Supplemental Figures

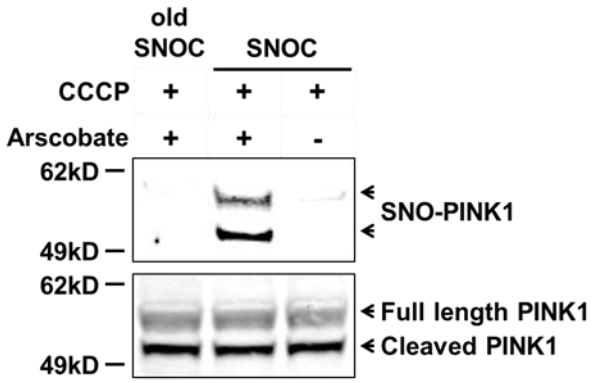

Figure S1 (Related to Figure 1). Full-length PINK1 is S-Nitrosylated

SH-SY5Y cells were pre-treated with 10 μM CCCP for 4 hr and then incubated for 20 min in 200 μM fresh SNOC or, as a control, old SNOC from which NO had been dissipated. Cell lysates were subjected to biotin-switch assay and immunoblotted with anti-PINK1 antibody.

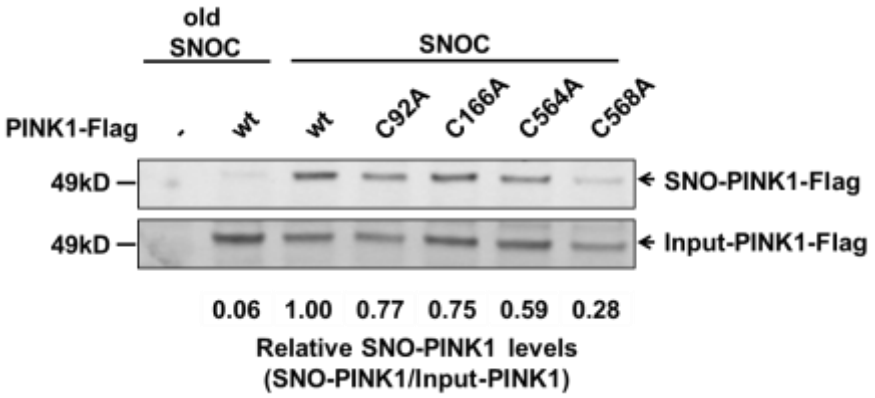

Figure S2 (Related to Figure 1). Cysteine 568 is the Predominant Site of S-Nitrosylation on PINK1

HEK293 cells were transfected with wt or various Cys mutant PINK1-Flag constructs. After 1 day, cells were pre-treated with 10 μM MG132 for 4 hr and then incubated for 20 min in 200 μM fresh or old SNOC. Cell lysates were subjected to biotin-switch assay and immunoblotted with anti-Flag antibody.

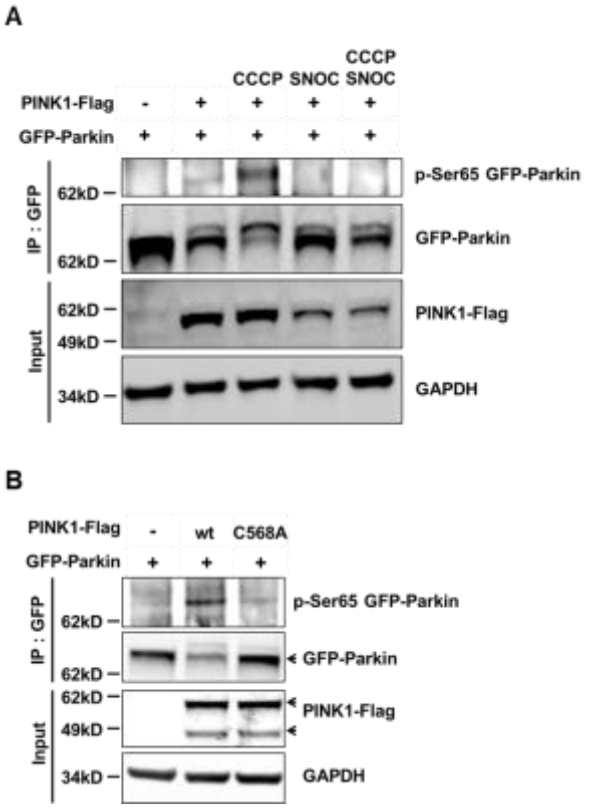

Figure S3 (Related to Figure 2). SNOC Decreases PINK1-Dependent Parkin Phosphorylation, and PINK1(C568A) Mutant Inhibits Kinase Activity

(**A**) SH-SY5Y cells were transfected with GFP-Parkin and wt PINK1-Flag constructs. The next day cells were exposed to 200 μM SNOC in the presence or absence of 10 μM CCCP for 90 min. Cell lysates were immunoprecipitated with anti-GFP antibody, and proteins were immunoblotted with anti-Parkin pSer65 antibody. Total cell lysates were immunoblotted with anti-Flag and anti-GAPDH antibodies for the input lanes.

