Supporting Information

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SI Materials and Methods

Plasmids. *MitoDsRed* was subloned from pDsRed2-Mito vector (Clontech) into the pAct-PPA expression plasmid. *EGFP* was cloned into pDsRed2-Mito vector in place of the *DsRed* gene and subcloned into the pAct-PPA expression plasmid. N-terminal Flag tag Drp1 and Fis1 were obtained by amplification from cDNA clones (AT04516 and RE29957, respectively) and cloned into the pAct-PPA expression plasmid. C-terminal Flag tag Mfn and OPA were obtained by amplification from cDNA clones (RE04414 and GH13793, respectively) and subcloned into the pAct-PPA expression plasmid. *parkin* was amplified from a cDNA clone (GM10489) and subcloned into a pMK33 copper-inducible expression plasmid. EGFP was cloned at the C-terminal end of the *parkin* gene. The HA-Ub construct was obtained from Y. Wang (1).

Real-Time Quantitative PCR. Real-time PCR was performed using the SYBR Green Master Mix method (Sigma) with a Bio-Rad MyiQ system. Reactions were performed in a 25-μl reaction mixture containing 12.5 μl Master Mix, 50–900 nM of each primer depending on the primers, 1 μl DNA sample from the reverse-transcription reaction, and nuclease-free water. The PCR protocol used consisted of a 30-s denaturation at 95 °C followed by 30 s annealing at 60 °C and 30 s at 72 °C for 40 cycles. The housekeeping gene *GAPDH* or *Rpl21* was used as the internal control. PCR primers for *PINK1*, *parkin*, *Mfn*, *Drp1*, *Fis1*, and *Opa1* were designed using GenScript real-time PCR (TaqMan) primer design and are listed below. Primers were optimized to find the concentration that had the lowest C_T value. Relative quantitation was performed using the comparative C_T method. Data were normalized by subtracting the mean C_T value of the housekeeping gene (reference gene) from the mean of the C_T value of the gene of interest (ΔC_T) for each experimental condition. The ΔC_T value at the different times of treatment was compared to that of the control untreated sample ($\Delta\Delta C_T$). The amount of gene expression, normalized to an endogenous reference, was then determined using $2^{-\Delta\Delta C}{}_T$. The efficiencies of target and reference amplifications were found to be the same.

Live Imaging. Cells were grown on imaging dishes (Chamber slide Lab-tek II 8; Fisher). After appropriate treatment, cells were treated with the selective mitochondrial dye rhodamine 123 (200 μM; Invitrogen) for 40 s, washed three times with either PBS or Schneider's medium, and imaged live in growing medium on a Deltavision DV microscope (×100 objective; NA:1.40), using standard epifluorescence. Ten separate fields per well were imaged, each field containing four to seven cells.

Immunocytochemistry. S2R+ cells were cotransfected with *parkin-GFP* and mitoDsRed constructs as described above. Two days after transfection, cells were plated on imaging dishes (Chamber slide Lab-tek II 8; Fisher) and subjected to appropriate RNA interference (RNAi) probe treatment for 2 days before being treated with 10 mM paraquat (6 h) or 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (2 h). Bafilomycin A1 (Sigma) was used at 20 nM (2 h). After treatment, the medium was removed and the cells were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. Cells were permeabilized with 0.2% TritonX-100 (Sigma) in PBS (10 min at room temperature) and then incubated with blocking solution (1% BSA in PBS, 0.2% TritonX) for 30 min. Cells were incubated in blocking solution for 1 h at room temperature using specific primary antibodies. An appropriate secondary fluorochrome-conjugated antibody was added in blocking solution for 1 h at room temperature (1:400; Molecular Probes). Images were acquired on a DeltaVision DV microscope (100× objective; NA:1.40) by using standard epifluorescence and deconvolved to improve clarity.

