Supporting Information

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SI Materials and Methods

Molecular Cloning and Transfections. Human PINK1 cDNA (OriGene) was subcloned into mammalian expression vector pIRES2-EGFP and its derivative with IRES-EGFP was removed. An HA epitope tag was inserted into the frame at the C terminal of PINK1 by PCR. Human DJ-1 and parkin cDNAs (OriGene) were subcloned into pRK5-myc vector. PINK1 point mutations ($PINK1^{A217D}$, $PINK1^{G309D}$, $PINK1^{L347P}$, and the PINK1 dead kinase $PINK1^{K219M}$) were generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). Full-length PINK1 cDNA was cloned into a pshuttle-1–3XFlag-IRES-GFP vector (Stratagene) to generate Flag-tagged PINK1. To generate V5-tagged PINK1, WT full-length PINK1 cDNA was cloned into a pcDNA3.1 vector (Invitrogen). The parkin RING2 deletion construct ($Parkin^{\Delta R2}$) was a gift from Hardy Rideout (Columbia University, New York). PINK1-ECFP, Parkin-EYFP, and EGFP-Parkin were constructed by inserting PINK1 and Parkin cDNA into the frame of the mammalian expression vectors pAmCyan1-N1, pZsYellow1-N1, and pEGFP-C2 (Clontech), respectively. All constructs were confirmed by sequencing. DNA transfections were performed using FuGENE 6 (Roche Diagnostics GmbH) or Lipofectamine 2000 (Invitrogen), following the manufacturer's guidelines.

Generation of PINK1 N27-Inducible Cells. The expression of human WT and mutant PINK1 in the $1RB_3AN_{27}$ (N27) neurons (provided by Anumantha Kanthasamy, Iowa State University, Ames, IA) was accomplished using an ecdysone-inducible system (Stratagene) with some modifications to the manufacturer's instructions: First, N27 were stably transfected with the repressor pERV3 vector to express constitutively the ecdysone receptor and the retinoid-X-receptor heterodimer. Transfected cells were selected for a couple of weeks by the addition of G418 (500 μg/mL; Gibco). The most inducible clone was selected for the subsequent stable transfection of the modified pEGSH vector, which contains the ecdysone-responsive element that, in the absence of ponasterone A, tightly suppresses transcription. pEGSH vector (Stratagene) was modified by introducing a DNA fragment containing IRES-emerald GFP (provided by Pradip Roy-Burman, University of Southern California, Los Angeles). The cDNAs of full-length human PINK1, $PINK1^{L347P}$, and $PINK1^{W437\times}$ (1) were subcloned into the multiple cloning site. Stably transformed cells were selected and maintained in RPMI containing 10% FBS, G418 (500 μg/mL), and hygromycin (200 μg/mL). Higher expressors of GFP were selected by a FACSVantage cell sorter (BD Biosciences). Each cell type was sorted 3 to 4 times for the top 10% of cells with the highest GFP signal. The inducible expression of PINK1 was confirmed using immunocytochemistry, immunoblotting, and quantitative real-time PCR (with primers designed to amplify a segment that is common to both rat Pink1 and human PINK1: Fwd 5′- CTGTCAGGAGATCCAGGCAATT -3′ and Rev 5′- GCATGGTGGCTTCATACACAGC-3′).

Cell Culture. Human neuroblastoma SH-SY5Y and M17 cells were maintained in DMEM/Ham's F-12 medium (Invitrogen) supplemented with 10% FBS and 1% (vol/vol) penicillin-streptomycin. HeLa and HEK293T cells were maintained in DMEM supplemented with 10% FBS and 1% (vol/vol) penicillin-streptomycin. Primary cortical neuronal cultures were done as previously described (2).

Immunoblotting. For Western blot analyses, 50 μg of protein from whole-cell lysates were separated on 4–16% Bis-Tris gels and electroblotted. Immunoblots were probed with primary antibodies (see *Materials and Methods*). Incubation with fluorescent-conjugated secondary antibodies was followed by infrared detection (Odyssey Detection System; Li-COR). For isolated mitochondria, 25 μg of protein were loaded.

For protease protection assay, 50 μg of mitochondrial protein from HEK293T cells were treated with 0.2, 2, or 20 μg/mL proteinase-K for 20 min on ice. Then, the proteinase-K was inactivated with 2 mM phenylmethanesulphonylfluoride for 10 min on ice.