(**B**) SH-SY5Y cells were transfected with GFP-Parkin plus wt PINK1 or non-nitrosylatable PINK1(C568A)-Flag. The next day cell lysates were immunoprecipitated with anti-GFP antibody, and proteins were immunoblotted with anti-Parkin pSer65 antibody. Total cell lysates were immunoblotted with anti-Flag and anti-GAPDH antibodies for the input lanes.

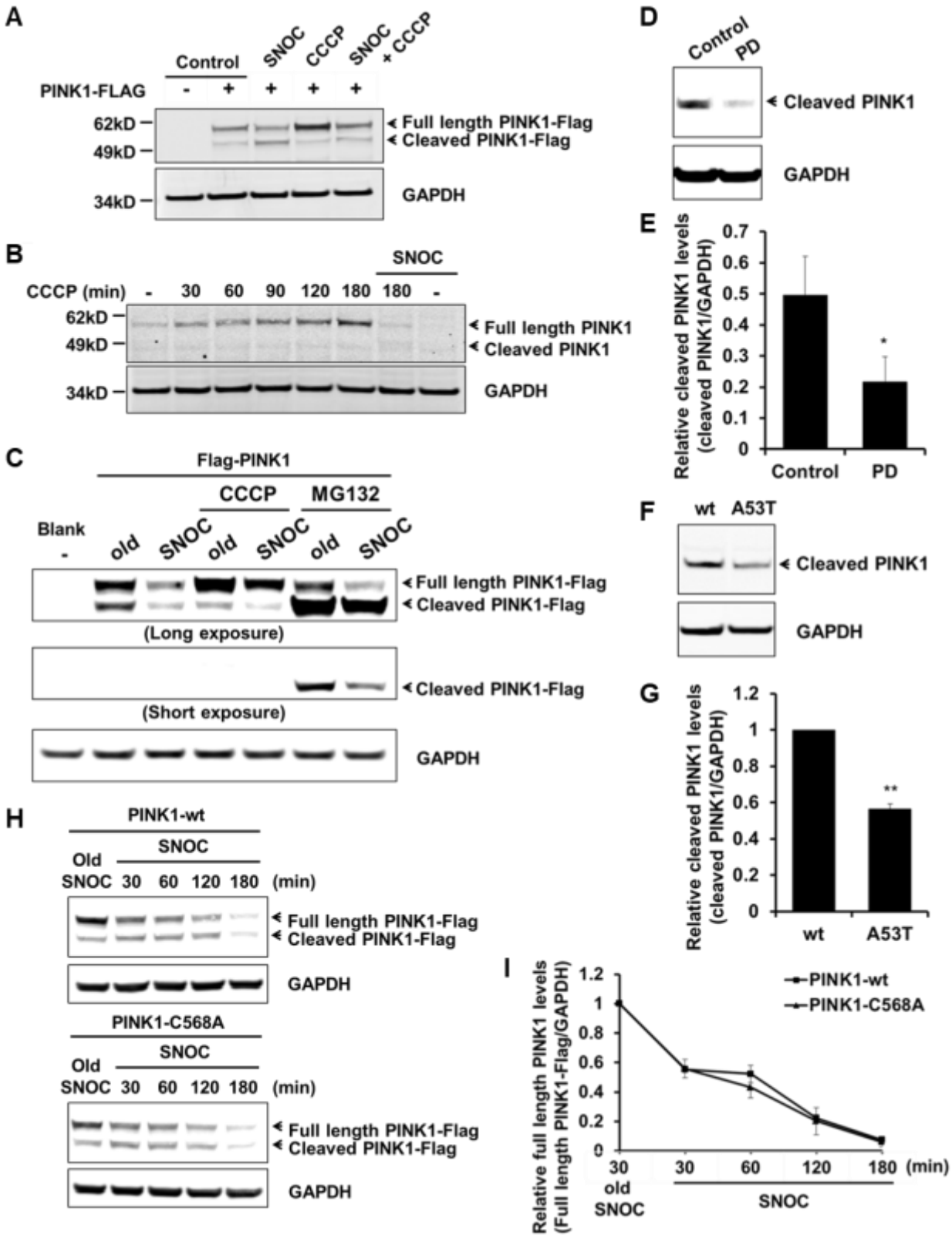

Figure S4 (Related to Figure 2). SNOC Downregulates PINK1-Flag Protein Levels

(**A**) SH-SY5Y cells were transfected with wt PINK1-Flag. After 1 day, cells were exposed

to 10 μM CCCP or 200 μM SNOC. After 90 min, cell lysates were prepared and immunoblotted with anti-Flag and anti-GAPDH antibodies.