Immunoprecipitation. Cells were transfected as described above. Cells were lysed in 50 mM Tris–HCl, pH 8, 150 mM HCl, 1 mM $MgCl_2$, 2 mM EGTA, 1% TritonX, 10% glycerol, 10 mM NEM, 50 μM MG132, and protease inhibitor mixture (Roche) were added fresh before harvesting cells. Protein A agarose beads were incubated with anti-Flag (1:50) or anti-GFP (1:) antibodies for 1 h at 4 °C. Immunocomplexes were collected by incubating previously prepared Protein A agarose beads with 1 mg of whole-cell extract, rocking at 4 °C overnight. Beads were then resuspended in 4× Laemmli buffer and centrifuged at 13,000 × *g* for 10 min. Cleared samples were subjected to Western blot with the indicated primary antibodies.

Primers. Each of the RNAi knockdown primers, listed below, also contained a T7 promoter sequence at the 5′ end:

PINK1: CAATGTGACTTCTCCAGCGA and TCGTAGCGTTTCATCAGCAG;
Parkin: CTGTTGCAATTTGGAGGGA and CTTTGGCACGGACTCTTTCT;
OPA1: GGAGACGCTGCAGACGGAGA and GGTCCTGCAGATCAGACTCC;
Drp1: ACATCATGGCCACGCAATT and CCTGCTGCACTTCGTTG;
Fis1: ATGATTTTGGAGGAACTGGC and GGGAGCGAACTTGATCATTT;
Mfn: GGAACCTCTTTATTCTCTAT and GGTTTGCTTTGCCCCAACAT.

dsRNAs for *Atg5* and *Atg8b* were obtained from the Sheffield RNAi screening facility.

The following real-time quantitative PCR primers were used:

GAPDH: GCGAACTGAAACTGAACGAG and CCAAATCCGTTAATTCCGAT;
Rpl21: GACGCTTCAAGGGACAGTATCTG and AAACGCGGTTCTGCATGAG;
Parkin: AGTACACCGTGGACCCAAAT and TGTGCTGACTTTGATGGTGA;
PINK1: AACAGTCCGGAGATCCTACAG and GACGACCCTCGCACATAA;
Mfn: ACGAATTGCTTCTGCCAAGT and TGATGTTCACCACATTTAGCTTC;
Drp1: CTCAGATTTCGTCGCACAGT and ACGTGGTTCTGCTCATGC;
OPA1: AGATTGCGCGAGTCTGGT and TTAGCGGAGATTTGCGAGA;
Fis1: TCGCTCCCTAGAGGAATATATTAAA and TAGCCATAGCAATGCCAAGT.

1. Wang Y, Price MA (2008) A unique protection signal in Cubitus interruptus prevents its complete proteasomal degradation. *Mol Cell Biol* 28:5555–5568.

