Online Supplemental Materials

Cell Culture, Transfection, and Pharmacological Treatment

HEK293T cells were cultured at 37°C with 5% CO_2 in DMEM with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin. Cells were transfected twice over 96 hrs with 10 nM siGENOME SMARTpool siRNA (Dharmacon, Lafayette, Colorado) using Lipofectamine RNAiMAX (Invitrogen, Inc.) according to manufacturer's instructions for reverse transfections. GFP-Parkin and OCT-DsRed were transfected using jetPRIME (Polyplus Transfection, Illkirch, France) according to manufacturer's instructions. Treatments with CCCP (Sigma-Aldrich, St. Louis, Missouri) or MG132 (Sigma-Aldrich) were performed prior to harvesting.

Confocal Microscopy / Image processing

Pictures were taken at the same intensities in the green and deep red channels and mitochondrial membrane potential was determined by the total MitoTracker Deep Red fluorescence/Total MitoTracker Green fluorescence ratio. Mitochondrial interconnectivity was analyzed using ImageJ (National Institute of Health). The MitoTracker Green fluorescence images were de-blurred and auto-thresholded. Particles over 1.0 µm were analyzed and interconnectivity was calculated by the Area/Perimeter ratio. All experiments were repeated at least 3 times in a blinded fashion.

Cell Fractionation

For mitochondrial isolation, cells were harvested in cold phosphate buffered saline (PBS) and pelleted by centrifuging at 1000xg for 10 min at 4°C. Cells were resuspended in 5 ml BIB (210 mM mannitol, 70 mM sucrose, 5 mM Tris pH 7.4, 0.2 mM EGTA, 0.1 mM EDTA, 0.1% bovine serum albumin) and homogenized with 10 strokes at 900 rpm using a teflon homogenizer and glass tube. Debris was cleared by an identical spin twice (600xg) and the resulting supernatant was subjected to centrifugation at 12,000xg for 10 min at 4°C to pellet mitochondria. The pellet was resuspended in 500 μl IB (210 mM mannitol, 70 mM sucrose, 5 mM Tris pH 7.4) and centrifuged again. The final, mitochondria-enriched pellet was resuspended in a small volume of IB and protein concentration was measured by BCA (Pierce, Rockford, Illinois). When both cytosolic and mitochondrial fractions were needed, the above protocol was modified as follows: 1.5 ml IB was used for the first resuspension and 500 μl BIB for the second; the supernatant from the first 12,000xg spin was conserved as the cytosol-enriched fraction. A cocktail of protease inhibitors (Benzamidine, PMSF, Aprotinin, and Leupeptin) was included in BIB and IB except when the mitochondria were used for subsequent *in organello* cleavage or protease protection assays. Tim23 and Rpn2 were used as mitochondrial and cytosolic marker proteins respectively.

Flow Cytometry

Cells were stained with JC-1 (5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzamidazol-carboncyanine; Invitrogen) or MitoTracker Green and analyzed by flow cytometry according to the manufacturer’s instructions. For JC-1, cells were treated with 0.05% Trypsin and 0.53 mM EDTA and harvested in culture medium, then incubated in suspension with DMSO or 10 μM

CCCP for 5 min at 37°C. JC-1 was then added at a concentration of 2 μM, and cells were incubated for a further 20 min. For the Mitochondrial mass experiment, cells were incubated in either DMSO or 10 μM CCCP for 24 hrs at 37°C, then stained for 30 min with 100 nM MitoTracker Green followed by a 30 min pure media chase, trypsinization, and harvesting. Cytofluorimetric analysis was performed using FACSCalibur (BD, Franklin Lakes, New Jersey) with an argon laser at 488 nm and band-pass filters at 530 ± 15 nm for green and 585 ± 21 nm for red. A minimum of 10,000 cells were used to calculate mean red and green fluorescence for each trial. The red to green ratio in JC-1 stained cells was used as a relative measure of mitochondrial membrane potential, while the MitoTracker Green to Forward Scatter ratio in MitoTracker Green stained cells was used as a relative measure of mitochondrial mass.