(**B**) SH-SY5Y cells were exposed to 10 μM CCCP or 200 μM SNOC. After the indicated time, cell lysates were prepared and immunoblotted with anti-PINK1 and anti-GAPDH antibodies.

(**C**) SH-SY5Y cells were transfected with wt PINK1-Flag. After 1 day, cells were exposed to 200 μM fresh or old SNOC in the presence or absence of 10 μM CCCP or 2.5 μM MG132. After 2 hr, cell lysates were prepared and immunoblotted with anti-Flag and anti-GAPDH antibodies.

(**D** and **E**) Postmortem human brain samples from PD and age-matched control (>70 yr old) were immunoblotted for PINK1 and GAPDH. Representative gel image (**D**) and ratio of cleaved PINK1/GAPDH in control or PD brains (**E**). Data are mean + SEM, n = 9; *p < 0.05 by two-tailed Student's t-test (see **Table S1** for patient information).

(**F** and **G**) Cell lysates from hiPSC-DA neurons bearing the A53T α-synuclein mutation or isogenic corrected control neurons (wt) were immunoblotted with anti-PINK1 and anti-GAPDH antibodies (**F**). Protein levels of cleaved PINK1 were normalized to GAPDH levels (**G**). Values are mean + SEM, n = 3 experiments; **p < 0.01 by two-tailed Student's t-test.

(H and **I)** SH-SY5Y cells were transfected with wt PINK1 or non-nitrosylatable PINK1(C568A)-Flag. The next day cells were exposed to 200 μM fresh or 'old' SNOC and then incubated for the indicate time. Cell lysates were prepared and immunoblotted with anti-Flag and anti-GAPDH antibodies (**H**). Protein levels of full-length PINK1 were normalized to GAPDH (**I**). Values are mean ± SEM, n = 3 experiments.

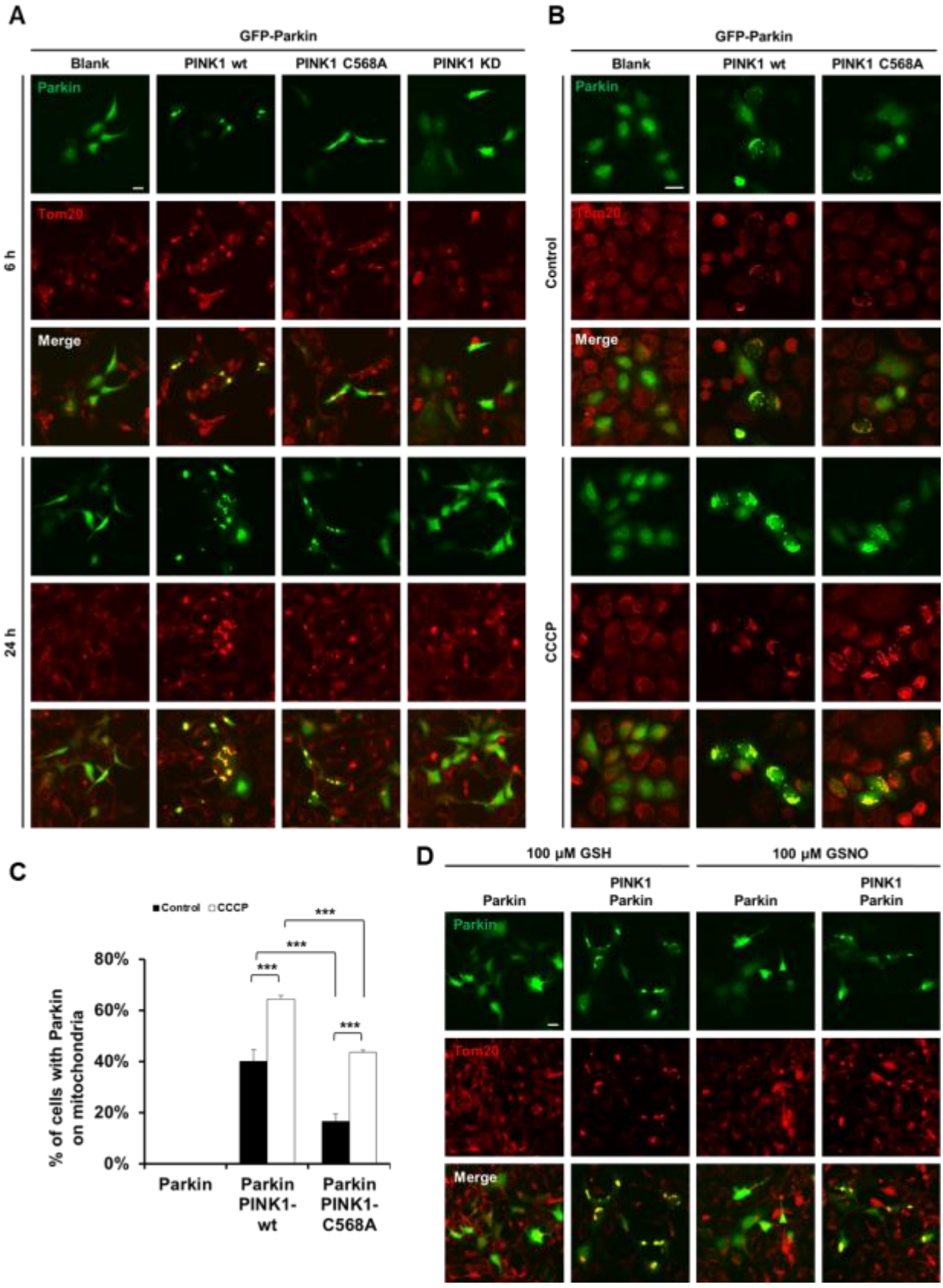
A
GFP-Parkin
Blank
PINK1 wt
PINK1 C568A
PINK1 KD
Parkin
Tom20
Merge
6 h
24 h
B
GFP-Parkin
Blank
PINK1 wt
PINK1 C568A
Control
CCCP
C
Control
CCCP
***
***
***
***
80%
60%
40%
20%
0%
% of cells with Parkin on mitochondria
Parkin
Parkin PINK1-wt
Parkin PINK1-C568A
D
100 μM GSH
100 μM GSNO
Parkin
PINK1 Parkin
Parkin
PINK1 Parkin
Parkin
Tom20
Merge

