Supplementary Figure 1. CCCP and valinomycin elicit different cell fate outcomes in a PINK1/Parkin-dependent manner (related to Figure 1).

(A and B) HeLa cells expressing Venus-Parkin-WT, RFP-Smac and CFP-LC3 were treated with 10μM CCCP in a 10-hour time course. Mitophagy response was visualized with fluorescent microscopy exhibiting translocation of Parkin and LC3 to the mitochondria (A) and quantified by collecting image pairs and analyzed via MATLAB to detect colocalization (B).
(C-F) HeLa cells expressing Venus-Parkin-WT and RFP-Smac were treated with 10μM CCCP in a 100-min time course (C and E) or 10μM Valinomycin in an 80-min time course (D and F). Parkin localization to Mitochondria was visualized with fluorescent microscopy (C and D) and quantified by collecting image pairs and analyzed via MATLAB to detect colocalization (E and F). CCCP and Valinomycin triggered similar phenotypic responses apropos of Parkin localization to mitochondria in HeLa cells.
(G and H) HeLa cells expressing Venus-Parkin-WT and stained with Hoechst 33258 were treated with 10μM Valinomycin in the presence or absence of Z-VAD in a 12-hour time course. Cells were incubated with valinomycin for 1.5h prior to image capture. Apoptotic cell death was visualized with fluorescent microscopy (G) and quantified by counting cells positive for condensed chromatin using MetaXpress as described in Figure 1 (H). Scale bar, 10 μm.

Supplementary Figure 2. Valinomycin induces Parkin-dependent suppression of Mcl-1 and activation of apoptotic initiator caspases in MEF cells (related to Figure 2).

(A) PINK1 null MEF cells were reconstituted by expression of human PINK1 (hPINK1) and human Parkin (hParkin) respectively and subjected to 10μM valinomycin in a 6-hour time course. Pro-apoptotic and anti-apoptotic proteins were monitored by western blotting using antibodies for their respective proteins. Upon valinomycin treatment, cells with restored PINK1-Parkin pathway exhibited a depletion of Mcl-1 levels, activation of Caspase-8 and -9, and cleavage of Caspase-3 and PARP. The corresponding apoptotic response was not detected in PINK1 null cells. (B) and (C) MEF cells expressing Venus-Parkin-WT and stained with Hoeschst 33258 were treated with 10μM valinomycin in the presence or absence of Z-VAD in a 9-hour time course. Cells were incubated with valinomycin for 1h prior to image capture. Apoptotic cell death was visualized with fluorescent microscopy (B) and quantified by scoring the percentage of apoptotic nuclei. Scale bar, 10 μm.

Supplementary Figure 3. Sequence alignment and structural highlights of Parkin and Ubiquitin (related to Figure 4).

(A) PRALINE multiple sequence alignment of Parkin and human ubiquitin. S65 residue in the Ubl domain of Parkin is highlighted with a star. Secondary elements in Ubl domain and ubiquitin are shown above the sequence alignment.
(B) Structure comparison of Ubiquitin and Parkin Ubl domain. Ubiquitin (PDB: 1UBQ) and Parkin (PDB:4K95) structures were displayed using Pymol.
(C) Crystal structure of rParkin (PDB:4K95). Key residues and the relevant functional domains are indicated.

Supplementary Figure 4. Phosphorylation of ubiquitin at Ser^{65} by PINK1 promotes Parkin activation and mitochondrial recruitment (related to Figure 4).