Oxygen Consumption Assay

Oxygen consumption assays were performed as in (Will et al, 2006)). Briefly, mitochondria-enriched fractions were prepared as above, but were resuspended in respiration buffer (250 mM sucrose, 15 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$, 30 mM K_2HPO_4, pH 7.4) instead of IB. Mitochondria were incubated at 0.2 μg/μl with 25 mM succinate, 1.65 mM ADP, and 50 nM mitoXpress in respiration buffer then topped with mineral oil. Oxygen consumption was monitored over 60 min as the increase in MitoXpress emission using Victor 3 (PerkinElmer, Waltham, Massachusetts) with 340 ± 40 nm excitation and 642 ± 10 nM emission. Mitochondria were omitted from reactions as negative controls and the resulting emission values were subtracted from those of the samples to give the final values shown. 5 μM of the complex III inhibitor Antimycin A was included with one set of mitochondria from non-targeting siRNA-transfected cells as a further negative control.

Protease Protection Assay

Mitochondria were incubated at a concentration of 0.8 μg/μl in IB containing the indicated concentration of proteinase K for 20 min at 4°C. Reactions were stopped by adding PMSF at a final concentration of 2mg/ml. Proteins were then precipitated by adding 1ml cold acetone. Precipitated proteins were centrifuged at 16,000xg and pellets were dried and resuspended in sample buffer.

Carbonate Stripping Assay

Mitochondria were incubated at a concentration of 0.8 μg/μl in IB with or without 0.1M Na_2CO_3 for 30 min at 4°C, followed by centrifugation at 144,000xg for 1 hr at 4°C (Fujiki et al, 1982). Supernatants were removed and set aside and pellets were resuspended in an equal volume of IB.

Immunoblotting

Samples were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk, washed, and incubated overnight at 4°C with primary antibody in 3% BSA. Membranes were blocked again with 5% milk and washed, then incubated with peroxidase-conjugated secondary antibody (Jackson, West Grove, Pennsylvania) at a 1:5000 dilution. After washing again, membranes were exposed with Enhanced Chemiluminescence Substrate (PerkinElmer). All wash, blocking, and staining steps were performed with PBS containing 0.1% Tween. Densitometric quantification was performed using ImageJ and normalized to signal from Tim23.

Statistical Tests

All statistical analyses were performed by one-way, parametric analysis of variance (ANOVA) using the Bonferroni *t*-test for post-hoc comparison and a requisite p-value of 0.05 or less. All error bars represent standard error of the mean.

Antibodies

The antibodies used in this study and their dilutions are as follows:

PINK1, 1:5000 (Novus BC100-494, Littleton, Connecticut),

Parkin, 1:2000 (Santa Cruz sc-32282, Santa Cruz, California)

HTRA2, 1:1000 (Santa Cruz sc-15467, Santa Cruz, California)

Tim23, 1:100,000 (BD 611222, Franklin Lakes, New Jersey)

MPPα, 1:30,000 (Sigma-Aldrich HPA021648, St. Louis, Missouri)

MPPβ, 1:30,000 (Proteintech 160-64-1-AP, Chicago, Illinois)

PARL, 1:1000 (Abcam Ab-42638, Cambridge, Massachusetts)

AFG3L2, 1:1000 (GeneTex GTX102056, Irvine, California)

ClpP, 1:1000 (Santa Cruz sc-271284, Santa Cruz, California)

MFN2 (Sigma-Aldrich M6319, St. Louis, Missouri)

MnSOD, 1:2000 (Stressgen SOD-110, Belgium)

OPA1, 1:2500 (BD 612607, Franklin Lakes, New Jersey)

Rieske, 1:5000 (Mitosciences MS305, Eugene, Oregon)

