Supporting Information

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SI Materials and Methods

Western Blot. Cells were lysed in equal volumes of 1% Nonidet P-40 with protease inhibitors on ice for 25 min and then centrifuged at 10,000 × *g* for 10 min. Supernatants were protein assayed using the DC Protein Assay (Bio-Rad), SDS-Laemmli buffer was added and samples were heated at 65 °C for 5 min. Equal concentrations of each sample were loaded onto Novex Tris-Glycine (Invitrogen) or Criterion Tris-HCl (Bio-Rad) precast gels and transferred onto polyvinylidene fluoride membranes (Millipore) for Western blotting.

Antibodies for Western Blots. Blots were probed for parkin (PRK8; Santa Cruz Biotechnology), Bax (2772; Cell Signaling), Bid (sc-11432; Santa Cruz Biotechnology), Bak (06-536; Millipore), Bcl-2 (sc-7282; Santa Cruz Biotechnology), cytochrome *c* (sc-13560; Santa Cruz Biotechnology), VDAC (PA1-954A; Cell Signaling, Danvers, MA), myc (9E10; Santa Cruz Biotechnology), actin (ab6276; Abcam), and/or Tom20 (sc-11415; Santa Cruz Biotechnology). Secondary antibodies and ECL-plus were purchased from GE Healthcare. Following ECL application, blots were exposed to HyBlot Cl autoradiography film (Denville Scientific, Inc.).

IP/Western Blot. Blots for IP studies were probed for ubiquitin (3936; Cell Signaling). Blots were stripped in 1× Stripping Buffer [62.5 mmol/L Tris, pH 6.8, 2% (wt/vol) SDS, 7.6% βME] at 55 °C for 10 min, washed 3 × 10 min in 0.1% PBS/Tween, and probed for Bax 6A7 (sc-23959; Santa Cruz Biotechnology). Aliquots of each IP were first analyzed to determine the individual Bax IP efficiency. Then, eluates were normalized to equal Bax IP on subsequent gels to examine for differences in Bax ubiquitination.

Bid was immunoprecipitated using a polyclonal Bid antibody conjugated to agarose beads (sc-11423AC; Santa Cruz Biotechnology). Following standard IP/Western blotting conditions for ubiquitin, blots were stripped and probed for Bid (sc-135847; Santa Cruz Biotechnology).

Cell Death and Apoptosis Assays. Following 48 h drug treatments, cells were incubated with MTT (M5655, Sigma) for 2 h at 37 °C, and the reaction was stopped by the addition of lysis buffer (50% dimethyl formamide and 20% SDS) overnight at 37 °C. Absorbance was read at 570 nm, with a 650-nm subtraction on a Synergy H1 (BioTek) plate reader. Data were analyzed using a one-way ANOVA followed by Tukey's post hoc analysis. Western blot analysis of apoptosis was performed by analyzing cleaved caspase 3 (9661S; Cell Signaling) 18 h after drug treatments.

Live Cell Imaging. Parental MES and CHO cells, or those stably expressing or transiently transfected with WT parkin, were transfected with GFP-Parkin or GFP-Bax (kindly provided by R. Youle, NIH, NINDS, Bethesda, MD) along with mito-mCherry. Twenty-four hours after transfection, cells were treated with DMSO, CCCP (20 μM, 6 h), or staurosporine (1 μM, 5 h). The medium was removed, and images from live cells were captured on a Zeiss Axiovert 200 inverted microscope.