For immunoprecipitation, cells were harvested and resuspended in lysis buffer (50 mM Tris, pH 7.4/150 mM NaCl/1 mM EDTA/0.1% Triton-100) supplemented with 2× protease inhibitor (Roche) and homogenized with a Dounce homogenizer (Kontes Glass Co, Vineland, NJ). The cell homogenates then were centrifuged at 11,000 × *g* for 10 min, and cell lysates were collected. Cell lysates first were precleared with protein A & G agarose beads for 1 h at 4 °C and then were incubated with rabbit anti-Flag or rabbit anti-V5 antibody for at least 2 h at 4 °C with constant agitation, followed by 4 washes of 20 min each with the lysis buffer. The beads that captured PINK1 complexes were mixed with an equal amount of 2× SDS sample buffer and heated at 95 °C for 10 min to elute the complex proteins. Eluents were used for SDS/PAGE, followed by Western blot analyses with mouse anti-Flag antibody (Ab; Sigma). Human Parkin antibody (sc-32282; Santa Cruz) was used for the detection of endogenous Parkin.

Immunostaining. Cells were grown on coverslips, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and blocked with 5% BSA. For primary antibodies used, see *Materials and Methods*. Samples were analyzed using a Zeiss LSM510 Laser Scanning Confocal Microscope (Carl Zeiss Microimaging, Inc.). Z-sections were acquired (0.7-μm interval), and final images were obtained after merging planes.

To label mitochondria, 25 nM MitoTracker Red CMXRos or MitoTracker Deep Red 633 (Invitrogen) was added to the medium for 25 min at 37 °C, followed by a quick wash in PBS and fixation. To study mitochondrial morphology and distribution, images from at least 20 random fields from 3 independent experiments were taken and analyzed.

Live-Cell Organelle Marker and Immunofluorescence Microscopy. To study the subcellular distribution of Parkin-eYFP and eGFP-Parkin on induction of PINK1 expression, N27-stable cells were grown on 4-welled chamber slides (Nalgene Nunc International). Single XY scans of live cells were acquired in sequential scanning mode on a Zeiss LSM510 Laser Scanning Confocal Microscope (Carl Zeiss Microimaging, Inc.) equipped with a 63×/1.25 NA oil lens. Z-sections were acquired (0.7-μm interval), and final images were obtained after merging planes. All signals were acquired in the same order with all laser lines at 10% intensity and with the following parameters: excitation 488 nm, emission read 513–527 nm for Parkin-GFP; excitation 543 nm, emission read 603–750 for MitoTracker Red; and excitation 515 nm, emission read 406–480 nm for Parkin-eYFP. Samples with 1 of the fluorophores omitted were scanned first to establish gain and offset acquisition settings to ensure that each acquired signal was caused by the emission by individual fluorophores only. All scans were acquired using identical settings and were compiled into panels and

processed identically by adjusting image brightness and contrast in Adobe Photoshop 7.0 (Adobe Systems, Inc.).

Live-Cell $\Delta\Psi_m$ Microscopy. Cells grown on 4-welled chamber slides were incubated in regular growth medium with 10 nM TMRM (Invitrogen) for 20 min. Images were acquired on a Zeiss LSM510 Laser Scanning Confocal Microscope (Carl Zeiss Microimaging) equipped with live-imaging station (CTI-Controller 3700 and incubator S; Leica) with temperature controller (heating insert P; PeCon). Acquisition parameters were chosen to allow the use of minimal laser intensity to prevent photodamage. TMRM signal was acquired at 37 °C using excitation and emission wavelengths of 543 and 575 nm, respectively. A series of optical z-sections was taken at 0.5-μm intervals capturing the entire signal of each cell. Imaging following mitochondrial depolarization with the uncoupling agent CCCP (100 nM) was used to confirm minimal background fluorescence. All imaging settings were kept constant for all experiments to allow direct comparison between control and treated cells expressing or not expressing Parkin-GFP.

TMRM Fluorescence Quantification. Single-cell shapes were outlined and selected for each field. The integrated morphometric analysis feature in Image J (National Institutes of Health) was used to quantify the fluorescence intensity of each individual z-plane, yielding a numerical value representing signal intensity. The sum of all z-plane signals yielded the TMRM intensity value for individual cells. Averages of total TMRM values are provided in Fig. 1 and Fig. S2.