Figure S5 (Related to Figure 3). S-Nitrosylation of PINK1 Decreases Parkin Translocation to Mitochondria

(**A**) SH-SY5Y cells were transfected with GFP-Parkin plus wt-, non-nitrosylatable C568A, or kinase dead (KD) PINK1-Flag and incubated for 6 or 24 hr. Scale bar, 20 μm.

(**B** and **C**) PINK1 null HeLa cells were transfected with GFP-Parkin plus wt PINK1 or non-nitrosylatable PINK1(C568A). Seven hours later, cells were exposed to 100 μM CCCP for 3 hr. Positive cells were scored as exhibiting translocation of GFP-Parkin to the mitochondrial membrane. Data are mean + SEM; ***$p < 0.001$ by ANOVA; n = 3 experiments. Scale bar, 20 μm.

(**D**) SH-SY5Y cells were transfected with GFP-Parkin plus wt PINK1-Flag. After 6 hr, cells were exposed to 100 μM GSNO or GSH control, and then incubated an additional 24 hr. Scale bar, 20 μm. For each panel, Parkin was identified by the presence of GFP (green) and Tom20 with a specific anti-Tom20 antibody (red).

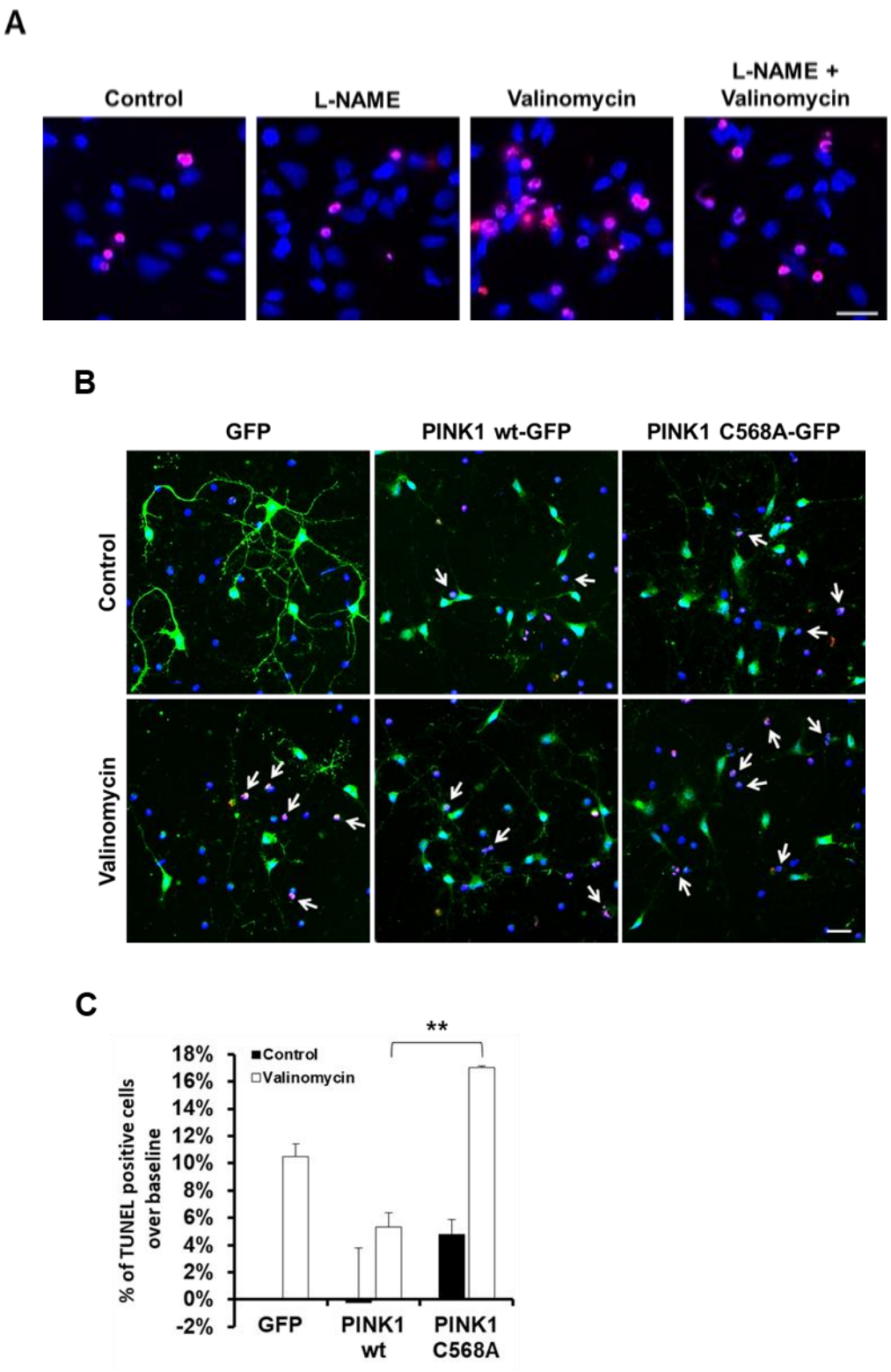

Figure S6 (Related to Figure 6). NOS Inhibition with L-NAME Decreases Cell Death in Response to Valinomycin, while Expression of Non-Nltrosylatable PINK1(C568A) Increases Valinomycin-Induced Cell Death

