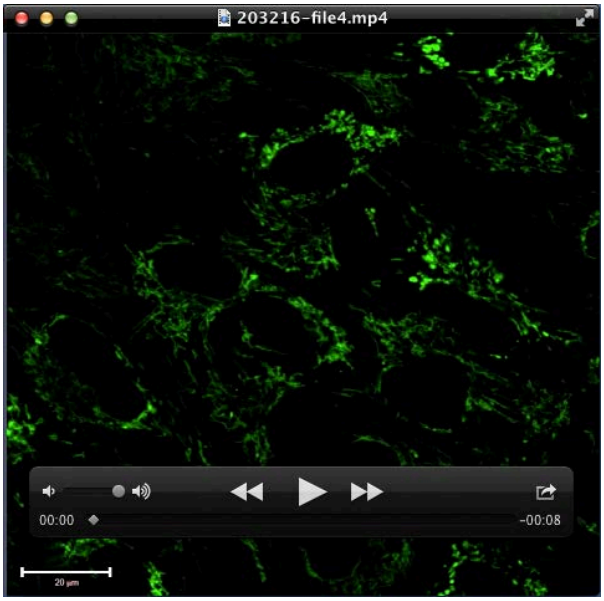
Supplemental Movie 1 – Mito-roGFP cells treated with 4 mg/mL nocodazole for 2 hrs prior to the start of filming show a near complete loss of mitochondrial motility due to depolymerization of microtubules.



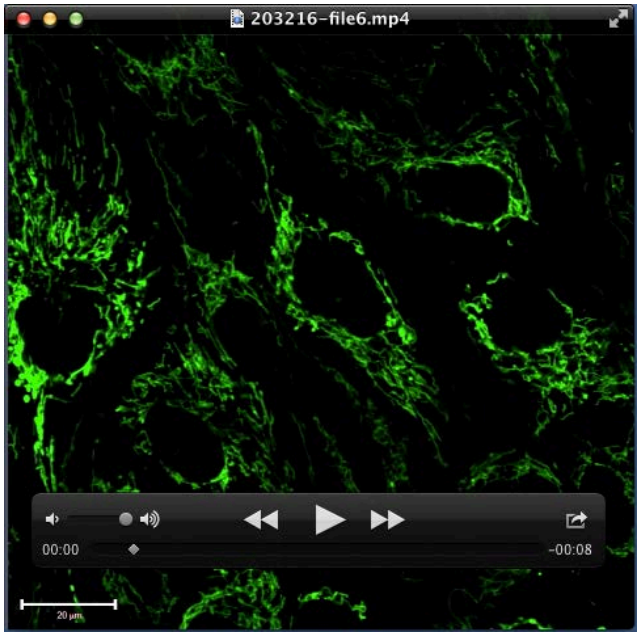
Supplemental Movie 2 – Mito-roGFP cells matched to those in Supplemental Movie 1 were pre-treated with nocodazole for 2 hrs prior to the addition of 10 mM MG132 and show no discernible retrograde trafficking and perinuclear accumulation of mitochondria due to depolymerization of microtubules.



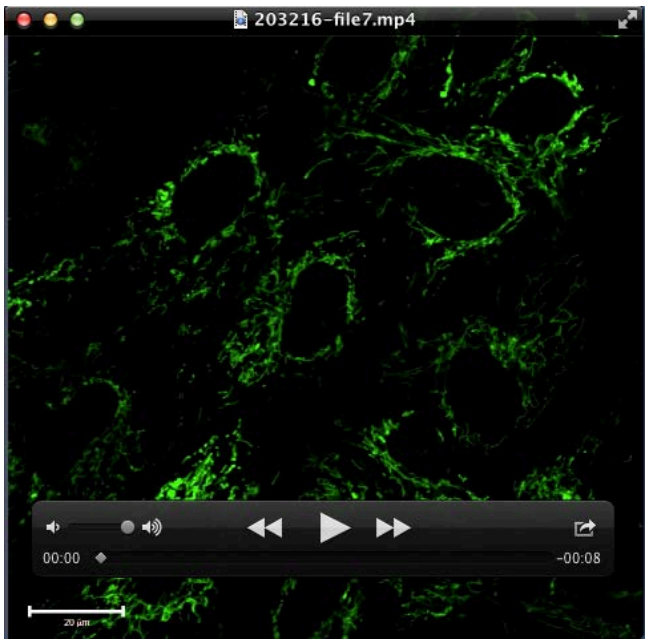
Supplemental Movie 3 – Vehicle (DMSO)-treated mito-roGFP cells show rapid mitochondrial trafficking, as well as fission and fusion activity.



