Supporting Information

Ordureau et al. 10.1073/pnas.1506593112

SI Materials and Methods

Antibodies and Other Reagents. The following antibodies were used in this study: α-MFN2 ([NIAR164]; Abcam), α-CISD1 (16006-1-AP; Proteintech), α-proliferating cell nuclear antigen (PCNA) (sc-56; Santa Cruz Biotechnology), α-TOMM20 (sc-11415; Santa Cruz Biotechnology), α-PARKIN (sc-32282; Santa Cruz Biotechnology and [EPR5024(N)]; Abcam), α-p-S65-PARKIN (ab154995; Abcam), α-UB (Dako), α-HA (11867423001; Roche), α-GFP (11814460001; Roche), α-Flag M2 (F1804; Sigma), and α-p-S65-UB (ABS1513; Millipore). UB (U6253), antimycin A (A8674), and OA (75351) were from Sigma. Phos-Tag gels were from Wako Chemicals. GFP-trap_A was from Chromotek. Recombinant HA-UB-vinyl sulfone, E1 UB-activating enzyme, and UB-conjugating enzyme UBE2L3, as well as K48-linked ($K48^{2-7}$) and K63-linked ($K63^{2-7}$) UB oligomers were generously provided by Brad Brasher (Boston Biochem, Cambridge, MA).

Cell Culture, Immunoblotting, and Mitochondrial Protein Isolation. U2OS parental UB-replacement cells were generously provided by Z. James Chen (University of Texas Southwestern Medical Center, Dallas, TX). All UB-replacement cells were made as previously described (1). Untagged or GFP-PARKIN was stably expressed in UB-replacement cells using a pLenti-Zeocin vector. Cells with low levels of untagged PARKIN were isolated by single-cell cloning, whereas those cells expressing GFP-PARKIN were isolated by fluorescence-activated cell sorting by selecting twice the lowest 5% of GFP expression. To induce protein expression, cells were treated with 2 μM DOX (renewed every 48 h) for 5 d unless stated otherwise to induce UB shRNA expression and expression of the shRNA-resistant replacement UB (WT, S65A, K6R, K11R, K48R, or K63R). Cells were either left untreated or depolarized with a mixture of antimycin A (10 μM) and OA (5 μM) for the indicated time period. At the indicated times, cells were washed twice with ice-cold PBS and lysed in lysis buffer [50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium 2-glycerol 1-phosphate, 1 mM sodium orthovanadate, 0.27 M sucrose, 1% (vol/vol) Nonidet P-40, 1 μg/mL leupeptin/aprotinin, 0.5 mM 4(2-aminoethyl)benzenesulfonyl flouride (AEBSF)] to produce whole-cell extracts.

Crude mitochondria were purified after two washes in ice-cold PBS by scraping cells in PBS containing 200 mM chloroacetamide (3 mL per 15-cm dish). Cells were then collected and centrifuged at 450 × *g* for 5 min at 4 °C. The cell pellet was resuspended in 5 mL (per 15-cm dish) of mitochondrial isolation buffer [MIB; 50 mM Tris·HCl (pH 7.5), 70 mM sucrose, 210 mM sorbitol, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium 2-glycerophosphate, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μg/mL leupeptin/aprotinin] plus 100 mM chloroacetamide and was centrifuged at 1,400 × *g* for 5 min at 4 °C. The cell pellet was resuspended in 5 mL of MIB buffer plus 100 mM chloroacetamide and sonicated four times for 15 s at the lowest settings. Sample were spun for 10 min at 1,400 × *g* to remove unbroken cells/debris, and supernatant was collected; this supernatant corresponds to the "total protein" fraction, and was transferred into a round-bottomed tube before centrifugation for 10 min at 10,000 × *g*. Supernatant corresponding to the cytosolic fraction and the crude endoplasmic reticulum (ER) fraction was removed, and the pellet corresponding to the crude mitochondria fraction was resuspended in 10 mL of MIB buffer plus 100 mM chloroacetamide before centrifugation for 10 min at 9,000 × *g*. The mitochondrial pellet was washed two more times, and the pellet was then lysed in lysis buffer plus 100 mM chloroacetamide and 0.5 mM AEBSF.

Whole-cell extracts or mitochondrial extracts were sonicated and clarified by centrifugation (16,000 × *g* for 10 min at 4 °C), and protein concentrations were determined by the Bradford assay. Cell extracts (25 μg) or mitochondrial extracts were separated by SDS/PAGE and transferred to PVDF membranes, and proteins were detected by immunoblotting.

p-S65 Measurement in Free Unanchored UB in HeLa Cells. PINK1 WT or KO HeLa Flp-In T-REx cell lines (Life Technologies) expressing various forms of PARKIN were generated as described before (2). To induce protein expression, cells were treated with 0.5 μM DOX for 16 h to induce low levels of PARKIN proteins. Cells were either left untreated or depolarized with a mixture of antimycin A (10 μM) and OA (5 μM) for the indicated time period. At the indicated times, plates were washed twice with ice-cold PBS and lysed in lysis buffer [50 mM Tris·HCl (pH 7.5), 8 M urea, 1 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium 2-glycerol 1-phosphate, 1 mM sodium orthovanadate, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 0.5 mM AEBSF, 200 mM chloroacetamide, phosphatase inhibitor mixture (Roche)] to produce whole-cell extracts. Whole-cell extracts were sonicated and clarified by centrifugation (16,000 × *g* for 10 min at 4 °C) and filtered through 0.45 μ filters (Sartorius), and protein concentrations were determined by the Bradford assay. Proteins with a molecular mass between 3 and 10 kDa were separated from cell extracts (2 mg) using a 50-kDa molecular weight cutoff (MWCO) spin concentrator (Millipore), followed by 30-kDa and 10-kDa MWCO spin concentrators. During the isolation, lysates and flow-through were diluted when needed in 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM sodium 2-glycerol 1-phosphate, 1 mM sodium orthovanadate, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 0.5 mM AEBSF, and phosphatase inhibitor mixture. Final flow-through from the 10-kDa MWCO spin concentrator was concentrated using a 3-kDa MWCO spin concentrator, and samples were reduced [10 mM tris(2-carboxyethyl)phosphine (TCEP)], alkylated (20 mM chloroacetamide), and precipitated with 20% (vol/vol) TCA. TCA-precipitated proteins were then subject to the UB-AQUA/PRM proteomic workflow as described below.