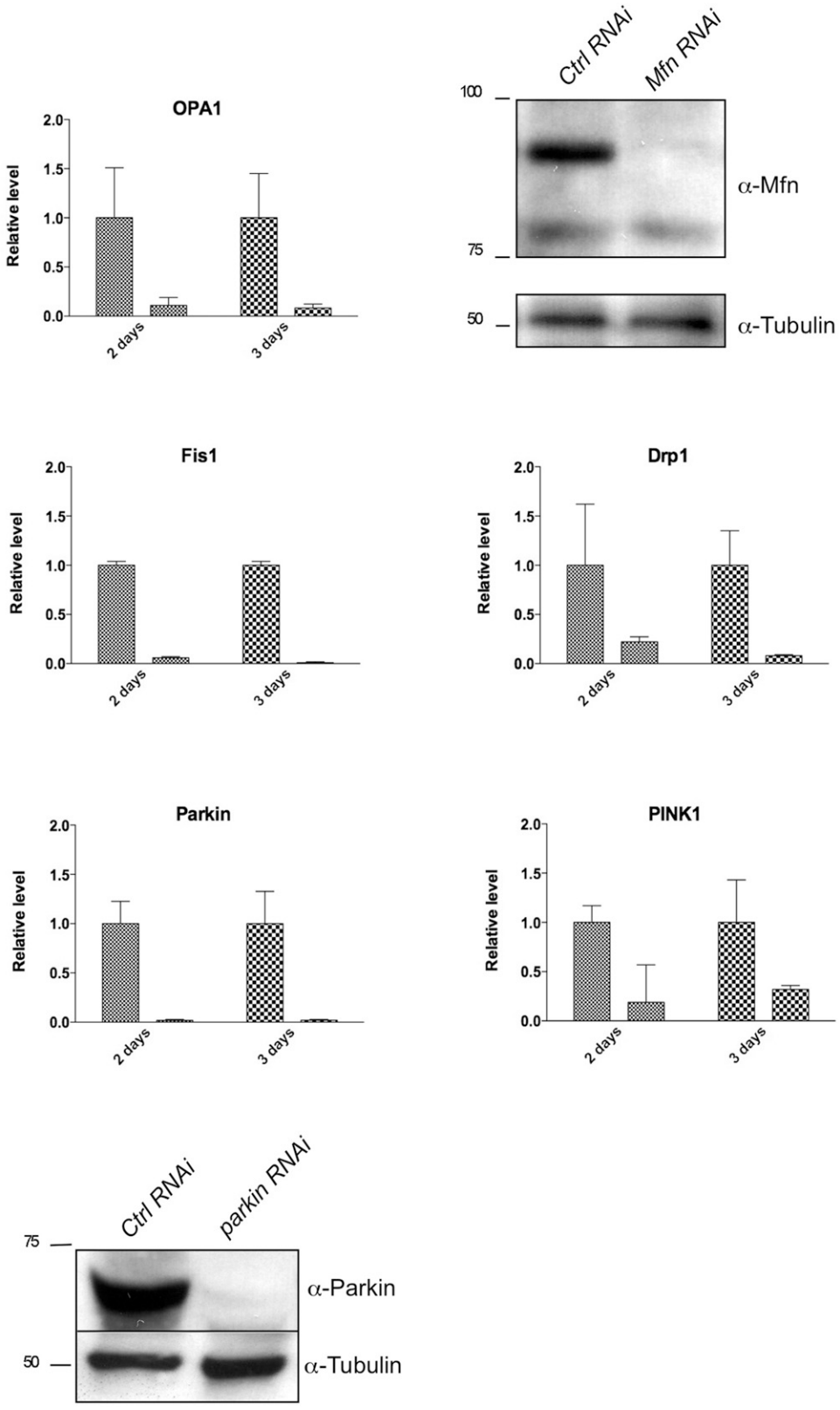

Fig. S1. Analysis of RNAi knockdown efficiency in *Drosophila* S2R+ cells. Quantitative real-time PCR was performed for target genes to determine the degree of RNAi-induced knockdown of endogenous message after 2 and 3 days. Knockdown efficiency was preferentially assessed by Western blotting where possible for Parkin and Mfn.