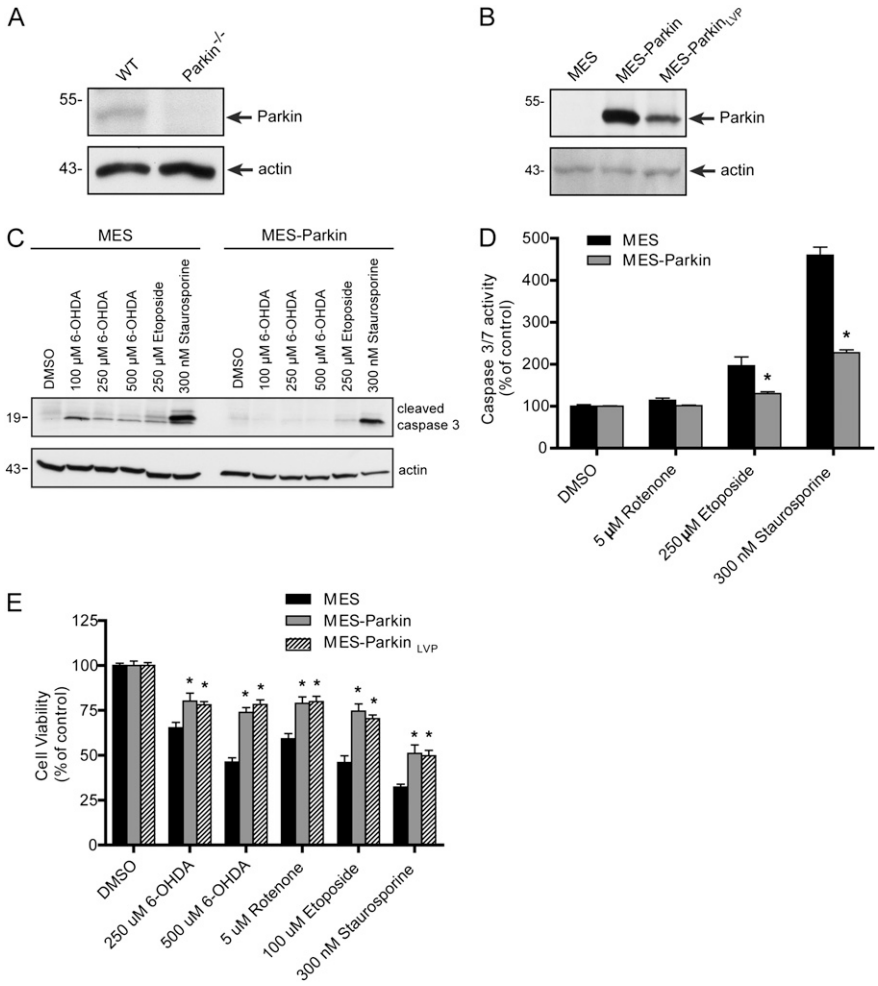

Fig. S1. Parkin prevents the induction of apoptosis and subsequent cell death. (*A*) Representative Western blot of parkin expression in primary cultured cortical neurons from WT and parkin$^{-/-}$ mice. (*B*) Representative Western blot of parkin expression in MES cells and MES cells stably overexpressing human myc-tagged parkin monoclonally or through a polyclonal lentiviral overexpression (MES-Parkin and MES-Parkin$_{LVP}$, respectively). (*C* and *D*) MES and MES-Parkin cells were treated with various apoptotic and nonapoptotic stressors for 18 h. Cleaved caspase 3 was measured by Western blot (*C*) or caspase 3/7 activity was measured using a commercially available kit (*D*; mean ± SEM, $n = 12$ from three independent experiments; $^*P < 0.05$ compared with their respective controls). (*E*) MES, MES-Parkin, or MES-Parkin$_{LVP}$ cells were treated with various apoptotic and nonapoptotic stressors for 48 h. Cell viability was determined using an MTT reduction assay (mean ± SEM, $n = 18$ from three independent experiments; $^*P < 0.05$ compared with MES).