Core2, 1:5000 (Mitosciences MS304, Eugene, Oregon)

HSP60, 1:10,000 (Sigma-Aldrich H4149, St. Louis, Missouri)

Tom20, 1:50,000 (Santa Cruz sc-11415, Santa Cruz, California)

RPN2, 1:5000 (Enzo 112-11, Plymouth Meeting, Pennsylvania)

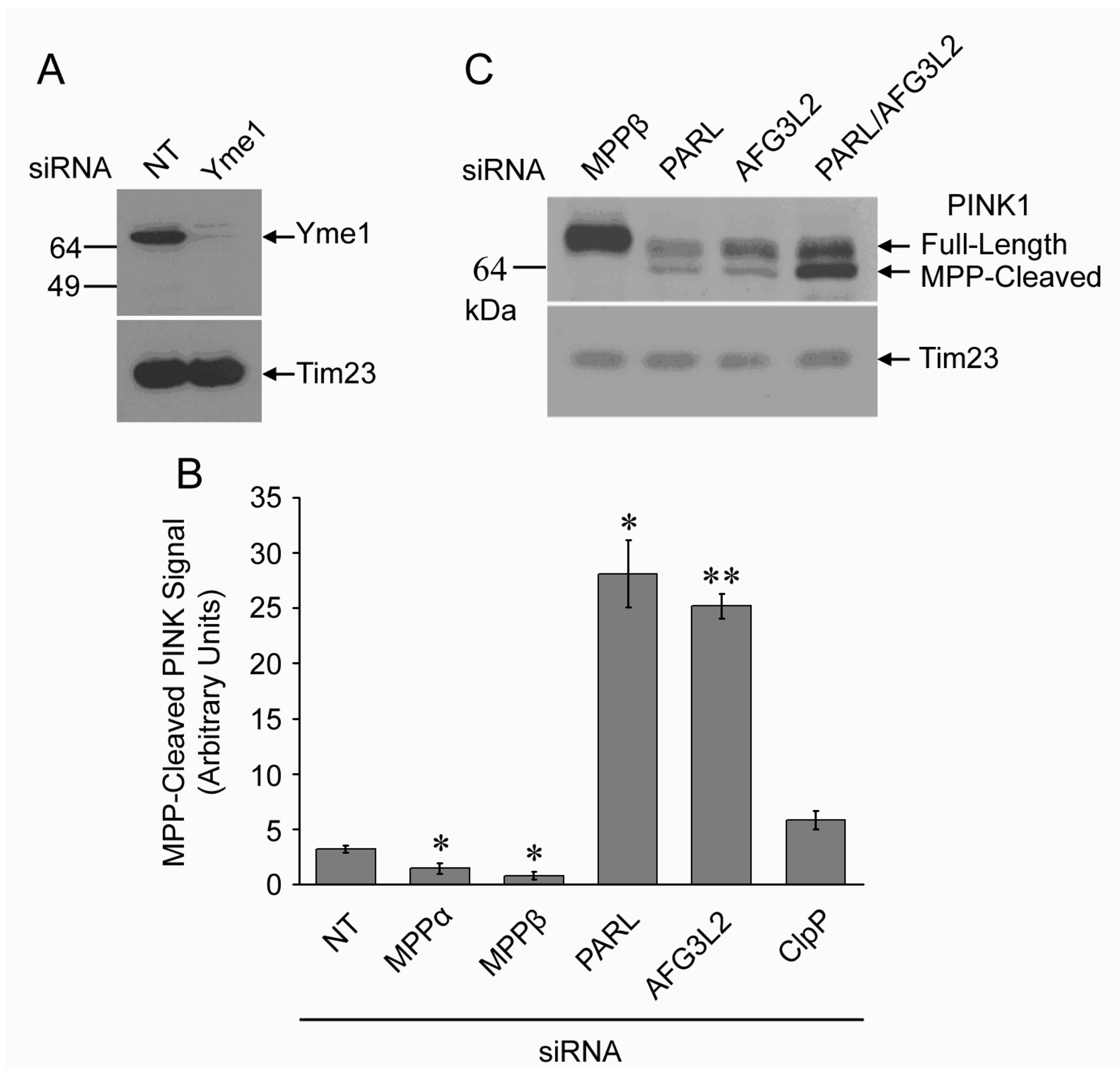
Cytochrome C, 1:10,000 (BD 556433, Franklin Lakes, New Jersey)