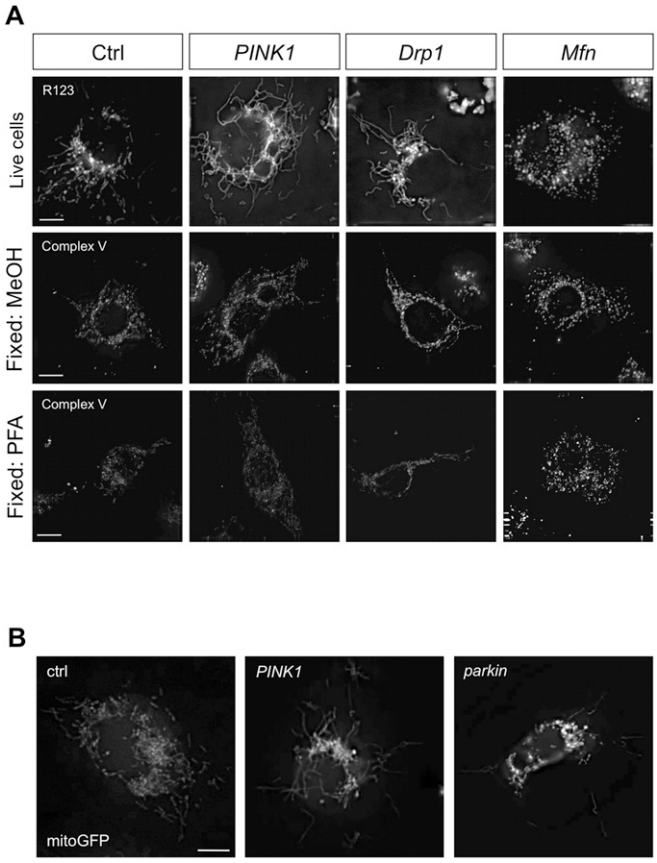

Fig. S2. Sample preparation methods for comparing mitochondrial morphology. (*A*) S2R+ cells were treated with the indicated dsRNAs [control (Ctrl) dsRNA is targeted against *DsRed*] and prepared for imaging using standard protocols. One sample were stained with the mitochondria-selective vital dye rhodamine 123 and imaged live. Other samples were fixed with either methanol (MeOH) or paraformaldehyde (PFA) by standard methods. Fixed cells were immunolabeled with anti-Complex Vα antibody to mark mitochondria. *Mfn* RNAi causes fragmentation that is not changed by fixation, whereas *Drp1* RNAi results in long mitochondria in live cells consistent with other reports. *PINK1* RNAi also reveals elongated mitochondria. Fixation by either method dramatically reduces the appearance of long mitochondria. (*B*) S2R+ cells treated with control, *PINK1*, or *parkin* RNAi as before. Cells are transfected with plasmids to express *mitoGFP* to visualize mitochondria. (Scale bar: 5 μm.)

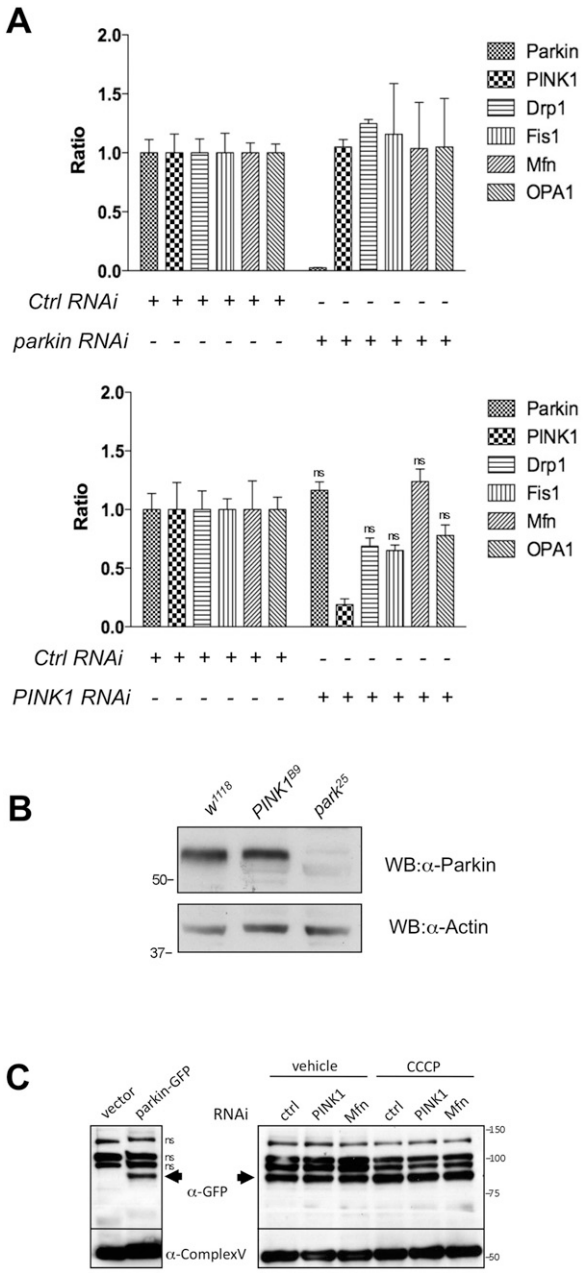

Fig. S3. Analysis of gene expression changes upon *PINK1* or *parkin* RNAi knockdown. (*A*) Quantitative real-time PCR was performed for key mitochondrial fission and fusion regulators after 3 days of RNAi treatment of *PINK1* or *parkin* compared with control. Statistical significance was determined by one-way ANOVA with Bonferroni correction, but no significant changes were detected. (*B*) The level of Parkin protein was also assessed in the *PINK1*^{B9} null mutant. (*C*) Western blot of S2R+ cells transfected either with empty vector (lane 1) or *parkin-GFP*–expressing plasmid (lanes 2–8) and treated with the indicated RNAi probe. A total of 10 μM CCCP or vehicle was added 2 h before harvesting cells.