Mitochondrial UB and Poly-UB Capture and Proteomics. Mitochondrially derived ubiquitylated proteins were purified using Halo-4× UBA^{UBQLN1} as described (2). Briefly, whole-cell extracts (0.5 mg) or mitochondrial extracts (0.5 mg) that were lysed in lysis buffer containing 100 mM chloroacetamide were incubated at 4 °C for 4 h or 16 h with 30–50 μL of Halo-4× UBA^{UBQLN1} beads (pack volume). Following four washes with lysis buffer containing 1 M NaCl and one final wash in 10 mM Tris (pH 8.0), proteins were released from Halo-4× UBA^{UBQLN1} beads using sample buffer before analysis by SDS/PAGE and immunoblotting or by 6 M guanidine HCL when analyzed by MS. For immunoblotting, samples were separated on 4–12% Bis-Tris gradient gels. For MS, samples were subjected to reduction (10 mM TCEP) and alkylation (20 mM chloroacetamide) followed by TCA precipitation. Samples were digested overnight at 37 °C with Lys-C and trypsin [in 100 mM tetraethylammonium bromide, 0.1% Rapigest (Waters Corporation), 10% (vol/vol) acetonitrile (ACN)]. Digests were acidified with an equal volume of 5% (vol/vol) formic acid (FA) to a pH of ~2 for 30 min, dried down, resuspended in 5% (vol/vol) FA, and subjected to C18

StageTip (packed with Empore C18; 3M Corporation) desalting. Samples were analyzed by liquid chromatography (LC)/tandem MS or used for AQUA as described below.

UB-AQUA Proteomics. UB-AQUA was performed largely as described previously but with several modifications (2, 3). A collection of 19 heavy-labeled reference peptides (2), each containing a single $^{13}C/^{15}N$-labeled amino acid, was produced at Cell Signaling Technologies and quantified by amino acid analysis. UB-AQUA peptides from working stocks [in 5% (vol/vol) FA] were diluted into the digested sample [in 5% (vol/vol) FA] to be analyzed to an optimal final concentration predetermined for individual peptide such that each peptide's intensity would range between 10^6 and 10^8. Samples and AQUA peptides were oxidized with 0.1% hydrogen peroxide for 30 min, subjected to C18 StageTip and resuspended in 5% (vol/vol) FA. Both in vitro and in vivo experiments were performed in triplicate and analyzed sequentially by LC/MS on an Exactive-Orbitrap mass spectrometer (Thermo Scientific) equipped with a Thermo Fisher nanospray source, a PAL HTC (ThermoScientific) autosampler for sample handling, and an Accela HPLC pump (ThermoScientific) for LC separations. Nanoliter flow rates across the column were achieved using a flow-split method. Peptides were separated on a 125-μm × 18-cm hand-pulled fused silica microcapillary column with a needle tip diameter of <10 μm and were packed with 3 μm of 200-Å Maccel C18 AQ beads (The Nest Group). The column was equilibrated with buffer A [3% (vol/vol) ACN + 0.125% FA]. Peptides were loaded onto the column in 100% buffer A. Separation and elution from the column were achieved using a 60-min or 90-min 0–30% gradient of buffer B [100% (vol/vol) ACN + 0.125% FA]. The length of time required for the total LC method with loading, separation, and column equilibration was 75 or 105 min. The Exactive-Orbitrap mass spectrometer was operated using the following parameters: automatic gain control (AGC) of 3×10^6, resolution of 5×10^4, m/z range of 350–1,000, maximum ion time of 500 ms, and lock mass of 371.1012 m/z. LC/MS data analysis was performed using Pinpoint software (Thermo Fisher Scientific) with manual validation of peptide masses (2). MS^1 intensities from Pinpoint were exported as a .CSV file for further analysis in Microsoft Excel and GraphPad Prism. Total UB was determined as the average of the total UB calculated for each individual locus, unless specified otherwise. In experiments using the UB replacement cells, the femtomole values of the AQUA peptides starting with Met1 of UB were multiplied by 2 to reflect the fact that half of the RNAi-resistant UB reexpressed under DOX treatment contains an N-terminal HA tag preventing the measurement of these peptides. For UB chain synthesis kinetics, data were further analyzed in Prism and linear regression analysis was performed to fit slopes with quantitative MS data. R^2 is the coefficient of determination, and the 95% confidence intervals are indicated by dotted lines (Fig. S4*C*).

UB-AQUA/PRM Proteomics. Our MS data were collected using a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled with a Famos Autosampler (LC Packings) and an Accela600 LC pump (Thermo Fisher Scientific). Peptides were separated on a 100-μm i.d. microcapillary column packed with ~0.5 cm of Magic C4 resin (5 μm, 100 Å; Michrom Bioresources) followed by ~20 cm of Accucore C18 resin (2.6 μm, 150 Å; Thermo Fisher Scientific). Peptides were separated using a 30-min gradient of 7–30% acetonitrile in 0.125% FA with a flow rate of ~300 $nL \cdot min^{-1}$. The scan sequence began with an Orbitrap full MS^1 spectrum with the following parameters: resolution of 70,000, scan range of 200–1,000 Thomson (Th), AGC target of 1×10^6, maximum injection time of 250 ms, and profile spectrum data type. This scan was followed by 12 targeted MS^2 scans selected from a scheduled inclusion list with a 5-min retention time window. Each targeted MS^2 scan consisted of high-energy collision dissociation (HCD) with the following parameters: resolution of 35,000, AGC of 1×10^6, maximum injection time of 200 ms, isolation window of 1 Th, normalized collision energy (NCE) of 27, and profile spectrum data type. Raw files were searched, and precursor and fragment ions were quantified using Skyline version 3.1 (4). The UB-AQUA peptides used for quantitation were previously listed by Ordureau et al. (2) (Fig. S1*G*). Data generated from Skyline were exported into an Excel spreadsheet and Prism for further analysis as previously described (2).

Immunoprecipitation of PARKIN and p-S65-PARKIN Quantification. Cells at 80% confluence expressing catalytically inactive GFP-$PARKIN^{C431S}$ mutant were treated with DOX (2 μg/mL renewed every 48 h) for 5 d and then treated with antimycin A (10 μM) and OA (5 μM) for 1 h. Cells from 2 × 15-cm dishes were washed twice with ice-cold PBS and lysed in 3 mL of lysis buffer supplemented with protease and phosphatase inhibitors. Whole-cell extracts were sonicated, clarified by centrifugation (16,000 × *g* for 10 min at 4 °C), and filtered through 0.45-μm filters (Sartorius), and protein concentrations were determined by the Bradford assay. GFP-PARKIN from whole-cell extracts (10 mg) was affinity-purified with 10 μL of GFP-trap_A resin (Chromoteck) for 1 h at 4 °C. Beads were washed three times with lysis buffer containing 500 mM NaCl and four times with 10 mM Tris (pH 8.0). Elution with 6 M guanidine HCL was performed twice (50 μL for 10 min), and eluates were combined, reduced (10 mM TCEP), and alkylated (20 mM chloroacetamide) and then precipitated with 20% TCA. TCA-precipitated proteins were then subject to the AQUA proteomic workflow.