Electron Microscopy. SH-SY5Y cells cotransfected with PINK1 and myc-Parkin were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde for 20 min at room temperature, stained sequentially in 2% osmium oxide and 1% uranyl acetate, dehydrated in a series of ethanol washes, and embedded in Spur resin for sectioning and analysis. Samples were analyzed with an electron microscope (Bio-Imaging Resource Center, Rockefeller University, New York). For immuno-EM, anti-PINK1 antibody (100-494) and anti-myc antibody (9E10) were used for overnight labeling at 4 °C; then HRP-labeled secondary antibodies were applied for 1 h at room temperature. The 3,3′-diaminodbenzidine substrate was used for color development, followed by EM procedure.

Fluorescence Lifetime Imaging Microscopy. FLIM was performed as described previously (3).

Two-Dimensional Gel Electrophoresis. Two-dimensional electrophoresis was performed following the manufacturer's protocol (Bio-Rad) followed by Western blot analyses using a polyclonal anti-parkin antibody (Abcam). One hundred micrograms of protein were processed using a Ready Prep 2D Cleanup kit (BioRad) and were resuspended in 125 μL of rehydratation buffer (BioRad). Samples were applied to 7–10 IPG strips (BioRad) and incubated overnight at room temperature. Isoelectric focusing and 2D SDS/PAGE were run under standard conditions, and proteins were transferred onto PVDF filters.

Glutathione S-transferase (GST)-Fused Proteins. To generate the GST-fusion protein PINK1 WT and PINK1 KD, *Escherichia coli* strain BL-21 was transformed with plasmids. After 4 h of induction with 0.1 mM isopropyl thiogalactopyranoside (Sigma), the GST-PINK1 fusion proteins were purified with glutathione-Sepharose 4B (BD Biosciences) according to the manufacturer's instructions. Protein preparations were analyzed by Coomassie staining of SDS/PAGE under standard conditions.

Parkin in Vitro Phosphorylation Assay. The in vitro kinase assay was performed as described by Casari et al. (4). Parkin phosphorylation by GST-PINK1 protein was performed in a 40-μL reaction buffer containing 20% glycerol, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM DTT, 40 mM Hepes (pH 7.4), protease inhibitor mixture (Sigma), 100 μM ATP, and without and with 1 μCi [γ-^{32}P]ATP, GST-PINK1 proteins, and His-Parkin (Boston Biochem) at 30 °C for 30 min. The reaction was initiated by the addition of GST-PINK1 protein and terminated by adding SDS sample buffer. Phosphorylated proteins were separated on an 8% SDS/PAGE gel and transferred to nitrocellulose membrane (BioRad) by standard Western blotting technique. Radiolabeled His-Parkin was exposed to Phosphor Screen (Packard).

Statistical Analysis. Differences among means were analyzed using 1- or 2-way ANOVA, followed, when showing significant differences, by pair-wise comparisons between means using Newman–Keuls post hoc testing. When only 2 groups were compared, Student's *t* test or Mann-Whitney test was used. In all analyses, the null hypothesis was rejected at the 0.05 level.

1. Zhou C, et al. (2008) The kinase domain of mitochondrial PINK1 faces the cytoplasm. *Proc Natl Acad Sci USA* 105:12022–12027.
2. Nagai M, et al. (2007) Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat Neurosci* 10:615–622.
3. Grailhe R, et al. (2006) Monitoring protein interactions in the living cell through the fluorescence decays of the cyan fluorescent protein. *ChemPhysChem* 7:1442–1454.
4. Casari G (2005) Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. *Hum Mol Genet* 14:3477–3492.