(**A**) Representative images of TUNEL staining, as assessed quantitatively in Figure 6E. (**B** and **C**) hiPSC-DA neurons were electroporated with GFP, wt PINK1-GFP, or non-nitrosylatable mutant PINK1(C568A)-GFP. Subsequently, transfected cells were exposed to 250 nM valinomycin for 9 hr, fixed with 4% PFA and assayed for apoptotic neurons by TUNEL. Representative images of TUNEL staining (**B**) and quantification of TUNEL assay (**C**) are shown. Scale bar, 20 μm. Data are mean + SEM from five random fields in each experiment; **$p < 0.01$ by ANOVA; n = 3 experiments.

Table S1. Clinical Features of Human Subjects Used in this Study

Subject	CNS Diagnosis	Brain Area	PMI (hr)	Age (yr)	Gender
Patient 1	Parkinson's disease	Frontal cortex	12	73	M
Patient 2	Parkinson's disease	Frontal cortex	24	74	M
Patient 3	Parkinson's disease	Frontal cortex	5	82	F
Patient 4	Parkinson's disease	Frontal cortex	N/A	74	M
Patient 5	Parkinson's disease	Frontal cortex	6	82	M
Control 1	Normal	Frontal cortex	8	74	F
Control 2	Normal	Frontal cortex	72	83	F
Control 3	Normal	Frontal cortex	18	93	F
Control 4	Normal	Frontal cortex	2	71	M