Yme1, 1:1000 (Proteintech 115-10-1-AP, Chicago, Illinois)

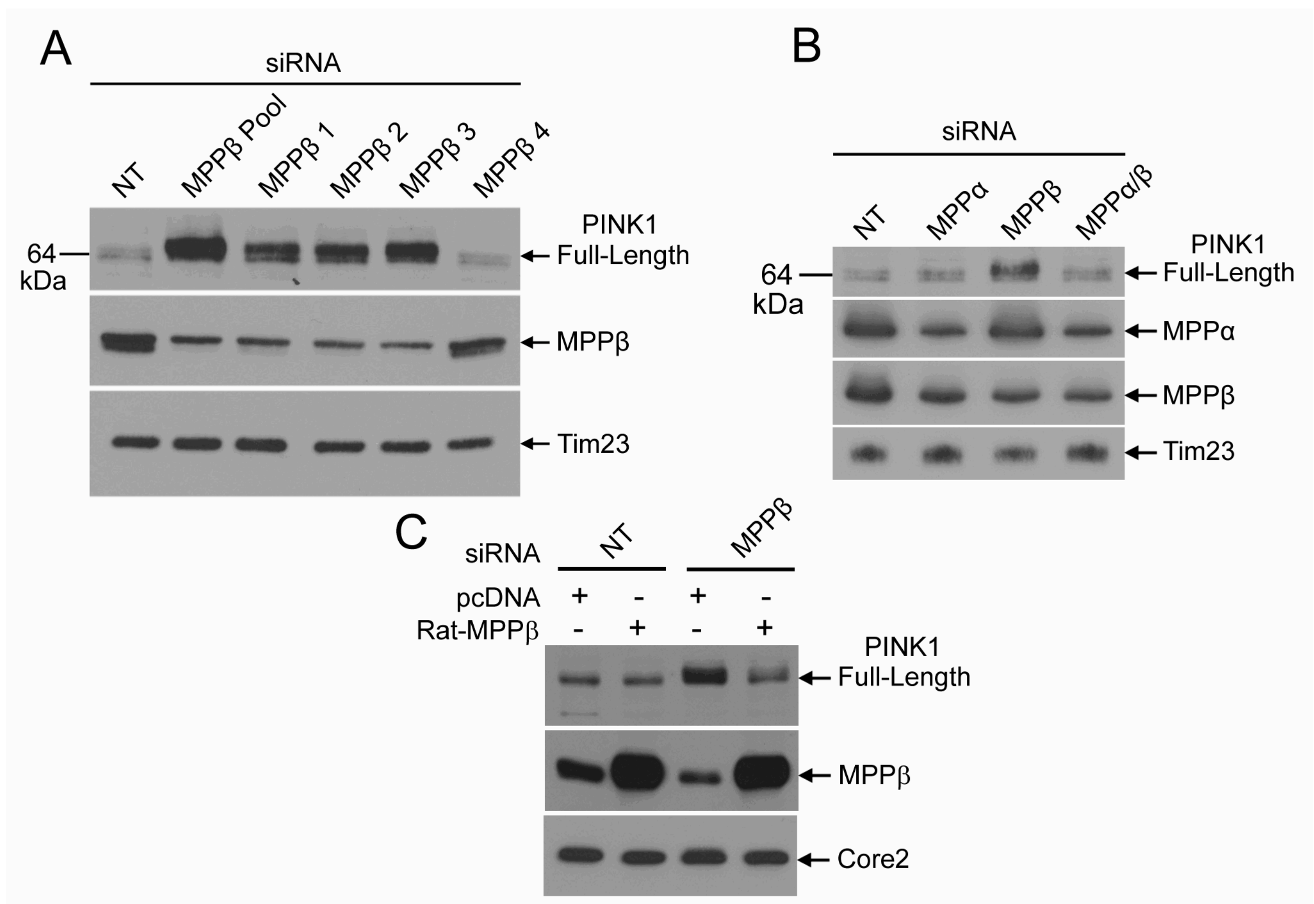
Supplemental References

Fujiki Y, Hubbard AL, Fowler S, Lazarow PB (1982) Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J Cell Biol* **93:** 97-102