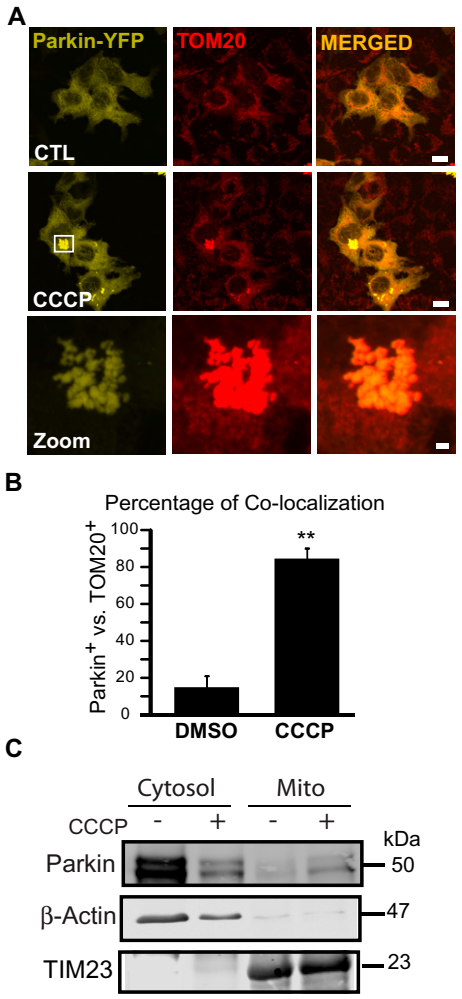


Fig. S1. CCCP exposure alters the subcellular distribution of Parkin from the cytosol to the mitochondria. (*A*) Relocation of Parkin-YFP in response to 1-h incubation with 10 μM CCCP (*Middle row*), but not with vehicle (0.1% DMSO) (*Top row*), in HEK293T cells studied 24 h posttransfection. Mitochondria, immunolabeled with the mitochondrial protein TOM20, show colocalization of Parkin-YFP and mitochondria. (*Bottom row*) A 6× magnification of the region outlined by the box in the image on the left of the middle row. (Scale bars, 10 μm for top and middle rows; 1 μM for bottom row.) (*B*) Percentage of Parkin signal that colocalizes with mitochondria (Parkin puncta that are $TOM20^{+}$) in transfected HEK293T cells incubated with DMSO or CCCP. This analysis is performed with the Image software J; values represent means ± SD of 10 independent experiments. **, Different from vehicle DMSO controls (Student t-test, $p < 0.01$). (*C*) Western blot analysis showing endogenous Parkin recruitment to mitochondria on CCCP exposure. Cytosolic (Cytosol) and mitochondrial (Mito) fractions of HEK293T cells incubated for 1 h with (+) and without (–) 10 μM CCCP. TIM23 and β-actin are used as mitochondrial and cytosolic markers, respectively.

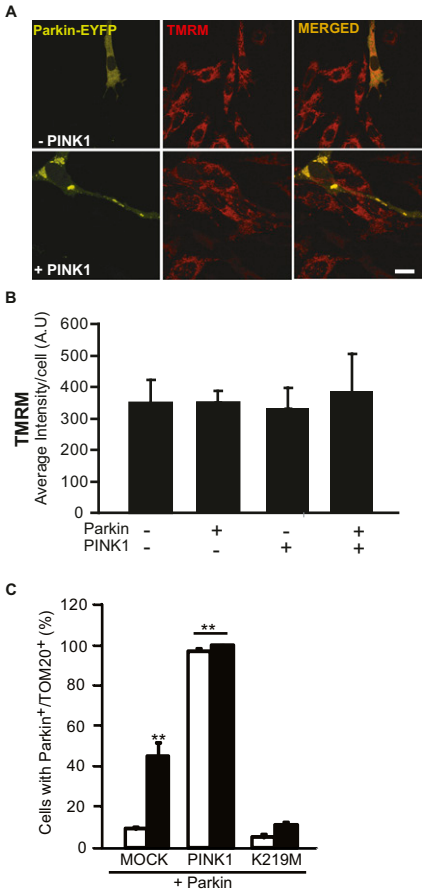


Fig. S2. PINK1-dependent recruitment of Parkin to mitochondria is independent of $\Delta\Psi_m$ but requires PINK1 kinase activity. (*A*) Representative changes in $\Delta\Psi_m$ assessed by TMRM fluorescence (red) in living Parkin-YFP–transfected N27 cells without (– PINK1) and with (+ PINK1) PINK1 induction. (Scale bar, 10 μM.) (*B*) Quantification of TMRM fluorescence showing the lack of effect of overexpression of PINK1, Parkin-YFP, or both on $\Delta\Psi_m$ (1-way ANOVA, $F(3, 8) = 0.40$, $P = 0.75$). Values represent means ± SD of 3 independent experiments performed on 15 cells per group and per experiment. (*C*) HeLa cells were cotransfected with Parkin-YFP and with either mock, WT PINK1, PINK1 or with PINK1 dead-kinase mutant K219M. Twenty-four hours after transfection, cells were incubated with vehicle (DMSO; white bars) or with 10 μM CCCP (black bars) for 1 h prior to fixation. Bars represent percent of cells showing Parkin-YFP colocalization with TOM20 ($Parkin^+/TOM20^+$) ± SD of 3 independent experiments, determined by confocal microscopy. **, Different from vehicle incubated, *Parkin*/*Mock* co-transfected cells.