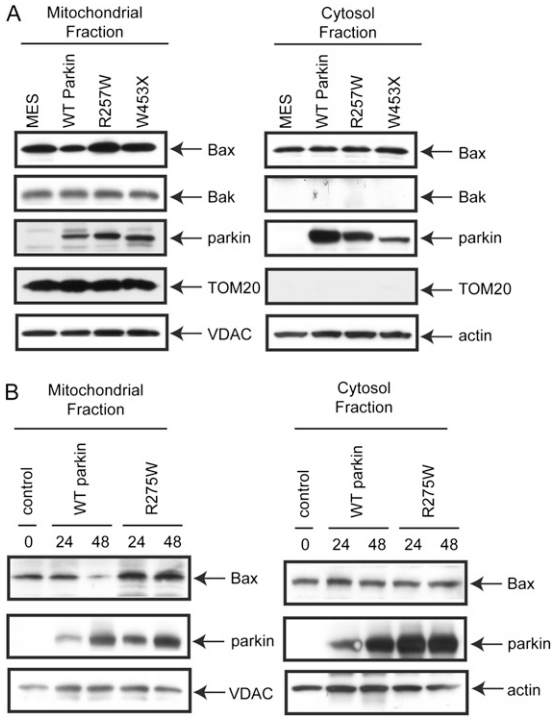

Fig. S2. Parkin alters Bax levels and distribution. (*A*) Soluble (cytoplasmic) and mitochondrial fractions from MES cells and MES cells stably expressing WT parkin or PD-linked parkin mutants R275W and W453X were protein normalized and analyzed by Western blot. (*B*) Soluble and mitochondrial fractions were collected after transient transfection to express WT parkin or R275W for 24 or 48 h, protein normalized, and analyzed by Western blot.

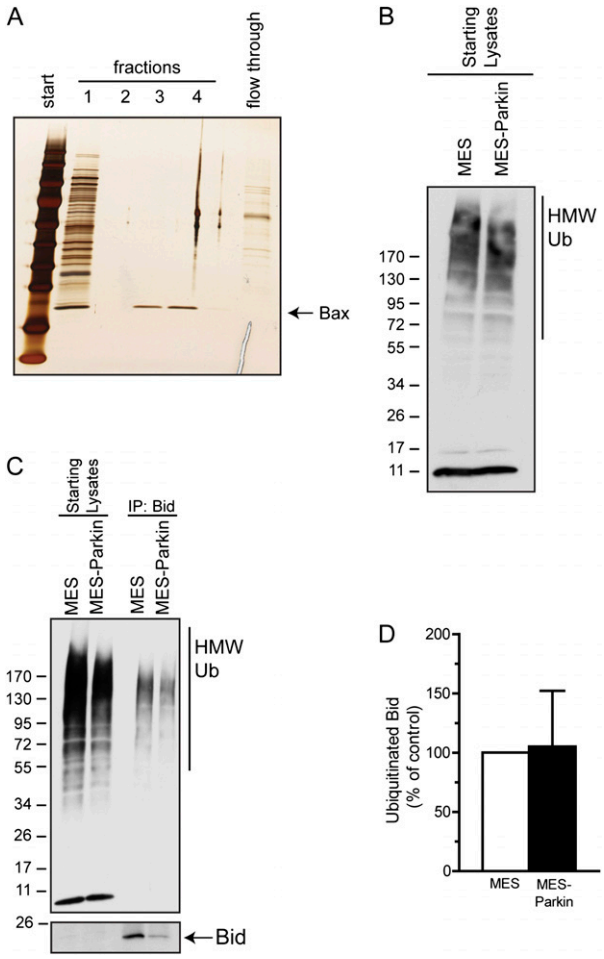

Fig. S3. (*A*) Bax was grown in bacterial culture, purified, and subjected to FPLC. The fractions containing Bax were run on SDS/PAGE and subjected to silver stain to examine purity. (*B*) A shorter exposure from Fig. 2*A*, demonstrating equal polyubiquitinated proteins in the protein-normalized starting lysates from MES and MES-Parkin cells. (*C*) MES and MES-Parkin cells were treated with proteasome inhibitor (MG-132, 10 μM) for 6 h and lysed in 1% Nonidet P-40, and Bid was immunoprecipitated and probed for ubiquitin by Western blot. (*D*) Densitometric analysis of ubiquitinated Bid relative to IPed Bid from MES and MES-Parkin cells demonstrates no trend of parkin influencing Bid ubiquitination (mean ± SEM, $n = 3$).