Supplemental Movie 4 – 10 mM MG132-treated mito-roGFP cells matched to vehicle-treated cells in Supplemental Movie 3 show rapid fission and fusion, with a net retrograde mitochondrial trafficking leading to perinuclear clustering of the mitochondria. Arrows denote cells displaying juxtanuclear mitochondrial rings induced by proteasome inhibition.



Supplemental Movie 5 – Mito-roGFP cells transfected with siNrf2 and then treated with DMSO show no discernable defects in mitochondrial fission, fusion, or trafficking.



Supplemental Movie 6 – Mito-roGFP cells transfected with Nrf2 siRNA and then treated with 10 mM MG132 show rapid fission and fusion, but attenuated mitochondrial retrograde trafficking and perinuclear clustering.

Supplemental Table: *Antibodies and Chemicals*

Protein	Species	Company	Catalog #	Immunofluorescence/Western Dilutions
Tom20	Mouse	BD Bioscience	612278	1:1000/NA
β-tubulin	Mouse	Sigma-Aldrich	T4026	1:2500/1:1000
PGAM5	Rabbit	Novus	NBP1-92257	1:1000/1:500
HA (12CA5)	Mouse	Univ of Virginia	Hybridoma Facility	NA/1:1000
γ-tubulin	Rabbit	Sigma-Aldrich	T5192	1:1000/NA
Drp1	Mouse	BD Transduction	611113	NA/1:1000
Bcl-XL	Rabbit	Cell Signaling	2762	NA/1:1000
SDHA	Mouse	Santa Cruz	sc-377302	NA/1:500
OPA1	Mouse	BD Transduction	612606	NA/1:500
Hsp60	Goat	Santa Cruz	sc-1052	1:1000/1:1000
GFP	Mouse	Rockland	600-301-215	NA/1:500
Hsp70	Mouse	Enzo	ADI-SPA-810	NA/1:1000
α-tubulin	Mouse	Sigma-Aldrich	T9026	NA/1:1000
Nrf2	Rabbit	Plafker lab	In-house antibody	1:300/1:2000
p62/SQSTM1	Mouse	Abcam	Ab56416	NA/1:1000
KEAP1	Rabbit	Proteintech	10503-2-AP	NA/1:1000
Mfn2	Rabbit	Weissman lab	In-house antibody	NA/1:1000
Miro1*	Mouse	Abcam	Ab55035	NA/1:500
KGDH and PDH	Rabbit	Szweda Lab	In-house antibody	NA/1:1000

*Detects Miro1 and Miro2

Chemical	Company	Concentration
MG132	Boston Biochem	10 μM
Epoxomicin	Cayman Chemicals	1 μM
Nocodozole	Sigma-Aldrich	4 μg/ml
Actinomycin D	Gibco	1 mM
Cycloheximide	Sigma-Aldrich	50 mM
FCCP	Cayman Chemical	5 mM
H_2O_2	Sigma-Aldrich	1 mM
DTT	Sigma-Aldrich	1 mM
Oligomycin A	Sigma-Aldrich	5 μM

siRNA oligomers

Gene	Company	Catalog #
Control (siCON)	Dharmacon	D-001220-01
Nrf2	Dharmacon	L-003755-00-0005
PGAM5	Ambion	4392420
KEAP1	Cell Signaling	5289
p62/SQSTM1	Cell Signaling	6399
Miro1	Dharmacon	L-010365-01-0005
Miro2	Dharmacon	L-008340-01-0005
Drp1	Dharmacon	L-012092-00-0005
Mfn2	Dharmacon	L-012961-00-0005
Cul3	Qiagen	SI02225685