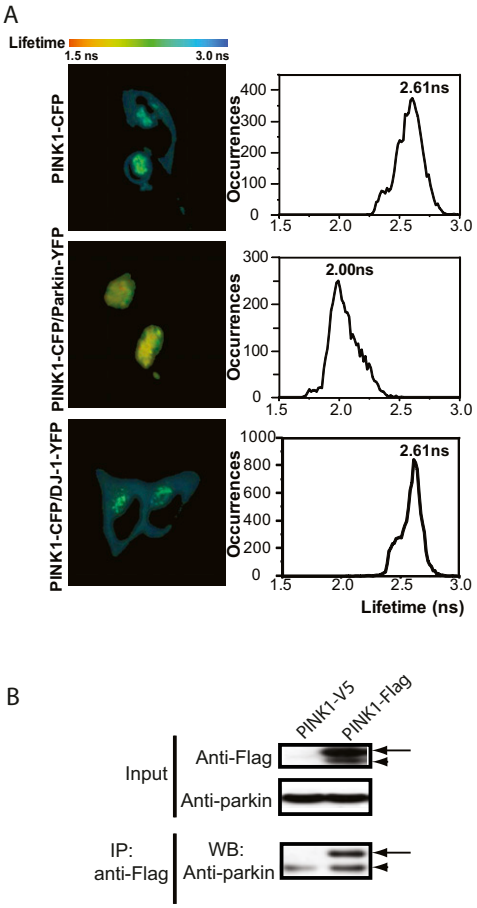


Fig. S3. Parkin interacts with PINK1 in mammalian cells. (*A*) FLIM analysis of the Parkin-PINK1 interaction. CFP-tagged PINK1 (PINK1-CFP) is transfected alone or in combination with YFP-tagged Parkin (Parkin-YFP) or with YFP-tagged DJ-1 (DJ-1-YFP) in HEK293 cells. Donor fluorophore lifetimes are color-coded according to the scale indicated at top of the figure. "Warmer" color is indicative of shorter donor fluorophore lifetime (2.00 ns vs. 2.61 ns) for the interaction between PINK1 and Parkin, compared with the control interaction between DJ-1 and PINK1. (*B*) Lysates prepared from SH-SY5Y cells transfected with full-length WT PINK1 tagged with Flag or with V5 tags were subjected to immunoprecipitation with anti-parkin antibody, followed by anti-Flag immunoblotting. Arrow indicates full-length PINK1; arrowhead indicates cleaved PINK1.