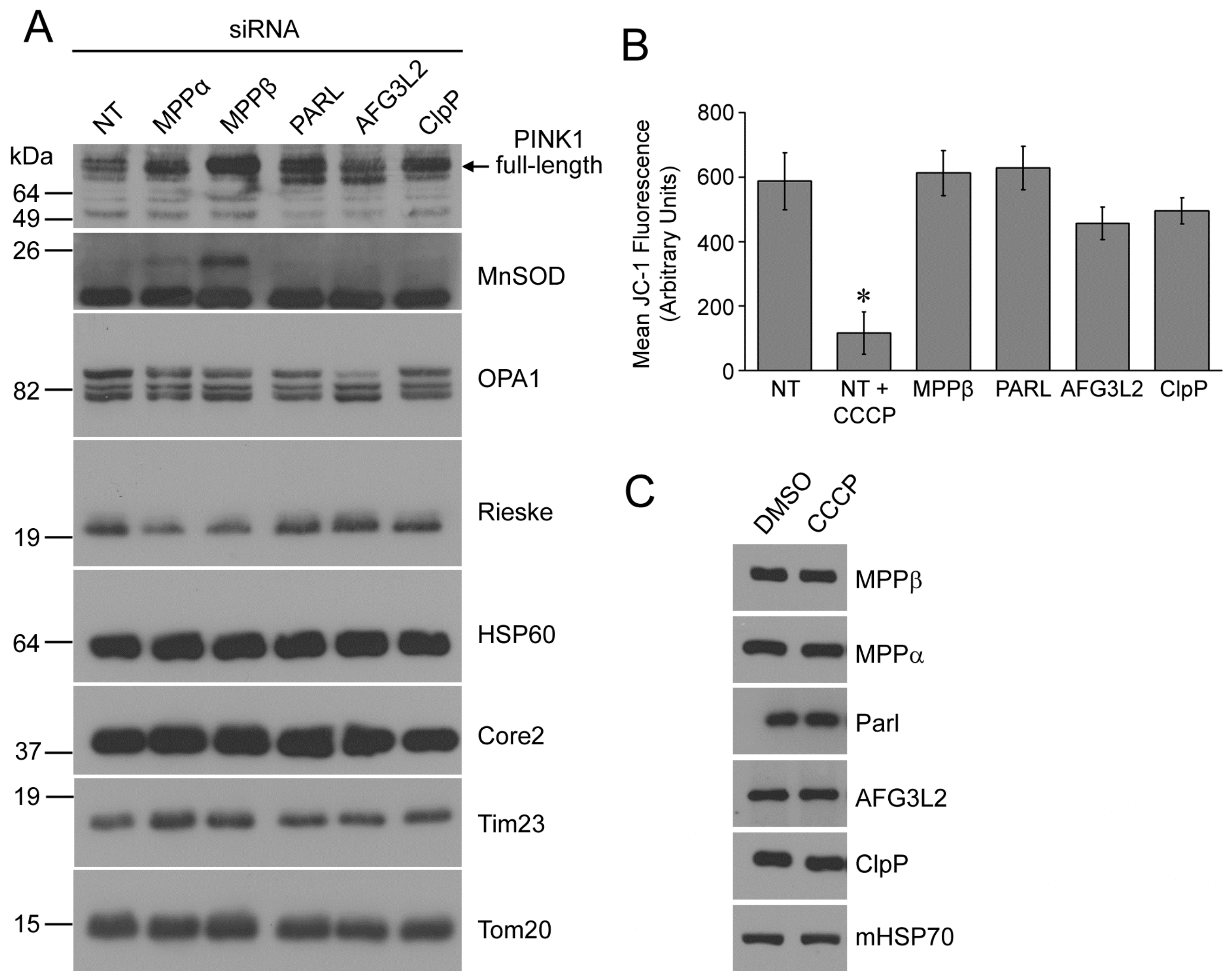
Will Y, Hynes J, Ogurtsov VI, Papkovsky DB (2006) Analysis of mitochondrial function using phosphorescent oxygen-sensitive probes. *Nat Protoc* **1:** 2563-2572