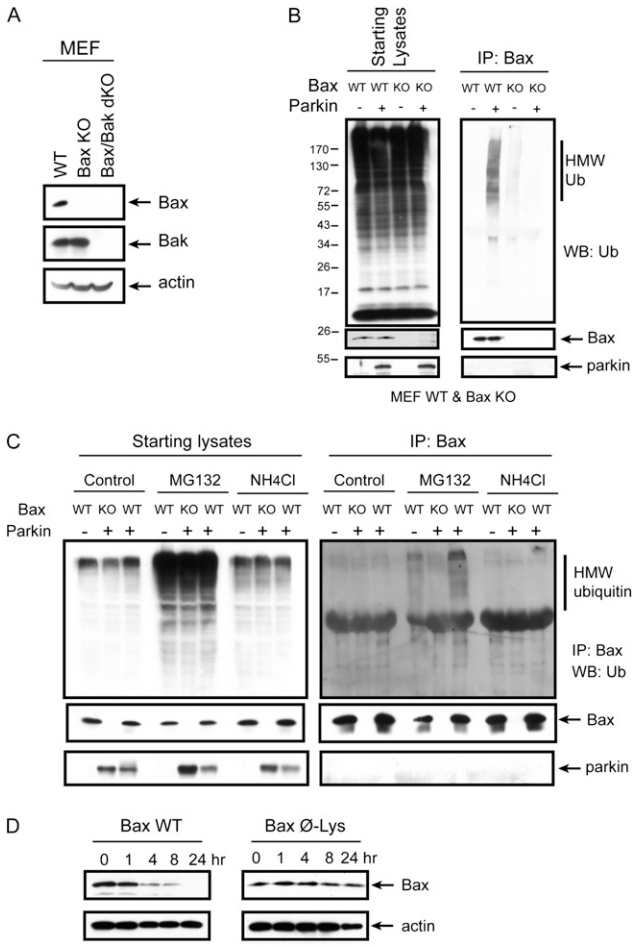

Fig. S4. Parkin expression may target a pool of Bax to the proteasome. (*A*) WT, Bax$^{-/-}$, and Bax/Bak dKO MEFs were lysed in 1% Nonidet P-40, and subject to a Bax or Bak Western to confirm genotype. (*B*) WT and Bax$^{-/-}$ MEFs were stably transduced to express parkin, treated with proteasome inhibitor (MG-132; 10 μM) for 4 h, lysed in 1% Nonidet P-40, and subjected to Bax IP. The eluates were then probed for ubiquitin, to visualize the levels of ubiquitinated-Bax as a function of parkin expression. (*C*) WT and Bax$^{-/-}$ MEFs were stably transduced to express parkin, treated with vehicle (DMSO), proteasome inhibitor (MG132; 10 μM), or lysosomal inhibitor (NH4Cl; 20 mM) for 4 h, lysed in 1% Nonidet P-40, and subjected to Bax IP. The eluates were then probed for ubiquitin, to visualize the levels of ubiquitinated Bax. (*D*) Bax/Bak dKO MEFs transiently transfected with Bax WT or Bax Ø-Lys were treated with cycloheximide (60 μg/mL) and collected at 0, 1, 4, 8, and 24 h. Whole-cell lysates were prepared and analyzed to examine the turnover of each Bax variant.