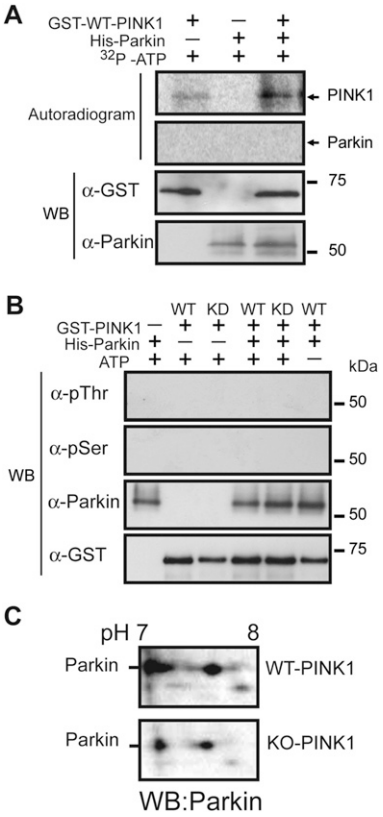


Fig. S4. PINK1 does not phosphorylate Parkin. (*A*) Autoradiography showing no phosphorylation of Parkin by PINK1 using an in vitro kinase assay with GST-PINK1, His-Parkin, and [γ-^{32}P]ATP. (*B*) Same negative findings as in (*A*) using a nonradioactive in vitro kinase assay with GST-PINK1, His-Parkin, and phosphoserine- and phosphothreonine-specific antibodies (kinase death PINK1^{K219M} mutant, KD). (*C*) Phosphorylation of Parkin is unchanged in PINK1-knockout mice as shown by 2D gel electrophoresis using an anti-Parkin antibody. In the isoelectric focusing gel, the more phosphorylated Parkin migrates to the more acidic left end, whereas the less or nonphosphorylated Parkin migrates to the more basic right end.

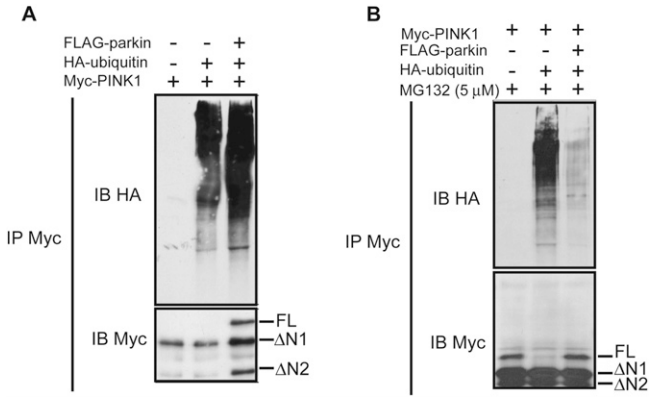


Fig. S5. Parkin fails to ubiquitinate PINK1. (*A*) Coexpression of Myc-PINK1, FLAG-Parkin, and HA-ubiquitin in SH-SY5Y cells followed by immunoprecipitation with anti-Myc antibody and probed with anti-HA or anti-Myc antibodies. PINK1 associates with but is not covalently modified by HA-ubiquitin. The increase in PINK1-associated HA-ubiquitin in the presence of Parkin reflects the noncovalent interaction of PINK1 with high-molecular-weight Parkin-ubiquitin conjugates. Also, Parkin expression does not decrease the normal steady-state levels of PINK1, suggesting that Parkin does not promote the degradation of PINK1 via the proteasome. Furthermore, Parkin expression seems to lead to an accumulation of full-length (FL) PINK1 (lane 3, *Lower panel*). ΔN1 and ΔN2, N-terminally truncated species (lanes 1 and 2, *Lower panel*). *, IgG heavy chain. (*B*) Parkin does not promote the proteasomal degradation of PINK1. SH-SY5Y cells coexpressing myc-PINK1, FLAG-Parkin, and HA-ubiquitin are treated with the proteasomal inhibitor MG132 (5 μM) for 24 h before immunoprecipitation with anti-myc antibody and probing with anti-HA and anti-myc antibodies. PINK1 interacts noncovalently with ubiquitin as reflected by the distinct absence of PINK1-ubiquitin conjugates (lane 2, *Lower panel*). Coexpression of Parkin fails to promote the accumulation of PINK1-ubiquitin conjugates (lane 3, *Lower panel*) following proteasome inhibition, suggesting that Parkin does not promote the degradation of PINK1 via ubiquitination and proteasomal targeting. Instead, Parkin expression promotes the accumulation of the full-length immature PINK1 species (lanes 1 and 3, *Lower panel*), possibly through retention of PINK1 in the cytoplasm and prevention of mitochondrial targeting/processing.