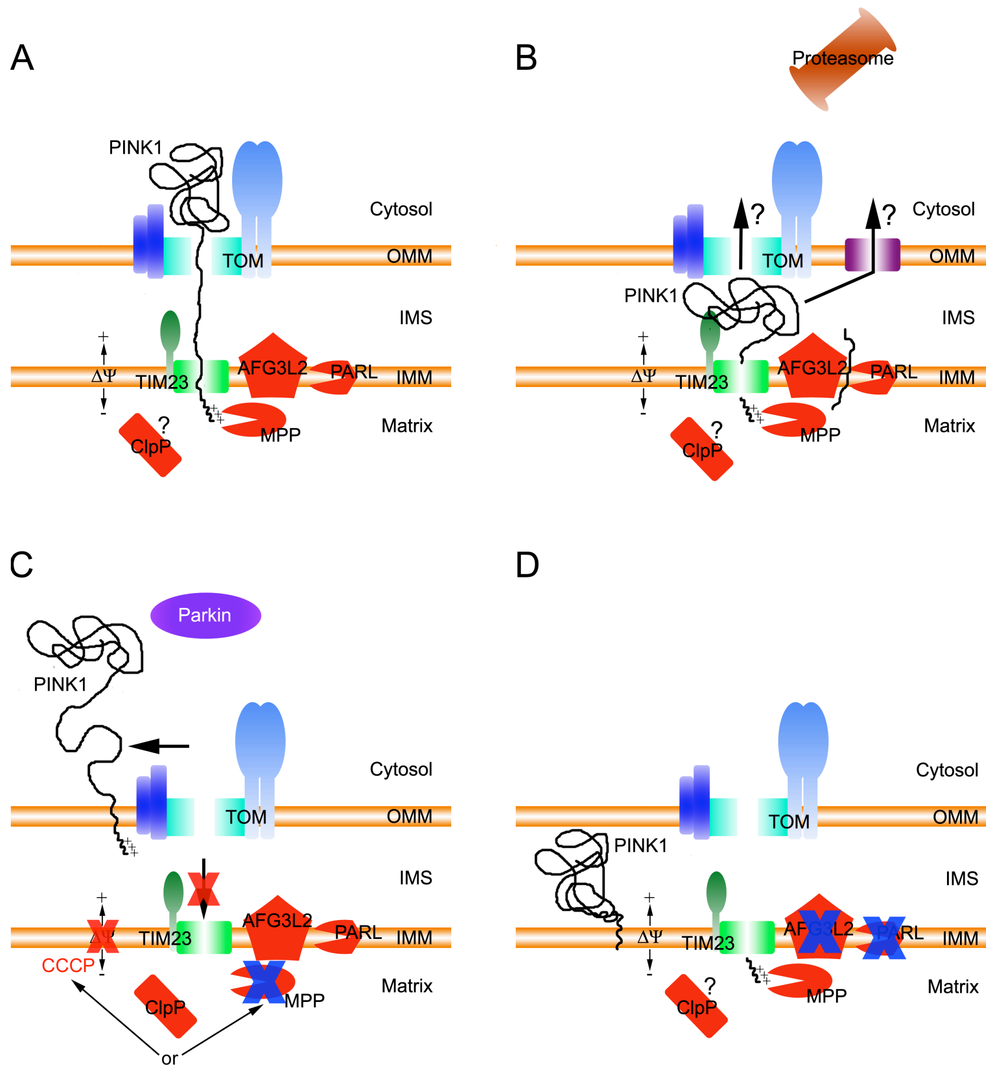
Supplemental Figure 1. (A) Mitochondria-enriched fractions from HEK293T cells transfected with non-targeting (NT) or Yme1-specific siRNA were probed for Yme1 and for Tim23 as a loading control. These mitochondria were from the same experiment as those used in Figure 1A. (B) Densitometric quantification of the putative MPP-cleaved PINK1 band normalized to Tim23 levels from mitochondrial fractions in experiments performed as in figure 3A (with MG132). n = 3; *, P<0.05; **, P<0.001 compared to NT. Error bars represent SEM. (C) Mitochondria-enriched fractions from cells transfected with the indicated siRNA were probed for PINK1 and for Tim23 as a loading control.