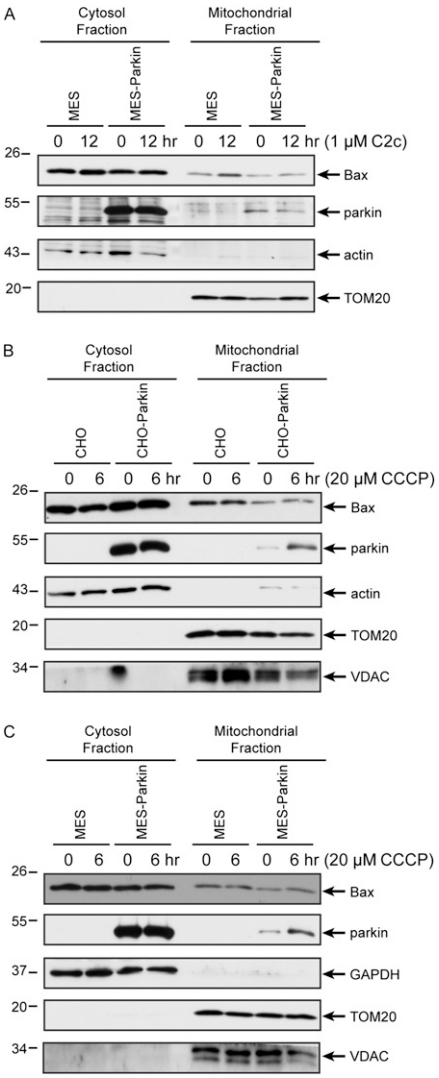

Fig. S5. Differential effects of the induction of apoptosis or mitophagy on parkin and Bax cellular localization. (*A*) Parkin inhibits C2 ceramide-induced Bax translocation to the mitochondria. MES and MES-Parkin cells were treated with DMSO or 1 μM C2 ceramide for 12 h and subjected to subcellular fractionation. Cytosolic and mitochondrial fractions were then probed for Bax and parkin, to visualize parkin-dependent Bax translocation as a function of apoptotic stress. C2 ceramide induced a twofold increase in Bax at the mitochondria, which was completely inhibited by parkin expression ($n = 3$). (*B* and *C*) Mitochondrial depolarization with CCCP induces parkin, but not Bax, translocation to the mitochondria. CHO and CHO-Parkin cells (*B*) or MES and MES-Parkin cells (*C*) were treated with DMSO or 20 μM CCCP for 6 h and subjected to subcellular fractionation. The cytosolic and mitochondrial fractions were then probed for Bax and parkin to visualize Bax and parkin translocation as a function of mitophagic stress. CCCP induced 436% and 509% increases in parkin at the mitochondria in CHO-Parkin and MES-Parkin cells, respectively, whereas Bax levels remained relatively unchanged (increased 32% and 60%, respectively; $n = 3$). Images are representative of three independent experiments.