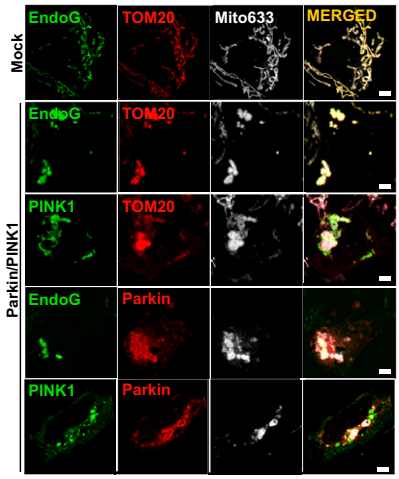


Fig. S6. Parkin and PINK1 co-overexpression induces the formation of perinuclear mitochondrial clusters in SH-SY5Y cells. Transfected cells with empty vector (Mock) have even mitochondrial distribution throughout the cytoplasm, as shown with the mitochondrial markers endonuclease-G (EndoG), TOM20, and MitoTracker Deep Red 633 (Mito633). By contrast, in the Parkin/PINK1 double-transfected cells, mitochondria are clustered at the perinuclear area. Dual immunostaining with PINK1 and TOM20 and EndoG and myc (for myc-Parkin) show that both PINK1 and Parkin are enriched at the periphery of the mitochondrial clusters. Moreover, PINK1-Parkin-myc dual immunostaining showed that indeed Parkin and PINK1 co-localize at the periphery of mitochondrial clusters.

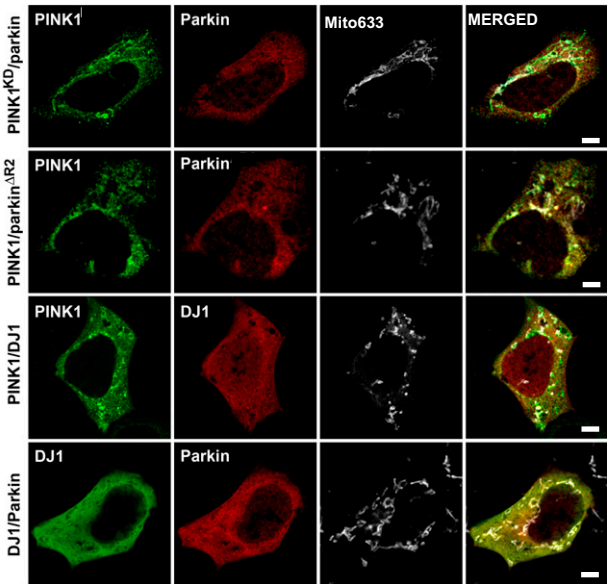


Fig. S7. Co-overexpression of PINK1^{K219M} (KD)/Parkin, PINK1/Parkin$^{\Delta R2}$ (ΔR2), PINK1/DJ-1, and DJ-1/Parkin in SH-SY5Y cells do not produce perinuclear mitochondrial clustering. MitoTracker Deep Red 633 (Mito633) is used here to label mitochondrial. (Scale bar, 5 μm.)

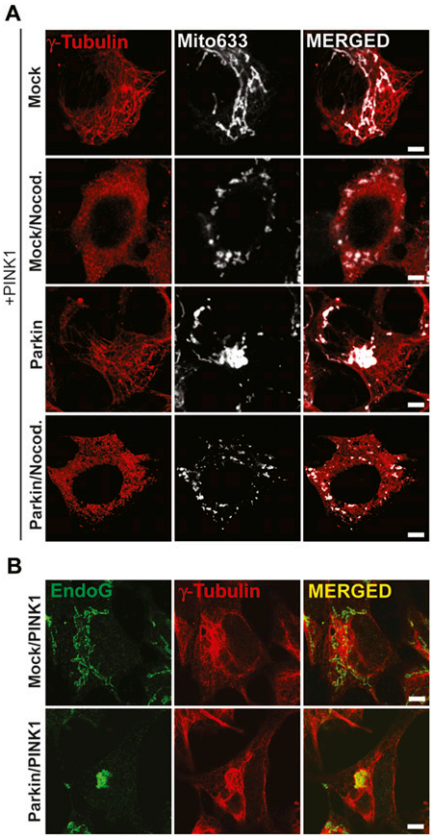


Fig. S8. Mitochondrial clusters dissipate upon microtubule depolymerization. (*A*) In empty vector-transfected (Mock) SH-SY5Y cells stably overexpressing PINK1, treatment of 1 μM nocodazole (Nocod.) for 1 h depolymerizes the microtubule network, resulting in the disruption of the mitochondrial network. In Parkin-transfected SH-SY5Y cells stably overexpressing PINK1, perinuclear mitochondrial clusters are observed at ~36 h posttransfection. However, the majority of perinuclear mitochondrial clusters are replaced by scattered mitochondrial aggregates on nocodazole treatment. (*B*) SH-SY5Y cells cotransfected with empty vector (Mock) plus PINK1 or with Parkin plus PINK1 and stained with the mitochondrial marker EndoG (green) and anti-γ-tubulin (red). As a highly conserved element of microtubule-organizing centers (MTOCs), γ-tubulin is used as a MTOC marker (*arrows*), revealing a strong immunostained zone in the perinuclear area. In Mock/PINK1-transfected cells, anti-EndoG staining shows widely distributed mitochondrial networks. In Parkin/PINK1-transfected cells, perinuclear mitochondrial clusters are colocalized with perinuclear γ-tubulin–positive MTOCs. (Scale bars, 5 μm.)