(A) Phosphorylation of ubiquitin by PINK1 at Ser^{65}. Ubiquitin is a novel substrate of PINK1 and Ser^{65} residue of ubiquitin is essential for phosphorylation by TcPINK1. (B) Ubiquitin phosphorylation enhances Parkin E3 ligase activity. Ubiquitin was incubated with TcPINK1 or kinase dead TcPINK1 for 60 min to allow phosphorylation to occur. Phosphorylated and unphosphorylated ubiquitin were used as input for Parkin auto- and trans-ubiquitination reactions *in vitro*. The enhanced auto-ubiquitination and trans-ubiquitination activity
were measured by immunoblotting with indicated antibodies and early appearance of ubiquitylated species. (C) The effect of 5 μM phospho-mimetic ubiquitin (Ub^{S65D}) and nonphosphorylatable ubiquitin (Ser^{S65A}) on Parkin auto-ubiquitination. Either phosphorylated rParkin or nonphosphorylated rParkin were used. Phosphorylated rParkin was made by incubating rParkin with TcPINK1 for 60 min prior to the ubiquitination reaction. Since auto-ubiquitination has a fast kinetics, the reaction was terminated at 10 min. (D) and (E) Expression of nonphosphorylatable ubiquitin ($HA\text{-}Ubiquitin^{S65A}$) suppresses valinomycin induced Parkin mitochondrial recruitment. Scale bar, 10 μm.

Supplementary Figure 5. Requirement of autocatalytic activity of Parkin for valinomycin-induced apoptosis (related to Figure 6).

(A) and (B) Immunoblotting analysis of $PINK1^{-/-}$ $Parkin^{-/-}$ MEF-hPINK1 cells expressing Venus-Parkin or $Venus\text{-}Parkin^{C431S}$ or Venus- $Parkin^{C431S}$ along with Tom70-4xUbi. $Parkin^{C431S}$ is catalytically inactive. (C) and (D) Mitochondrial recruitment of $Venus\text{-}Parkin^{C431S}$ with or without coexpression of Tom70-
4xUbi in response to valinomycin treatment. Scale bar, 10 μm. (E) $PINK1^{-/-}$ $Parkin^{-/-}$ MEF-hPINK1 cells expressing $Venus\text{-}Parkin^{C431S}$, $Venus\text{-}Parkin^{C431S}$ along with Tom70-4xUbi, $Venus\text{-}Parkin^{WT}$ and $mTurquoise\text{-}Parkin^{S65A}$ were treated with valinomycin for indicated time. Apoptotic cell death was quantified by scoring the percentage of apoptotic nuclei. (F) $PINK1^{-/-}$ $Parkin^{-/-}$ MEF-hPINK1 cells expressing $Venus\text{-}Parkin^{C431S}$, $Venus\text{-}Parkin^{C431S}$ along with Tom70-4xUbi, $Venus\text{-}Parkin^{WT}$ and $mTurquoise\text{-}Parkin^{S65A}$ treated with valinomycin for indicated time. Cells were fixed and stained with Hoechst 33258 to obtain 10x images. Scale bar, 10 μm.

Supplemental Experimental Procedures

Constructs. Venus-tagged Parkin constructs were generated by fusion of Parkin wild-type and mutant cDNAs to N-terminal epitope tags using gateway cloning vector pREX-Venus-DEST-IRES-Blasticidin, which is a derivative of a set of retroviral expression vectors described previously [S1]. For generation of PINK1 wild-type and mutant constructs, PINK1 was cloned into CSII-EF-DEST-IRES- Hygromycin lentiviral vectors (gift of Dr. Hiroyuki Miyoshi). The pMSCV-CMV-puro-IMS-RFP, a kind gift from Dr. Sabrina Spencer (Stanford University) was used as mitochondria marker as described previously [S2]. MBP-TcPINK1 and His_6-Sumo-Parkin were gifts of Dr. Miratul Muqit (University of Dundee) and Dr. Helen Walden (Cancer Research UK) respectively. pRK5-HA-Ubiquitin (17608), pGEX-Parkin (45969), GST-Parkin W403A (45974) and GST-Parkin C431SW403A (45977), pBabe-HA-mMcl-1 (25385) and pBabe-Flag-hMcl-1 (25371) were purchased from Addgene. GST-Parkin S65A, pRK5-HA-Ubiquitin S65A and Ubiquitin S65D were constructed by Quikchange mutagenesis (Agilent).