Supplemental Figure 2. (A) Immunoblots of PINK1, MPPβ, and Tim23 in mitochondria from HEK293T cells transfected with the Dharmacon siGenome anti-MPPβ SMARTpool or with the individual siRNA from the pool of 4. The catalogue numbers for the MPPβ siRNA labeled 1-4 in the figure are D-004747-01, D-004747-02, D-004747-04, and D-004747-21 respectively. Mitochondria from non-targeting (NT) siRNA-transfected cells were used as controls. (B) Mitochondria-enriched fractions from cells transfected with the indicated siRNA were probed for PINK1, MPPα, MPPβ, and Tim23. (C) Cells were transfected with rat MPPβ (resistant to the human directed siRNA) or vector as well as with the indicated siRNA. A mitochondria enriched fraction was harvested and probed for PINK1, MPPβ, and Core 2 (as a loading control).



Supplemental Figure 3. (A) Mitochondria-enriched fractions isolated from HEK293T cells with the indicated knockdowns were probed for the mitochondrial proteins shown. (B) Cytofluorimetric analysis of cells transfected with the indicated siRNA, treated with DMSO or 10 μM CCCP, and stained with JC-1 to measure relative mitochondrial membrane potential as the mean ratio of red to green fluorescence intensities. N = 3; *, P<0.05 compared to non-targeting (NT); Error bars represent SEM. (C) Mitochondria-enriched fractions from cells treated with DMSO or 10 μM CCCP were probed for the indicated proteases or for mHSP70 as a loading control.



Supplemental Figure 4. Model depicting putative steps in PINK1 proteolysis. (A) In healthy mitochondria, newly-synthesized PINK1 is imported through the TOM complex in the outer mitochondrial membrane (OMM) and then through the TIM23 complex of the inner mitochondrial membrane (IMM). (B) Imported PINK1 is cleaved first by MPP (possibly with cooperation from ClpP), then by PARL and AFG3L2 to generate the 52 kDa PINK1 fragment. This is exported to the cytosol via an as of yet unidentified mechanism and then degraded by the proteasome. (C) When PINK1 import is compromised by mitochondrial depolarization or MPPβ knockdown, full-length PINK1 accumulates at the mitochondrial surface (possibly within the lipid phase of the OMM), where it can recruit Parkin and induce mitophagy. (D) In the absence of PARL and AFG3L2, complete PINK1 processing cannot occur and the MPP-cleaved form of the protein accumulates within mitochondria, most likely in the intermembrane space (IMS), where it would be anchored to the IMM by its transmembrane domain.