PMI, postmortem interval; M, male; F, female; N/A, not available

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Dopaminergic Neural Induction and Electroporation of hiPSCs

hiPSCs were cultured, maintained, and differentiated into hiPSC-DA neurons as we previously described (Ryan et al., 2013). In brief, hiPSCs were maintained in a feeder free system in MEF-conditioned hiPSC medium (DMEM/F12 with glutamax [Life Technologies] plus knockout serum replacement [KSR] medium [20%, Life Technologies], non-essential amino acid solution [1%, Life Technologies], antibiotic-antimycotic additives [1%, Life Technologies], 2-mercaptoethanol [110 μM, Life Technologies], and FGF2 [8 ng/ml]). For dopaminergic neural induction, hiPSCs were disaggregated using TryplE Express for 3 min, washed with hPSC media and replated at 3.5×10^4–4.0×10^4 cells/cm^2 on Matrigel (BD)-coated plates in MEF-conditioned hPSC medium supplemented with 10 ng/ml of FGF2 and ROCK-inhibitor. After 24 hr, the ROCK inhibitor was withdrawn, and hiPSCs were allowed to expand for 3 days or until they were nearly confluent. Initial differentiation conditions consisted of KSR medium including LDN193189 (100 nM, Stemgent), SB431542 (10 μM, Tocris), sonic hedgehog (SHH) C25II (100 ng/ml, R&D), purmorphamine (2 μM, Stemgent), FGF8 (100 ng/ml, R&D), and CHIR99021 (CHIR; 3 μM, Stemgent). At day 5 of differentiation, KSR medium was gradually shifted to N2 medium, with increasing amounts of N2 medium (25%, 50%, 75%) added to KSR medium every 2 days. The culture medium was then switched to Neurobasal/B27/Glutamax supplemented with CHIR between days 11 to 13, and then to Neurobasal/B27/Glutamax supplemented with BDNF (brain-derived neurotrophic factor, 20 ng/ml; R&D), GDNF (glial cell line-derived neurotrophic factor, 20 ng/ml; R&D), TGFβ3 (transforming growth factor type β3, 1 ng/ml; R&D), ascorbic acid (0.2 mM, Sigma), dibutyryl cAMP (0.5 mM; Sigma), and DAPT (10 μM; Tocris) between days 13 to 22. On day 22, cells were dissociated using Accutase (Innovative Cell Technology), replated under high-cell density conditions (3.0×10^5–4.0×10^5 cells per cm^2) on dishes pre-coated with polyornithine (PO; 15 μg/ml)/laminin (1 μg/ml)/fibronectin (2 μg/ml) in differentiation medium (Neurobasal/B27 + BDNF, ascorbic acid, GDNF, dbcAMP, TGFβ3, and DAPT), and incubated for an additional 4-6 days. For electroporation of mt-Keima plus wt PINK1-GFP or mutant PINK1(C568A)-GFP, hiPSC-DA neurons were dissociated on day 20 of differentiation using Accutase, and 1.0×10^7 cells were resuspended in solution A+B mixture (Lonza, VPH-5002). A total of 10 μg of the plasmids was mixed with the cells and subjected to electroporation using a Nucleofector™ 2b Device (Lonza, program A-033). After electroporation, 2.0×10^6 cells were plated in differentiation medium onto 35 mm glass bottom dishes (MatTek, P35G-0-20-C) pre-coated with polyornithine, laminin, and fibronectin. Cells were allowed to recover for 5 days prior to conducting mitophagy assays.

Expression Constructs

Wild-type (wt) pcDNA3-PINK1 containing a C-terminal Flag sequence was a gift from Dr. Zhuohua Zhang (Sanford Burnham Prebys Medical Discovery Institute). C92A, C166A, C564A, and C568A PINK1 mutants were generated using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's protocol. For the generation of C-terminal Tomato- or GFP-tagged PINK1 constructs, wt PINK1 and mutant PINK1(C568A) were cloned into ptdTomato-N1 (Clontech) or pEGFP-N1 (Clontech), respectively. The N-terminal GFP-tagged Parkin construct was cloned into pEGFP-C1 (Clontech). For quantification of mitophagy, pMT-mKeima-Red (Amalgaam) was used, as previously described (Bingol et al., 2014; Katayama et al., 2011). Full-length human PINK1 was cloned into the pET28a vector with a His-tag. The PINK1(C564R/C575R) double mutant was generated using a QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's protocol.

Protein Purification

Transformed E. coli BL21(DE3) cells expressing PINK1 were grown at 37 °C to an optical density measured at 600 nm (OD 600) of 0.6. Cells were induced with 500 μM IPTG (Sigma) and grown further at 20 °C for 12 hr. Cells were harvested and suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 100 mM NaCl, 0.1 mg/ml lysozyme, and 20 μM PMSF (Sigma) and lysed by sonication on ice. Samples were centrifuged at 5000 × g, and the supernatants containing PINK1 were incubated with Ni-NTA Superflow beads (Qiagen) for 2 hr at 4 °C. The beads were washed thoroughly in wash buffer, and PINK1 was then eluted with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 250 mM imidazole.