Cell culture, transfection and reagent treatment. HEK293T cells were obtained from the ATCC (American Type Culture Collection). HeLa was a gift of Sabrina Spencer [S2]. Parkin and PINK1 wild type and null MEF cells were described previously [S3]. HeLa and HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen), penicillin, streptomycin (100 IU/ml and 100 mg/ml, respectively), and L-Glutamine. MEFs were grown in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 U/mL), 1 mM l-glutamine, 1mM Na-pyruvate, and 1× nonessential amino acids. All the stable cell lines made by lentivirus and retrovirus packaging were selected with 100 μg/ml hygromycin (Alexis Biochemicals), 5 μg/ml blasticidin (Invitrogen), or 2 μg/ml puromycin (Sigma) based on the selection markers. Mitochondrial membrane potential was dissipated with 10 μM (for HeLa) or 10-20 μM (for MEF) carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma-Aldrich) and 10 μM Valinomycin (Cayman).

Antibodies. Antibodies used in this study for western blot (WB) were: mouse anti-Parkin (1:5000; clone PRK8, Sigma-Aldrich), rabbit anti-PINK1 (1:1000; BC100-494, Novus Biologicals), mouse anti-EZRIN (1:5000, Sigma-Aldrich). Anti-caspase-3, 8, 9, PARP, Mcl-1, Bcl2, Bcl-xL antibodies were purchased from Cell Signaling Technology. Parkin phospho-Ser65 antibody is a gift of Kevan Shokat [S4].

Cell death assays. Three independent cell death assays were used. For the HeLa cells stably expressing RFP-Smac, cell death was monitored by quantifying RFP-Smac release from mitochondria as described previously [S2,S5]. More than 500 cells per condition were inspected. Live cell imaging of cell apoptosis in MEF cells were performed using NucView™488 (Biotium). MEFs were grown on 96-well plates overnight to reach a density of 4×10^4 cells/well. 4 μM NucView caspase-3 biosensor was added. HCS microscope ImageXpress (Molecular Devices) was employed to collect images at indicated time. Typically 10x objective was used for cell death analysis and four independent sites per well of a 96 well plate were imaged. At least 1000 cells per well were examined and quantified. For quantitation of caspase-3 activation in MEF cells, 2 μg/ml of Hoechst dye was included in the media to obtain the total number of nuclei in the field of view. The number of NucView™488 positive cells relative to the total number of nuclei was determined and plotted.

Apoptotic cell death was also visualized and scored by staining with Hoechst 33258 which enters all cells regardless cell health. The average intensity of DNA labeling significantly increases in apoptotic cells as nuclei condense during apoptosis. Quantitation of cell death was performed using automated high content analysis cell heath application module in MetaXpress software (Molecular Devices).

Live cell imaging and immunofluorescence microscopy. To obtain high throughput images and movies, cells were grown on Costar 96-well plates. Molecular Devices ImageXpress XL is used to screen the plates and collect data. Live cell imaging was collected for 1-5 h (for mitochondrial localization of Parkin) and 6-20 h (for apoptosis and mitophagy) for HeLa and MEF cells. To get high resolution images and movies for Parkin mitophagy, cells were growing on 4 well glass bottom chamber (Lab-Tek). Confocal images were acquired on a Nikon A1R Confocal and TIRF using a 100X (NA 1.45) objective. For immunofluorescence microscopy, cells were fixed with 4% paraformaldehyde. Immunofluorescence microscopy was performed as described previously [S6].

Statistical analysis. The Parkin localization in mitochondria and HeLa cells apoptosis were assessed by visually scoring more than 500 cells per stable cell line, in at least three independent experiments. For quantification of MEFs apoptosis, more than 5000 cells were quantified per condition using MetaXpress Multiwavelength Cell Scoring Application Module (Molecular Devices). Standard deviations were calculated from at least three sets of data. The p values are determined using SigmaPlot.

Supplemental References

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