PARKIN S65 Phosphorylation Measurements by AQUA Proteomics. PARKIN phosphorylation was measured using AQUA proteomics as described (2). Heavy-labeled reference peptides for S65-PARKIN were obtained from JPT Peptides Technologies (SpikeTides_TQL Proteotypic Peptides). S65-PARKIN AQUA peptides (nonphospho and phospho heavy peptide) were reduced (10 mM TCEP) and alkylated (20 mM chloroacetamide), dried down, and resuspended in 5% FA. The desired amount of AQUA peptide was mixed with a previously TCA-precipitated sample before Lys-C and trypsin enzymatic digestion (in 100 mM TEAB, 0.1% Rapigest, 10% ACN). Samples were then handled as previously described in the section on UB-AQUA samples (*UB-AQUA/PRM Proteomics*). MS^1 signal for both the cleaved and miscleaved forms of S65-peptide (NDWTVQNCDLDQQSIVHIVQR and NDWTVQNCDLDQQSIVHIVQRPWR, respectively) was measured and monitored using Pinpoint software.

Mitophagy Assays. Each cell line was treated with DOX (2 μM renewed after 48 h) for 5 d and then treated with both antimycin A (10 μM) and OA (5 μM) as indicated. At the indicated time (16–20 h), cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 1% BSA, and immunostained with α-TOMM20 antibody followed by Hoechst staining. All images were then collected with a Yokogawa CSU-X1 spinning disk confocal lens on a Nikon Ti-E inverted microscope equipped with a 100× Plan Apo N.A. 1.4 objective lens in the Nikon Imaging Center at Harvard Medical School. For quantification, ~100 cells were counted based on the classification of nonaggregated mitochondria, aggregated mitochondria, and cleared mitochondria in biological triplicate.

Analysis of GFP-PARKIN Translocation. U2OS UB-replacement cell lines expressing GFP-PARKIN or the indicated mutants were treated on DOX (2 μM, renewed after 48 h) for 5 d, and cells were treated with both antimycin A (10 μM) and OA (5 μM). At the indicated time (45 min or 1 h), cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 1% BSA, and immunostained with α-TOMM20 antibody

followed by Hoechst staining. All images were then collected as described in the mitophagy assay. For quantification, ~200 cells were counted to determine the percentage of cells containing mitochondria-localized PARKIN in biological triplicate.

Cell Proliferation Assay. Cell proliferation assays were performed using CCK-8 (Dojindo Molecular Technologies), which indirectly measures the levels of intracellular NADH and NADPH. Briefly, the same numbers of cells were plated in a 96-well plate in DMEM with 10% FBS in the presence and absence of DOX (2 μM) to induce depletion of endogenous UB and expression of the UB transgenes. At the indicated time, CCK-8 reagent was added to each well and incubated at 37 °C with 5% CO_2 for 2 h, and the OD_{450} was measured using a SpectraMax M5 instrument (Molecular Devices).

Protein Expression, Purification, and Phosphorylation. PARKIN purification and phosphorylation by PINK1 were performed as previously described (2). Halo-tag-4× UBA^{UBQLN1} resin was prepared as described by Emmerich et al. (5) (plasmid was a generous gift from P. Cohen, University of Dundee, Dundee, UK) with the following modifications: a 6His-tag was added N-terminal to the Halo-tag; upon expression in Rosetta (DE3) *E. coli* cells, it was purified by nickel-nitrilotriacetic acid (Ni-NTA) and stored at −80 °C. Covalent coupling of Halo-4× UBA^{UBQLN1} to the resin was done by mixing 5 mg of purified Halo-4× UBA^{UBQLN1} to 1 mL of HaloLink resin (Promega).

DNA encoding for 5× UBA^{Dsk2} (6) was codon-optimized for *E. coli* expression (Invitrogen) and subcloned by Gibson cloning into a modified pFN18A vector (6His-HALO) for bacterial expression. 6His-Halo-tag-5× UBA^{Dsk2} resin was expressed, purified, and prepared in the same way as the Halo-tag-4× UBA^{UBQLN1} resin.

UB mutants (S65A, S65D, and S65E) were expressed and purified as previously described (2).

Human DNA encoding for OPTN, p62 (SQSTM1), and NDP52 (CALCOCO2) was subcloned into a pDEST-60 vector encoding for N-terminal GST-tag. GST-OPTN, GST-p62, and GST-NDP52 were produced in Rosetta (DE3) *E. coli* cells and purified by chromatography on glutathione-Sepharose and eluted with 20 mM reduced glutathione in 20 mM Tris·HCl, 150 mM NaCl, and 2 mM DTT (pH 7.5).

Human GST-$MIRO1^{180–581}$ was grown at 37 °C to an OD_{600} of 0.8, induced with 0.6 μM isopropyl-β-D-thiogalactopyranoside (IPTG) for 17 h at 18 °C. Pellets were lysed in 50 mM Tris (pH 8.0), 200 mM NaCl, and 5 mM DTT and then affinity-purified on a glutathione-Sepharose column and eluted with 50 mM Tris (pH 8.0), 200 mM NaCl, 5 mM DTT, and 10 mM reduced glutathione. Pooled fractions were further purified by ion exchange and size exclusion chromatography into a final buffer of 25 mM Tris (pH 7.6), 200 mM NaCl, and 1 mM DTT.

All purified proteins used in experiments were run on SDS/PAGE, followed by staining with Colloidal Coomassie Blue for quality control and phosphorylation stoichiometry measured by MS.

Preparation of Phosphorylated UB. Human UB was phosphorylated in vitro with *Tribolium castaneum* PINK1 and purified away from PINK1 as previously described (2). In some experiments, pre-phosphorylated UB was prepared using a suppressor tRNA system in bacteria, allowing for direct incorporation of phosphoserine at a desired residue (7, 8). Briefly, human UB DNA encoding for UB fused to a 6× His C-terminal tag was mutated by PCR to replace the Ser codon of interest (S20, S57, or S65) with an *Amber* stop codon (TAG), which is used for phosphoserine incorporation. UB-6His constructs of interest were then subcloned in the pCRT7-NT-Topo-tetR/pLtetO Amp vector (52053; Addgene) by Gibson assembly. EcAR7 strains (52055; Addgene) were cotransformed with the pCRT7 plasmid of interest and pKD-SepRS-EFSep-5× tRNASep (B40 OTS) plasmid (52054; Addgene). After transformation, 0.5 mL of super optimal broth (SOC) medium was added to the bacteria, which were left to recover for 2 h at 30 °C. After recovery, transformed EcAR7 strains were plated on an LB agar plate containing both carbenicillin (200 μg/mL) and kanamycin (50 μg/mL), and left to grow at 30 °C for the next 2–3 d. Colonies of transformed EcAR7 + B40 OTS + pCRT7 pLtetO/tetR were picked and grown at 30 °C in 5 mL (for a few hours) and then in 50 mL of LB supplemented with 0.08% glucose, carbenicillin (200 μg/mL), and kanamycin (50 μg/mL). On the next day, starter cultures were diluted into 2 L of media (LB supplemented with carbenicillin, kanamycin, 2 mM phosphoserine, 0.08% glucose) to an OD_{600} of ~0.1 and grown at 30 °C with shaking until the OD_{600} reached ~0.8. Protein expression was started by the addition to final concentration of 1 mM IPTG and 100 ng/mL anhydrotetracycline, and the temperature was lowered to 25 °C for the duration of the protein expression procedure (~24 h). The cells were collected and lysed in 50 mM Tris (pH 7.5), 250 mM NaCl, 1% Triton X-100, 15 mM imidazole, 0.5 mM TCEP, and 1 mM AEBSF. UB-6His WT and mutants were purified by Ni-NTA as previously described (2). Purified UB-6His was loaded on a gel filtration Superdex 75 10/300 GL system (GE Healthcare).