Mass Spectrometry

For this series of experiments, in order to avoid confusion as to which cysteine residue might be S-nitrosylated, we expressed a PINK1 construct mutated at cysteines near the candidate Cys568 site, yielding PINK1(C564R/C575R). Purified PINK1 was S-nitrosylated by exposure to SNOC (100 μM) followed by the biotin-switch assay to substitute a more stable biotin adduct for NO in order to allow detection by Mass Spectrometry (MS). Biotinylated peptides were then eluted from the neutravidin beads with 80% acetonitrile, 0.2% trifluoroacetic acid, and 0.1% formic acid. Eluted peptides were dried and resuspended with 5% acetonitrile and 0.1% formic acid. The eluted peptides and

unenriched peptides were analyzed on a Q Exactive MS (Thermo Scientific) coupled to a nano-Easy LC pump (Thermo Scientific). The peptides were separated on a 100 μm i.d. capillary with a 5-μm pulled tip packed with 15 cm of 4-μm Jupiter C18 material (Phenomenex) using a reverse phase gradient with an increasing concentration of acetonitrile. For the eluted peptides, the gradient was 2 hr and for the un-enriched peptides the gradient was 4 hr. A cycle of one full-scan mass spectrum (400-1800 m/z) at 70,000 resolution followed by 10 data-dependent higher energy collisional (C-trap) dissociation (HCD) MS/MS spectra at 17,500 resolution was repeated continuously throughout the gradient. Application of MS scan functions and HPLC solvent gradients were controlled by the Xcaliber data system. Protein identification was performed with the Integrated Proteomics Pipeline - IP2 (Integrated Proteomics Applications, Inc., San Diego, CA. http://www.integratedproteomics.com/) using ProLuCID and DTASelect2 (Cociorva et al., 2007, Xu et al., 2015). MS/MS spectra were searched against the human Uniprot database (v. 3-25-2014) concatenated to a decoy database in which the sequence for each entry in the original database was reversed (Peng et al., 2003). The mass of 428.1916 (biotinylated cysteine after 'biotin switch' for NO) was set as a differential modification on cysteine and a 5 ppm mass filter was employed. Fragmentation of the amide bond of the peptide resulted in formation of 'b' and 'y' ion series corresponding to the N-terminal and C-terminal fragments, respectively. Manual validation of the spectra was performed to confirm software results and to identify the source of large peaks that were un-assigned by the software.

Phos-Tag Assay

To detect phosphorylated proteins via SDS-PAGE, SuperSep™ Phos-tag™ gels or 7.5% polyacrylamide gels containing 100 μM phos-tag acrylamide (Wako Chemicals) and 100 μM $MnCl_2$ were used. After electrophoresis, phos-tag acrylamide gels were washed 3 times (10 min each) with transfer buffer containing 10 mM EDTA and gentle shaking, and then washed once with transfer buffer without EDTA for 10 min according to the manufacturer's protocol. Proteins were then transferred to PVDF membranes and analyzed by conventional immunoblotting.

Quantification of Mitophagy

Quantification of mitophagy was performed as described previously (Katayama et al., 2011). Cells were transfected with pMT-mKeima-Red (mt-Keima, Amalgaam) with/without wt PINK1 or mutant PINK1(C568A)-GFP, and then subjected to confocal imaging using an LSM 710 NLO Confocal Microscope (Zeiss) equipped with a 40x objective (C-Apochromat 40x/1.20), an argon laser (for 458 nm [mt-Keima at neutral pH] and 488 nm [GFP]), and a DPSS 561-10 laser (for 561 nm [mt-Keima at acidic pH]). For mt-Keima measurement, 610 nm emission filters were used, whereas for GFP detection a 530 nm emission filter was used. Ratio images of mt-Keima (561 nm/458 nm) were obtained and analyzed using MetaMorph software; the ratio values ranged from 0 to 0.85. High-ratio regions (561 nm/458 nm) were automatically segmented, and their areas were calculated as previously described (Katayama et al., 2011). The mitophagy index (high-ratio area [561 nm/458 nm]/total mitochondrial area) was determined as an index of mitophagy activity. Neutralization of the lysosomal lumen with NH_4Cl reversed the high-ratio (561 nm/458 nm) signal to a low-ratio signal in round structures corresponding to lysosomes, but did not affect the tubular-reticular signal representing cytoplasmic mitochondria (Bingol et al., 2014). This control confirmed that the 'high-ratio' signals were derived from the autolysosomal fraction.

DAF-FM Staining for NO and Reactive Nitrogen Species

As an index of intracellular NO, hiPSC-DA neurons were incubated with 2.5 μM DAF-FM (Invitrogen, D23844) in HEPES buffer (10 mM HEPES, 146 mM NaCl, 2.5 mM KCl, 5 mM glucose, 2 mM $CaCl_2$, pH 7.4) for 30 min, washed with HEPES buffer for 15 min, and then fixed with 4% PFA for 20 min in the dark. Images of fluorescent cells were acquired and quantified by deconvolution microscopy equipped with SlideBook 5.0 software (Intelligent Imaging Innovations, Inc.) as previously described (Ryan et al., 2013).