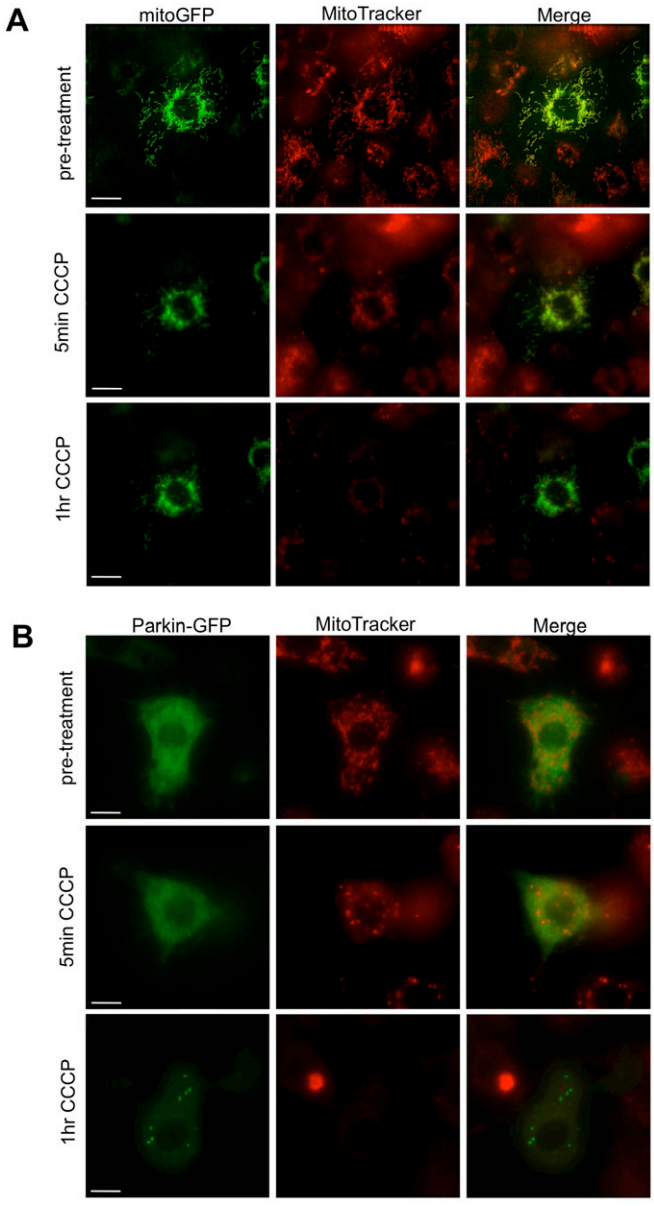

Fig. S4. Effect of CCCP treatment on mitochondrial membrane potential and Parkin-GFP localization over time. S2R+ cells were transfected with (*A*) *mito-GFP* or (*B*) *parkin-GFP*–expressing plasmids and incubated with the mitochondrial membrane potential sensitive dye MitoTracker Red before treatment with CCCP. Images were taken before CCCP treatment and after 5 min or 1 h. Mito-GFP labels all mitochondria in transfected cells whereas MitoTracker Red intensity reduces over time, indicating that membrane potential is lost. As membrane potential is lost, Parkin-GFP is recruited to a subset of mitochondria. (Scale bar: 5 μm.)