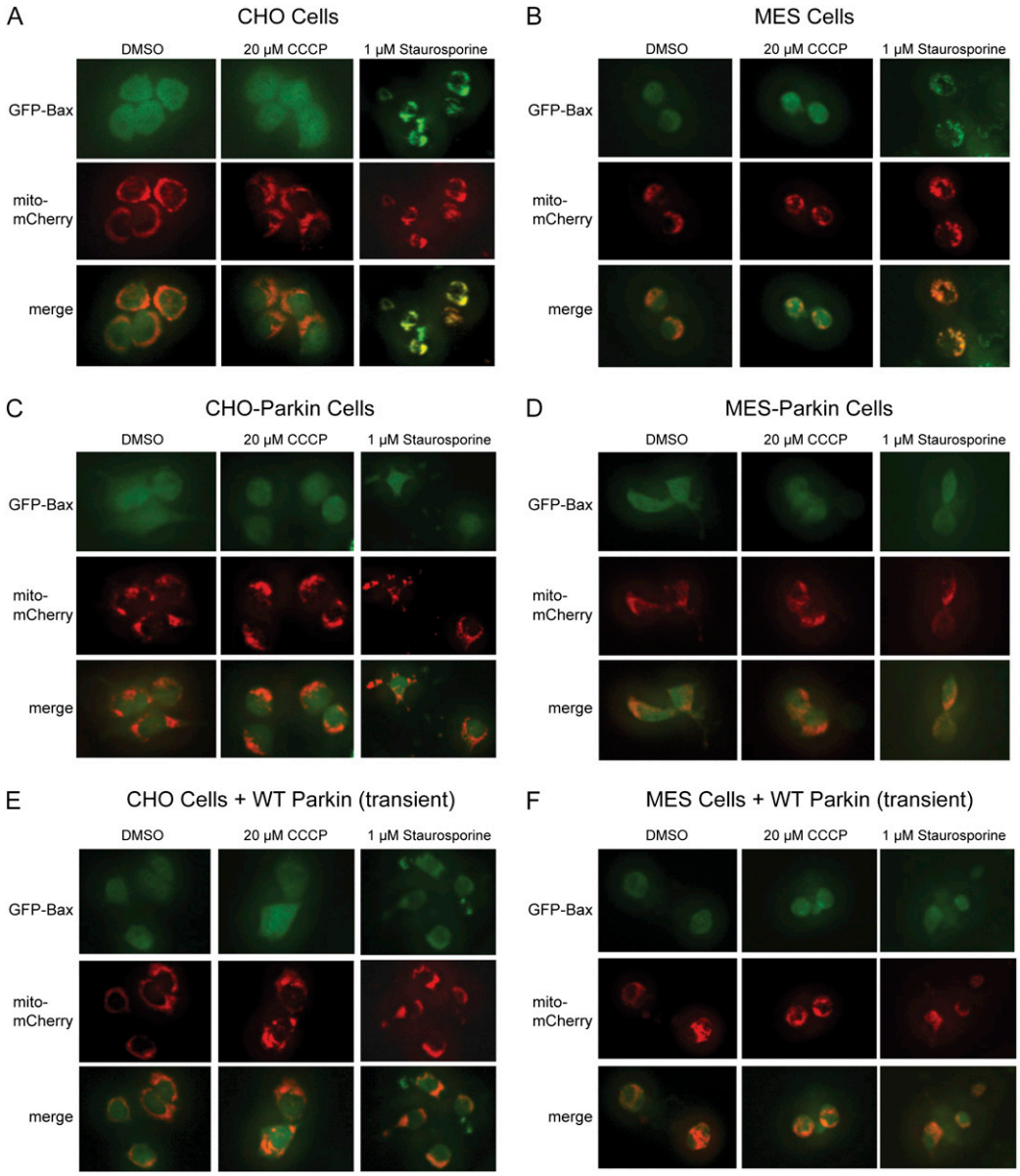

Fig. S6. Apoptotic stress, but not mitochondrial depolarization, induces parkin-dependent Bax translocation to mitochondria. Parental CHO and MES cells (*A* and *B*, respectively), and those expressing WT parkin stably (*C* and *D*) or transiently (*E* and *F*), along with GFP-Bax and mito-mCherry, were treated with DMSO, CCCP (20 μM, 6 h), or staurosporine (1 μM, 5 h). Fluorescent images of live cells were captured.

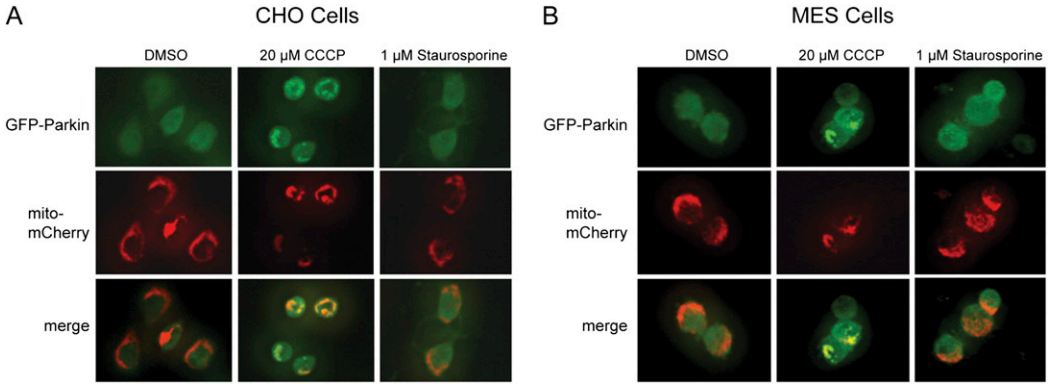

Fig. S7. Mitochondrial depolarization, but not apoptotic stress, induces parkin translocation to mitochondria. Parental CHO (*A*) and MES (*B*) cells transiently transfected with GFP-Parkin and mito-mCherry were treated with DMSO, CCCP (20 μM, 6 h), or staurosporine (1 μM, 5 h). Fluorescent images of live cells were captured.