Immunoblotting

Cultured cells were harvested and lysed in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate) for 10 min at 4 °C. Equivalent protein quantities were subjected to Bolt Bis-Tris Plus (Life Technologies) gel electrophoresis and transferred to PVDF membranes (Millipore). Membranes were blocked with Odyssey blocking buffer (Li-Cor) for 30 min at room temperature and then probed with primary antibodies against Flag (1:3000, Cell signaling, 2368), PINK1 (1:1000, Novus Biologicals, BC100-494), Parkin (1:3000, Abcam, ab77924, clone PRK8), GAPDH (1:5000, Millipore, MAB374, clone 6C5), ubiquitin (1:3000, Santa Cruz Biotechnology, sc-8017, clone P4D1), Ub-pS65 (1:1000, Boston Biochem, A-110). After incubation with secondary antibodies (IR-dye 680LT-conjugated goat anti-mouse [1:20,000; Li-Cor] or IR-dye 800CW-conjugated goat anti-rabbit [1:15,000; Li-Cor]), membranes were scanned with an Odyssey infrared imaging system (Li-Cor). The ImageJ program (http://imagej.nih.gov/ij/download.html) was used for densitometric analysis of immunoblots, and quantified results were normalized to loading controls.

To detect Parkin phosphorylation with anti-Parkin pSer65 antibody (Ubiquigent), cell lysates were prepared in RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo Scientific, 78441), and immuoprecipitated overnight at 4 °C with anti-GFP antibody (Santa Cruz Biotechnology, sc-8334). Immunoprecipitates were subjected to immunoblotting. PVDF membranes were blocked with 5% BSA in TBS-T1 (Tris-buffered saline containing 1 % Tween-20; 5%BSA/TBS-T1) for 1 hr, and incubated overnight with anti-Parkin pSer65 polyclonal antibody (1 μg/ml, Ubiquigent, 68-0056-100) in 5% BSA/TBS-T1 buffer. The non-phosphorylated form of the peptide immunogen (10 μg/ml, Ubiquigent, 68-1010-001) was also added to the immunoblot incubation medium to deplete non-phospho-specific polyclonal antibodies. Membranes were washed with TBS-T0.1 (TBS containing 0.1 % Tween-20) and incubated with HRP-conjugated anti-sheep secondary antibody in 5% BSA/TBS-T0.1. After repeated washes, the membranes were developed with SuperSignal WestDura Extended Duration substrate (Thermo Scientific) and imaged with a Chemidoc Touch Imaging System (Bio-Rad).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature, washed 3 times with PBS, and blocked (5% goat serum and 0.2% Triton X-100 in PBS) for 30 min at room temperature. Cells were incubated with primary antibodies overnight at 4 °C, and the appropriate Alexa Fluor (488, 555, 647)-conjugated secondary antibodies were used at 1:200. The following primary antibodies were used: Parkin (1:200, Abcam, ab77924, clone PRK8), LC3A (1:500, Biolegend, MMS-5238) and Tom20 (1:500, Santa Cruz, sc-11415). Cells were counterstained with 10 mg/ml Hoechst dye 33342 (Invitrogen). Cell images were acquired with an LSM 710 NLO Confocal Microscope (Zeiss) using a 63x/1.40 oil objective (0.132 μm per pixel resolution, 1 μm Z-stack). Colocalization of Parkin or LC3 and Tom20 in hiPSC-DA neurons was monitored using the Fiji program colocalization test module (http://fiji.sc/Downloads), and the data were analyzed with Pearson's correlation coefficient. To score Parkin translocation to the mitochondrial membrane in SH-SY5Y cells, we counted cells expressing GFP-Parkin (n > 450 cells per condition) that completely overlapped (Complete), partially overlapped (Partial), or did not overlap at all (None) with the Tom20 signal, as previously reported (for examples, see **Figure 3A**) (Shiba-Fukushima et al., 2012; Vives-Bauza et al., 2010).

Terminal-Deoxynucleotidyl-Transferase dUTP Nick End Labeling (TUNEL) Assay

Cells were fixed with 4% PFA for 20 min, washed with PBS, permeabilized for 2 min on ice with 0.1% Triton X-100 and 0.1% sodium citrate in PBS, and washed 3 times with PBS. Cells were labeled using an *in situ* cell death detection kit, TMR red (Roche) according to the manufacturer's protocol. Cells were counterstained with 10 mg/ml Hoechst dye 33342. Apoptotic cells were then imaged by deconvolution microscopy equipped with SlideBook 5.0 software and counted from three random fields with a minimum 600 cells scored in each experiment.