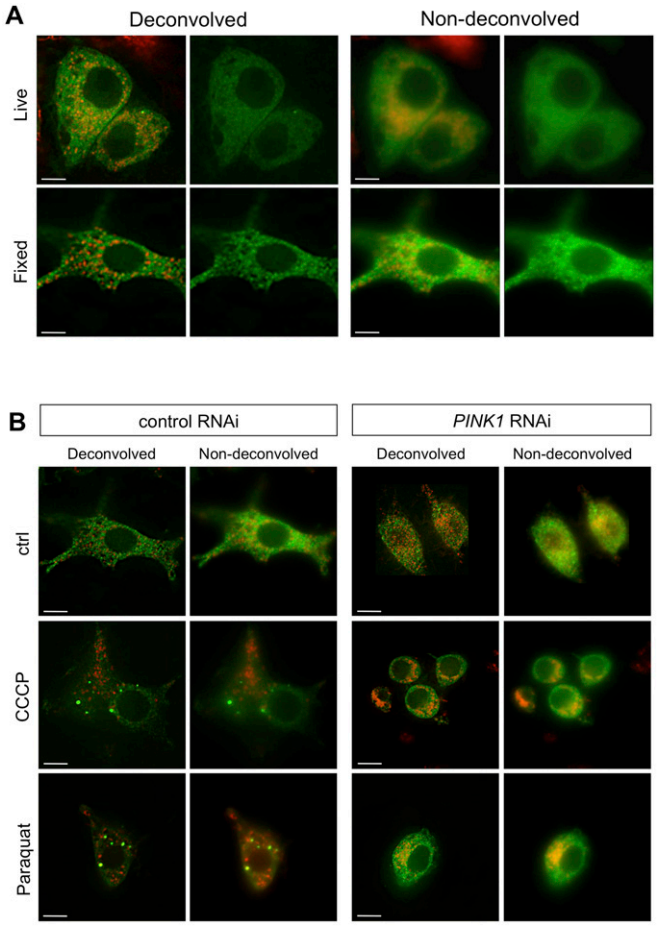

Fig. S5. Comparison of image acquisition and analysis methods for Parkin-GFP localization. S2R+ cells were cotransfected with *parkin-GFP* and *mitoDsRed* and imaged on a Deltavision DV epifluorescence microscope. (*A*) Untreated cells were either fixed or imaged live. Captured images are shown both deconvolved or raw (non-deconvolved). Parkin-GFP is largely cytoplasmic in untreated cells with occasional puncta. Parkin-GFP appears diffuse, although not entirely uniform, in live cells, whereas cytoplasmic Parkin-GFP appears more uneven or dappled in fixed samples. This dappled appearance is further enhanced by deconvolution of the image. (*B*) Comparison of images shown in Fig. 2 before and after deconvolution. Cells were fixed to maintain precise time exposure to the stressors CCCP or paraquat. (Scale bar: 5 μm.)