Analytical Size Exclusion Chromatography. To generate UBCH7-UB^{iso}, 150 μM UBCH7 active site C86K mutant, 450 μM His-UB, and 15 μM Uba1 were incubated for 20 h at 37 °C in 30 mM Tris (pH 8.8), 50 mM NaCl, 5 mM ATP, and 10 mM $MgCl_2$. The isopeptide-linked UBCH7-UB^{iso} was purified by size exclusion chromatography in 25 mM Tris (pH 7.6), 150 mM NaCl, and 1 mM DTT.

For analytical size exclusion chromatography, all proteins were diluted to 10 μM (20 μM for p-S65-UB) in sizing buffer for at least 30 min on ice before loading onto the column. Fractions were run on SDS/PAGE gels and visualized with Ruby Sypro dye.

In Vitro Ubiquitylation Analysis and UB-AQUA Proteomics. All in vitro ubiquitylation reactions were performed as described previously (2). Briefly, E1 (0.1 μM UBE1), E2 conjugating enzyme (1 μM UBE2L3), E3 ligase (1 μM PARKIN, unless stated otherwise), and total UB (30 μM, unless stated otherwise) were incubated for the indicated time at 30 °C in a 25-μL reaction mixture containing 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, and 0.2 mM TCEP. The reaction was started by the addition of 2 mM ATP and terminated by the addition of SDS sample buffer containing 10 mM EDTA when analyzed on lithium dodecyl sulfate (LDS)/PAGE or by the addition of 10 mM EDTA and TCA (20% final concentration) precipitated when the reaction was further analyzed by MS (*UB-AQUA/PRM Proteomics*). In some experiments, GST-$MIRO^{180–581}$ was used as a substrate and detected by Western blot analysis using α-GST antibodies.

di-Gly Proteomics. A total of 1.75 mg of purified mitochondria per condition was incubated with 40 μL of HALO-4× UBA^{UBQLN1} beads for 16 h to capture all chain linkages present in the sample (as well as some monoubiquitin upon long incubation). After incubation beads were spun down at low speed, supernatant was kept and set apart while beads were washed three times with 5 mL of lysis buffer followed by two washes with lysis buffer supplemented with 500 mM NaCl and, finally, five washes with 10 mM Tris (pH 8.0). Supernatant (after HALO-4× UBA^{UBQLN1} pull-down) was then incubated for 4 h with 15 μL of HALO-5× UBA^{DSK2} beads to capture residual monoubiquitylated protein left. HALO-5× UBA^{DSK2} beads were then washed as described before and combined with the HALO-4× UBA^{UBQLN1} beads, bound proteins were eluted from beads twice by the addition of 150 μL of 6 M guanidine HCL, and eluate was then spun down through a spin-X centrifuge tube filter (0.45 μm). Samples were subjected to reduction (10 mM TCEP) and alkylation (20 mM

chloroacetamide) followed by TCA precipitation (20% final). Samples were digested at 37 °C first for 4 h with Lys-C alone (in 100 mM TEAB, 0.1% Rapigest, 10% ACN), and trypsin was then added to samples and digested for a further 16 h at 37 °C. Digests were acidified with an equal volume of 5% FA to a pH of ~2 for 30 min, dried down, resuspended in 5% FA, subjected to C18 StageTip desalting, and dried down again.

For enrichment of di-Gly peptides, anti–K-ε-GG antibody (Cell Signaling Technology) was used. Before enrichment, the antibody was cross-linked to protein A beads using dimethyl pimelimidate (DMP). Briefly, the beads were washed three times with 1 mL of PBS, and antibody was added to the beads at a 1:1 ratio [micrograms to microliters (pack beads)] and incubated for 30 min in 1 mL of PBS. Beads were then washed four times with 10 vol of 0.1 M sodium borate (pH 9) and then resuspended in 10 vol of 0.1 M sodium borate (pH 9) containing freshly added DMP to a concentration of 20 mM and incubated for 30 min at room temperature with gentle mixing. The beads were pelleted, and this incubation with DMP was repeated. The beads were then washed three times with 10 vol of 50 mM glycine (pH 2.5) to remove all of the antibodies that were not covalently coupled to the beads. The covalently coupled antibody beads were washed once with 0.2 M Tris·HCl (pH 8) and then incubated in this buffer for a further 1 h at room temperature with gentle mixing to ensure that any residual DMP was broken down following reaction with the amine group of Tris. After blocking, the antibody-coupled beads were stored in PBS at 4 °C for future use.

Dried peptide was resuspended in 1.25 mL of di-Gly buffer [100 mM Hepes (pH 7.5), 10 mM Na_2HPO_4, 50 mM NaCl] and incubated with 7.5 μL (~7.5 μg of antibody) of cross-linked anti–K-ε-GG antibody beads for 6 h on a rotating unit at 4 °C. Antibody beads were washed twice with 1.5 mL of ice-cold di-GLY buffer containing 200 mM NaCl and 0.25% Nonidet P-40, twice with di-GLY buffer containing 150 mM NaCl, and three times with PBS. The final wash was in 1 mL of ddH_2O. di-Gly peptides were eluted twice by adding 20 μL of 0.15% TFA. Peptide supernatants were dried down and desalted using C18 StageTips, and eluted peptides were dried down and resuspended in 5% FA before MS analysis.

MS data were collected using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) coupled to a Proxeon EASY-nLC II LC pump (Thermo Fisher Scientific). Peptides were fractionated on a 100-μm i.d. microcapillary column packed with ~0.5 cm of Magic C4 resin (5 μm, 100 Å; Michrom Bioresources) followed by ~35 cm of GP-18 resin (1.8 μm, 200 Å; Sepax). Peptides were separated using a 2-h gradient of 4–27% acetonitrile in 0.125% FA at a flow rate of ~375 nL·min^{-1}. The scan sequence began with an MS^1 spectrum (Orbitrap analysis; resolution of 120,000, mass range of 400–1,400 m/z, AGC target of 2×10^5, maximum injection time of 100 ms). Precursors for MS^2 analysis were selected using a TopSpeed method of 3-s MS^2 analysis consisting of high energy collision-induced dissociation (quadrupole ion trap analysis; AGC of 2×10^4, NCE of 35, maximum injection time of 100 ms).