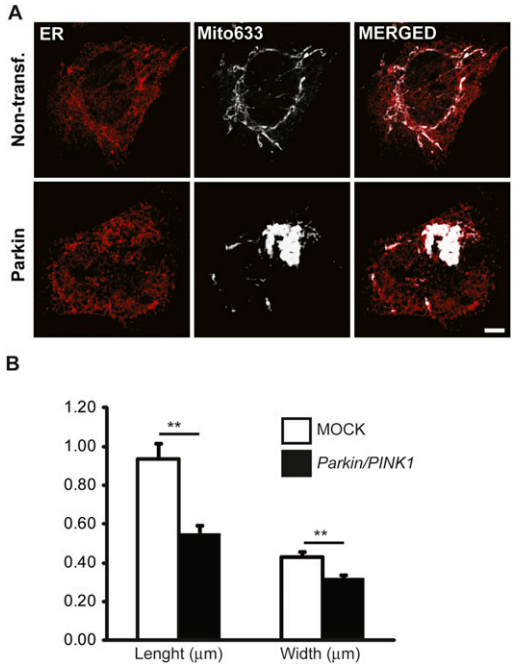
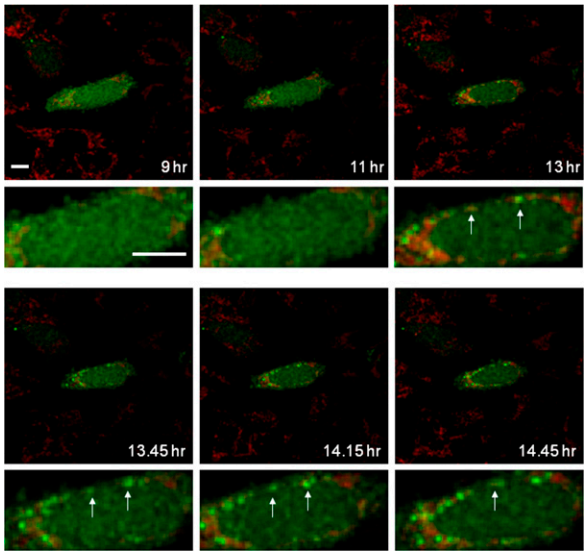


Fig. S9. (*A*) The endoplasmic reticulum (ER) network is not affected by Parkin-PINK1–induced mitochondrial clustering. In empty-vector (Mock)- and in Parkin-transfected SH-SY5Y cells stably overexpressing PINK1, the ER network, visualized by immunostaining with the ER marker calreticulin, spread throughout the entire cytosol and was not affected by the formation of perinuclear mitochondrial clusters shown by Mito633. (Scale bar, 5 μm.) (*B*) EM analyses revealed that *PINK1*/*Parkin*-cotransfected cells have smaller mitochondria than Mock-transfected control cells. Bars represent average counts ($n = 50$ cells) ± SD of 3 independent experiments. Mitochondrial length and size were measured using Image J software analyses. **, Different from mock-transfected cells (Mann-Whitney, $p < 0.001$).



Video S1. Parkin recruitment to mitochondria induces mitochondrial clustering that coalesces in the perinuclear area. N27-inducible cells were transfected with GFP-Parkin. Twelve hours after transfection, cells were incubated with 10 μM ponasterone for 9 h. Cells then were labeled with 25 nM MitoTracker Red (Invitrogen) for 25 min, followed by 2 washes in regular RPMI medium. Live-imaging recording was performed for 7 h while cells were incubated in their regular medium supplemented with 10 μM ponasterone. Ten hours after PINK1-induced expression, mitochondrial GFP-Parkin–positive aggregates become visible in the cytosol; 2 h later they coalesce in the perinuclear region.

Video S1.