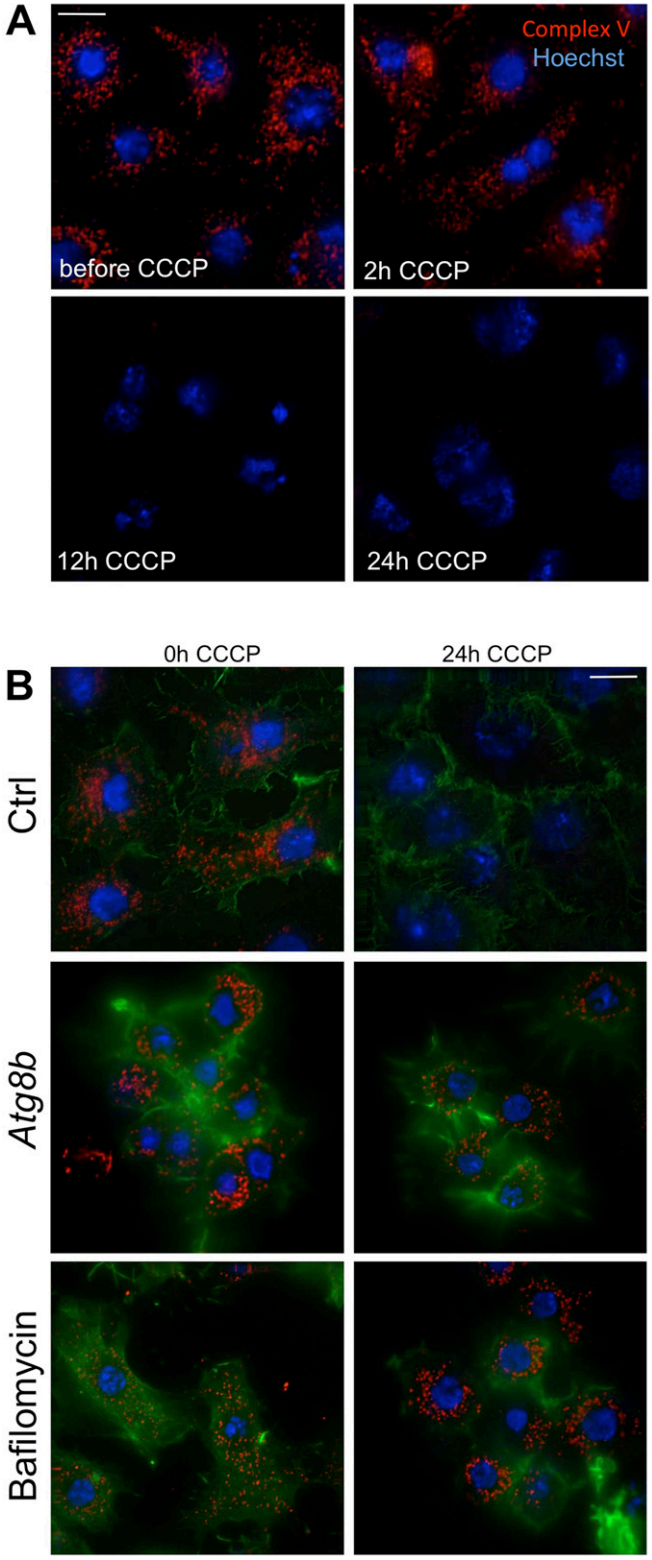

Fig. S6. CCCP-induced mitophagy. (*A*) S2R+ cells were treated with CCCP and fixed and imaged after the indicated time. (*B*) Cells were treated with dsRNAs against *DsRed* (Ctrl) and *Atg8b* or bafilomycin to inhibit autophagy and then treated with CCCP. Hoechst stain marks nuclei, anti-Complex Vα antibody labels all remaining mitochondria, and phalloidin-488 (green) marks the cell membrane. (Scale bar: 5 μm.)

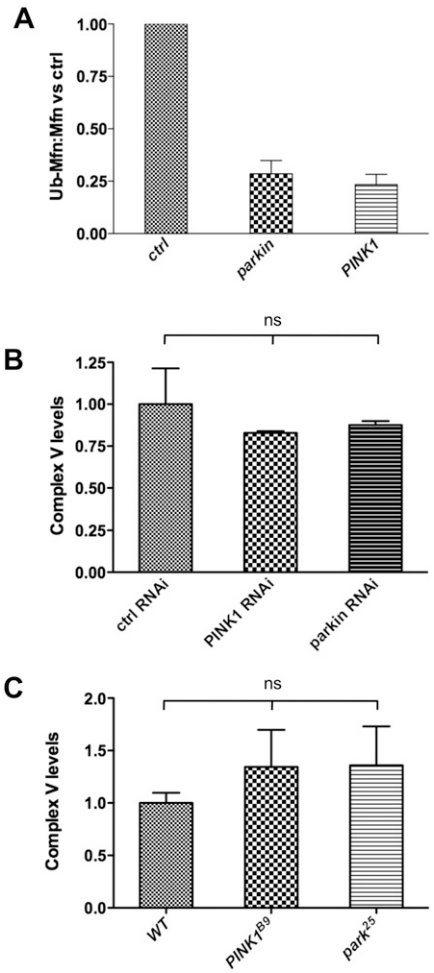

Fig. S7. Quantification of relative abundance of Mfn-Ub isoforms compared to unmodified. (*A*) S2R+ cells expressing Mfn-Flag were treated with either *DsRed* (ctrl), *PINK1*, or *parkin* dsRNAs and subjected to Western blot analysis as in Fig. 3 and Fig. 4. (*B*) S2R+ cells treated with dsRNAs, or (*C*) wild type or mutant animals were subjected to Western blot analysis for total Complex Vα levels normalized to actin levels (Fig. 5). No comparison showed significance $P > 0.05$, one-way ANOVA with Bonferroni correction. WT, wild type.