SEQUEST-based identification using a human UniProt database followed by a target decoy-based linear discriminant analysis was used for peptide and protein identification as described (9–11). Parameters used for database searching include the following: 20-ppm precursor mass tolerance, 0.03-Da product ion mass tolerance, and tryptic digestion with up to three missed cleavages. Carboxyamidomethylation of Cys was set as a fixed modification, whereas oxidation of Met and di-Gly modification of Lys were set as variable modifications. Localization of di-Gly sites used a modified version of the A-score algorithm (12) as described (9). A-scores of 13 were considered localized. Quantification of di-Gly sites based on spectral counts was done as described (9).

Single-Turnover Assays. For pulse–chase assays, 10 μM UBCH7 was mixed with 0.2 μM UBA1 and 15 μM UB^F, where all Lys in UB has been replaced with Arg and has been labeled with fluorescein (UB^F) in reaction buffer containing 50 mM Hepes (pH 7.0), 2.5 mM $MgCl_2$, 1.5 mM ATP, and 100 mM NaCl. The loading reaction proceeded for 15 min at room temperature before being quenched in 50 mM Tris (pH 7.6), 50 mM NaCl, and 50 mM EDTA for 5 min on ice. p-WT or p-C431S PARKIN at 0.4 μM was mixed with 0.2–0.4 μM p-UB in a final reaction volume of 50 μL in 25 mM Tris (pH 7.6), 50 mM NaCl, and 50 mM EDTA. The chase reactions were initiated by addition of UBCH7~UB^F and quenched at the indicated time points in SDS sample loading buffer. The di-UB synthesis assays contained 300 μM unlabeled UB^{WT} in the final reaction. GST-hMIRO1$^{180–581}$ ubiquitylation reactions contained MIRO at a final concentration of 0.6 μM in the chase reaction.

Capture of UB Chains by Poly-UB Binding Proteins. GST-tagged proteins (3 μg) were immobilized on glutathione-Sepharose [10 μL of packed beads equilibrated in 25 mM Hepes (pH 7.5), 1 mM EGTA, 2 mM $MgCl_2$, 0.5% (vol/vol) Triton X-100] plus 150 mM NaCl and incubated for 20 min at 4 °C with 0.5 mL of the same buffer. The beads were washed three times and then incubated with gentle agitation for 1 h at 20 °C with 0.5 mL of poly-UB chains (2 μg) in the same buffer. The beads were washed five times with buffer plus 150 mM NaCl and once with buffer without NaCl and Triton X-100. Bound proteins were released by denaturation with 2% LDS; the beads were removed by centrifugation for 1 min at 2,000 × g; the supernatants were heated for 5 min at 70 °C, subjected to SDS/PAGE, and transferred to PVDF membranes; and proteins were detected by immunoblotting.

1. Xu M, Skaug B, Zeng W, Chen ZJ (2009) A ubiquitin replacement strategy in human cells reveals distinct mechanisms of IKK activation by TNFalpha and IL-1beta. *Mol Cell* 36(2):302–314.
2. Ordureau A, et al. (2014) Quantitative proteomics reveal a feedforward mechanism for mitochondrial PARKIN translocation and ubiquitin chain synthesis. *Mol Cell* 56(3): 360–375.
3. Phu L, et al. (2011) Improved quantitative mass spectrometry methods for characterizing complex ubiquitin signals. *Mol Cell Proteomics* 10(5):M110.003756.
4. MacLean B, et al. (2010) Skyline: An open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* 26(7):966–968.
5. Emmerich CH, et al. (2013) Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains. *Proc Natl Acad Sci USA* 110(38):15247–15252.
6. Wilson MD, Saponaro M, Leidl MA, Svejstrup JQ (2012) MultiDsk: A ubiquitin-specific affinity resin. *PLoS ONE* 7(10):e46398.
7. Park HS, et al. (2011) Expanding the genetic code of Escherichia coli with phosphoserine. *Science* 333(6046):1151–1154.
8. Aerni HR, Shifman MA, Rogulina S, O'Donoghue P, Rinehart J (2015) Revealing the amino acid composition of proteins within an expanded genetic code. *Nucleic Acids Res* 43(2):e8.
9. Kim W, et al. (2011) Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell* 44(2):325–340.
10. Huttlin EL, et al. (2010) A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell* 143(7):1174–1189.
11. Eng JK, McCormack AL, Yates JR (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* 5(11):976–989.
12. Beausoleil SA, Villén J, Gerber SA, Rush J, Gygi SP (2006) A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat Biotechnol* 24(10):1285–1292.
13. Zheng N, Wang P, Jeffrey PD, Pavletich NP (2000) Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* 102(4):533–539.

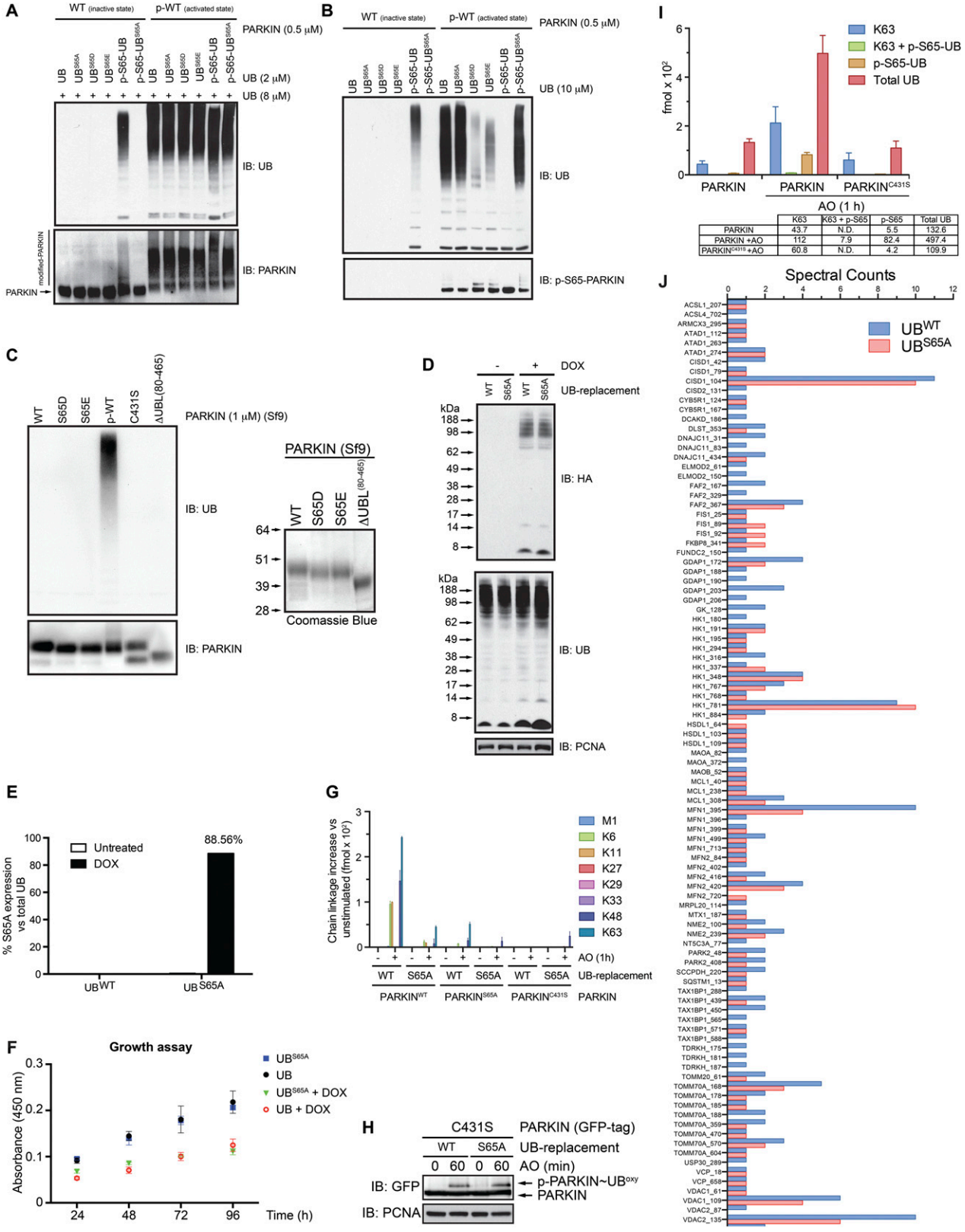

Fig. S1. UB-replacement system for studying the role of p-S65 UB in PINK1/PARKIN function. (*A*) Indicated PARKIN and p-WT PARKIN proteins (1 μM) were incubated with ATP, E1, UBCH7, UB^{WT}, and the indicated UB mimetic or phospho-UB. After 30 min, proteins were subjected to SDS/PAGE and blotting with α-UB or α-p-S65 PARKIN antibodies. IB, immunoblotting. (*B*) Unphosphosphorylated (inactive state) PARKIN or homogeneous p-S65 PARKIN was incubated with the indicated UB protein in the presence of UBCH7, ATP, and E1, and incubated for 30 min, prior to SDS/PAGE and immunoblotting. (*C*) Indicated PARKIN proteins were phosphorylated with PINK1, and PINK1 was removed before UB chain assembly assays in the presence of ATP, E1, UB, and UBCH7. (*Right*) Purified proteins used in this experiment. (*D*) Analysis of UB-replacement cells. The indicated cells were grown in the presence or absence of DOX for 5 d, and the levels

Legend continued on following page

of HA-UB and UB were measured by immunoblotting. PCNA was used as a loading control. (*E*) Analysis of UB-replacement cells using UB-AQUA. (*F*) Analysis of cell proliferation for UB-replacement cells. Error bars represent triplicate measurements (±SEM). (*G*) As in Fig.1*A*, but depicting individual chain linkage types observed by UB-AQUA. Error bars represent triplicate measurements (±SEM). (*H*) GFP-PARKIN activation occurs in vivo in UB^{S65A} cells in response to mitochondrial depolarization, as measured by formation of an oxy-ester between UB and GFP-$PARKIN^{C431S}$. The indicated UB-replacement cells stably expressing GFP-$PARKIN^{C431S}$ were depolarized for 1 h, and whole-cell extracts were probed with α-GFP to detect GFP-$PARKIN^{C431S}$ and its lower mobility oxy-ester (oxy) form. (*I*) Total abundance of UB (K63 locus), K63 linkage, and p-S65-UB in total mitochondria purified from the indicated U2OS cells 1 h after depolarization was determined using a UB-AQUA/PRM method. The number of femtomoles of the indicated species are shown. Error bars represent triplicate measurements (±SEM). (*J*) Mitochondria were isolated from the indicated cells, ubiquitylated proteins were isolated with HALO-UBA^{UBQLN1} and HALO-UBA^{DSK2}, proteins were digested with trypsin, and di-GLY–containing peptides were isolated using α–di-GLY antibodies before analysis by LC-tandem MS. The numbers of spectral counts identified for all MOM proteins identified in this analysis are shown (Dataset S1).

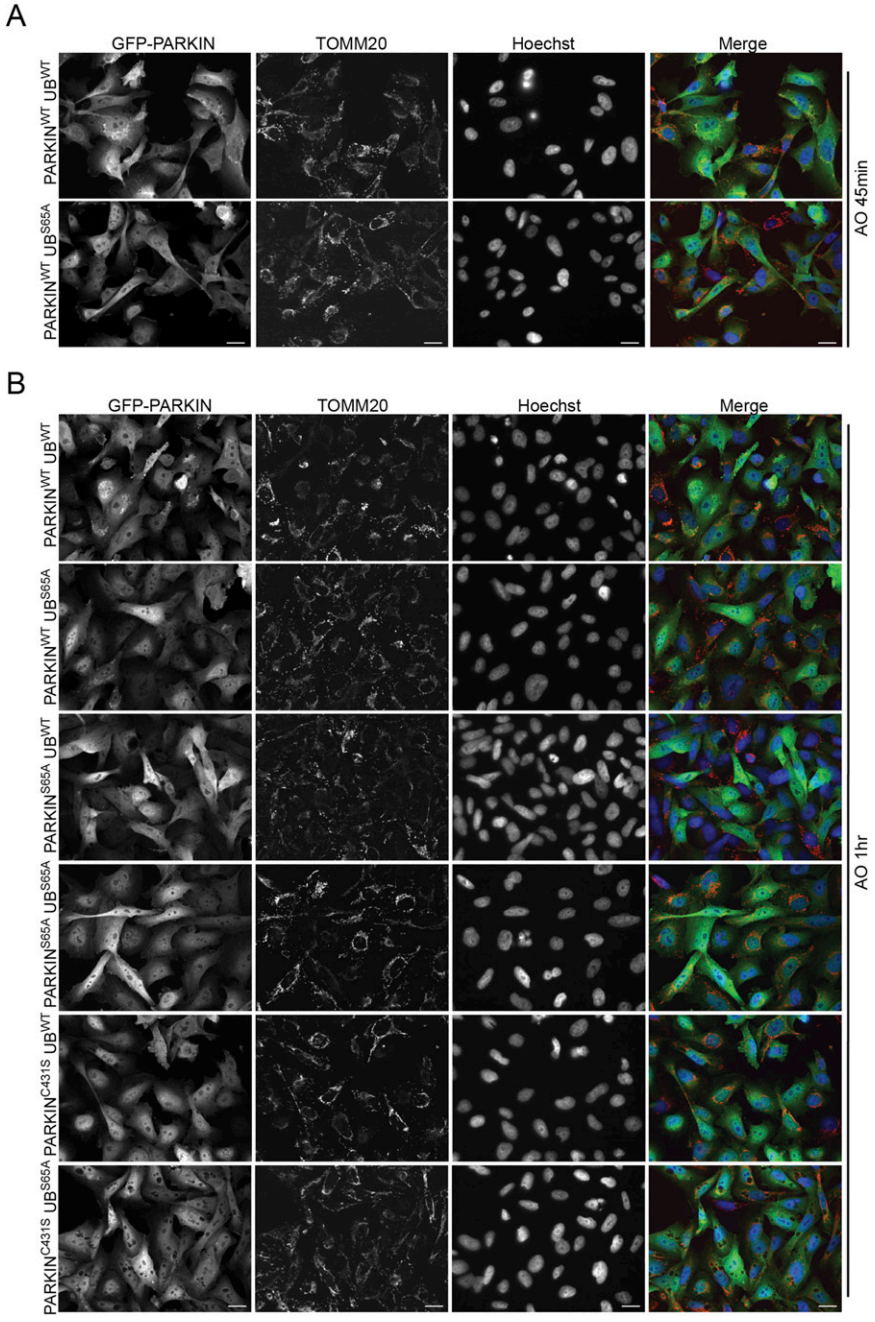

Fig. S2. PARKIN recruitment to mitochondria in UB^{S65A}-replacement cells. The indicated UB-replacement cells expressing GFP-$PARKIN^{WT}$, GFP-$PARKIN^{S65A}$, and GFP-$PARKIN^{C431S}$ were depolarized with AO for the indicated times, stained for mitochondria with α-TOMM20 antibodies, and imaged for GFP-PARKIN/mitochondria colocalization. Representative images were taken by confocal microscopy. (Scale bars, 20 μm.)

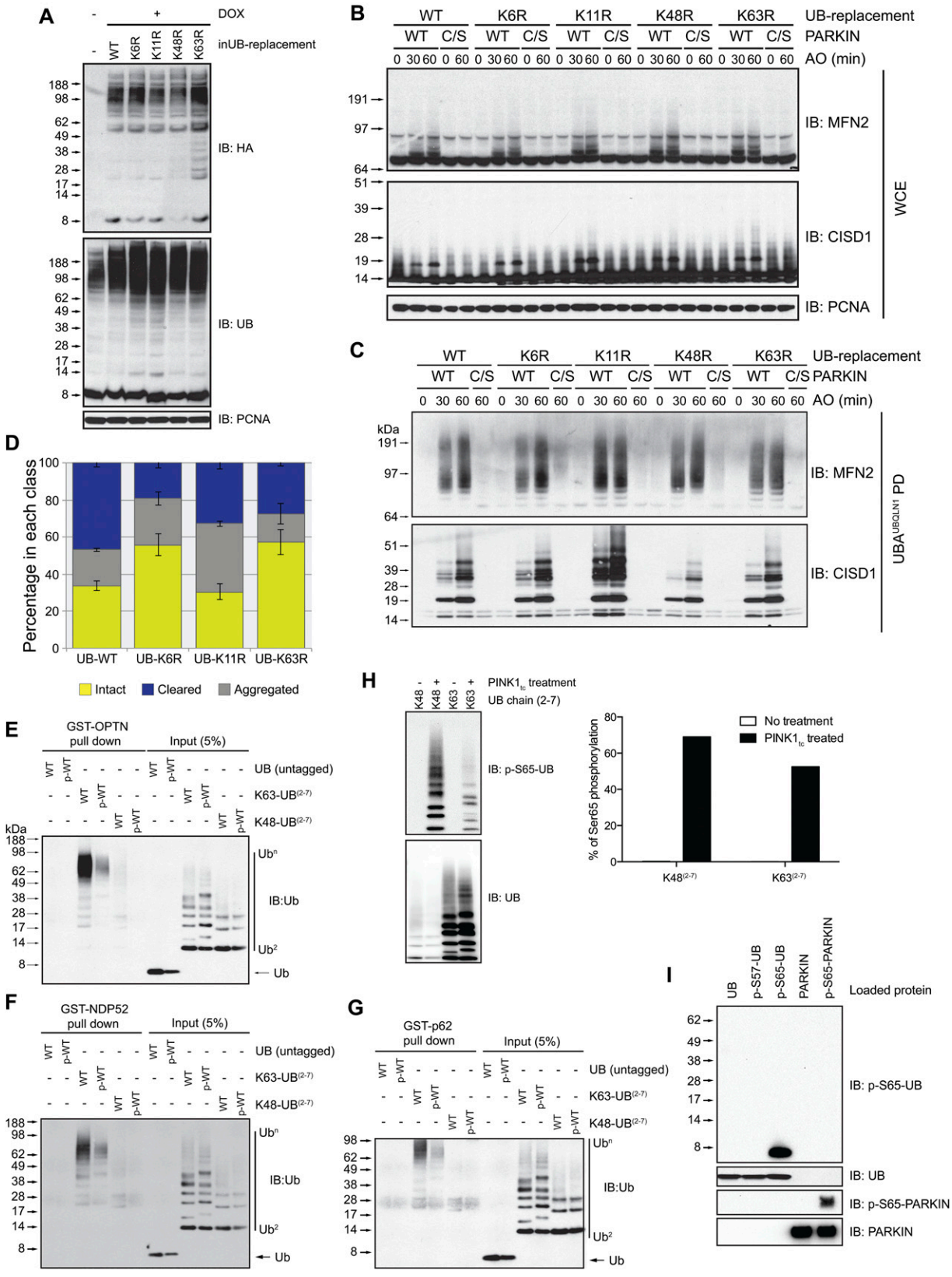

Fig. S3. Analysis of UB chain linkages in mitophagy and autophagy receptor binding. (*A*) Characterization of UB^{WT}-, UB^{K6R}-, UB^{K11R}-, UB^{K48R}-, and UB^{K63R}-replacement cells. Cells were grown with or without DOX for 5 d, and extracts were subjected to immunoblotting with the indicated antibodies. PCNA was used as a loading control. (*B*) UB chain assembly on MFN2 and CISD1 in vivo in UB-replacement cells in response to depolarization was examined by immunoblotting of whole-cell extracts. Blots were probed with α-MFN2, α-CISD1, and α-PCNA as loading controls for whole-cell extracts. (*C*) UB-replacement cells expressing individual K6R, K11R, K48R, and K63R UB mutants maintain polyubiquitylation of MFN2 and CISD1 in vivo. Mitochondria from UB^{WT}-, UB^{K6R}-, UB^{K11R}-,

Legend continued on following page

UB^{K48R}-, and UB^{K63R}-replacement cells grown with or without DOX for 5 d were subjected to UB capture using HALO-UBA^{UBQLN1}, and bound proteins were analyzed by immunoblotting using α-MFN2 and α-CISD1. This experiment was performed in parallel with the experiment shown in *B*. (*D*) UB^{K6R}- and UB^{K63R}-replacement cells display defects in depolarization-dependent mitophagy. Quantification of α-TOMM20 staining in UB^{WT}-, UB^{K6R}-, UB^{K11R}-, and UB^{K63R}-replacement cells expressing $PARKIN^{WT}$ 20 h after depolarization ($n > 100$). Error bars represent triplicate measurements (±SEM). GST-OPTN (*E*), GST-NDP52 (*F*), or GST-p62 (*G*) from bacteria was incubated with the indicated forms of UB in binding buffer and washed extensively, and the associated proteins were subjected to immunoblotting with α-UB antibodies. (*H*) Quantification of the extent of phosphorylation of K48 and K63 chains (*Left*) and immunoblot validation (*Right*) used in *E*–*G*. Note that the detection of K63 chains modified by phosphorylation of S65 is much weaker than detection of phosphorylated K48 chains. (*I*) Possible cross-reactivity of p-S65-UB antibody with p-S65-PARKIN or p-S57-UB was examined by immunoblotting of phosphorylated recombinant proteins (100 ng per lane; ∼12 pmol of UB and 2 pmol of PARKIN).

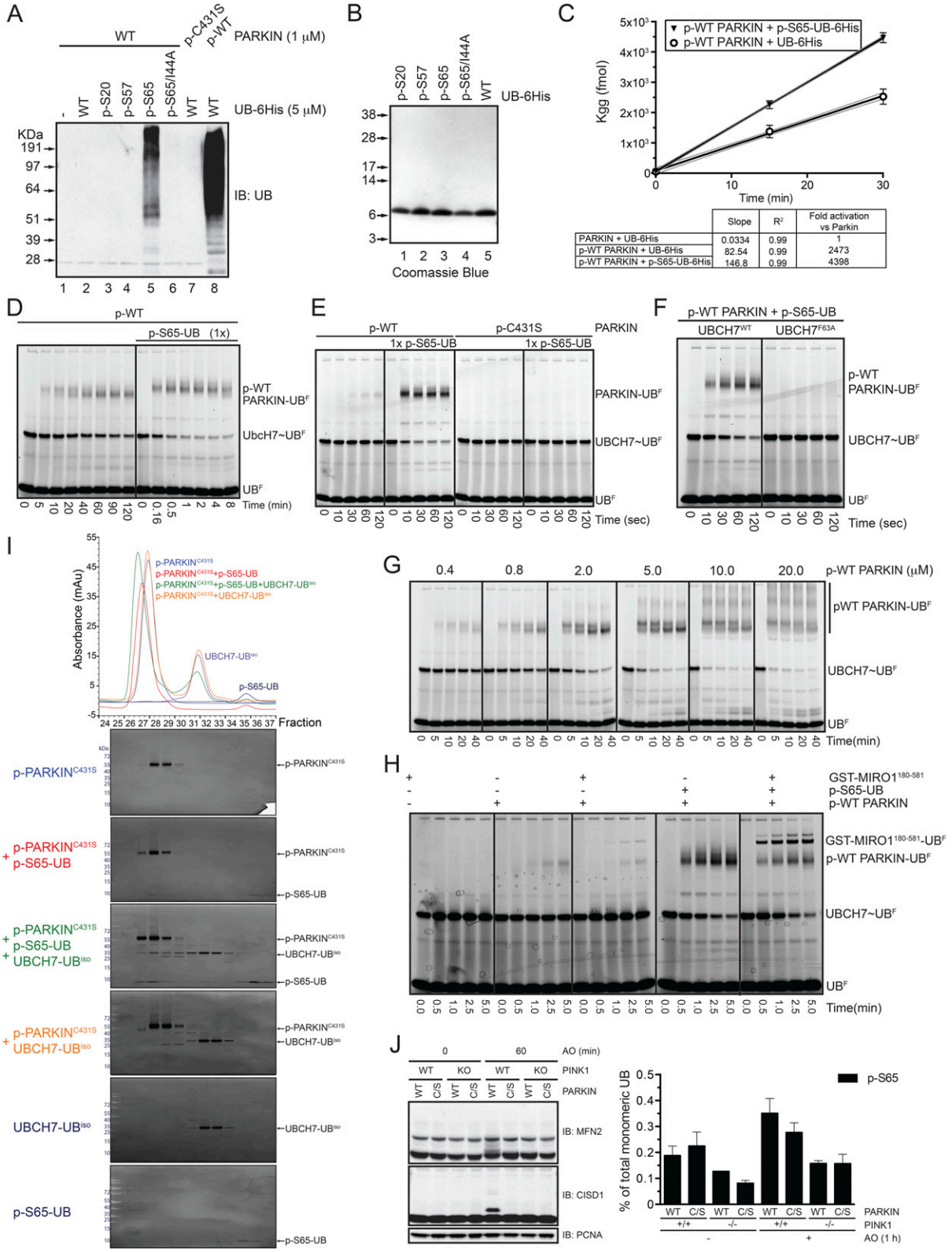

Fig. S4. Activation of UB transfer from PARKIN to substrates by p-S65-UB binding. WT, p-S20, p-S57, p-S65-UB, and p-S65-UB^{I44A} were produced using a genetically encoded *E. coli* system for production of phosphoserine-containing proteins (*B*) and used at 5 μM in UB chain assembly reactions with unphosphorylated PARKIN (1 μM) in the presence of ATP, E1, UBCH7, and UB (20 μM) (*A*). p-WT and p-C431S PARKIN were used as positive and negative controls, respectively. Reactions were analyzed by immunoblotting with α-UB antibodies. (*C*) Quantification of stimulation of p-WT PARKIN catalytic activity by p-S65-UB-6His using UB-AQUA. Error bars represent triplicate measurements (±SEM). (*D*) p-S65-UB greatly stimulates transfer of Lys-free UB from UBCH7 to PARKIN in single-turnover assays. UBCH7 was charged with E1, UB^F, and ATP, and purified by gel filtration. UBCH7~UB^F was mixed with p-WT PARKIN in the presence or absence of p-S65-UB, and reactions were quenched at the indicated times postmixing. (*Left*) Reaction mixtures were separated by SDS/PAGE, and UB^F was

Legend continued on following page

detected. Loss of UBCH7~UB^F was plotted. Related to Fig. 4*B*. (*E*) For single-turnover reactions to examine PARKIN auto-ubiquitylation, UBCH7 was charged with E1, UB^F, and ATP, and purified by gel filtration. UBCH7~UB^F was mixed with the indicated PARKIN protein in the presence or absence of p-S65-UB, and reactions were quenched at the indicated times postmixing. Reaction mixtures were separated by SDS/PAGE, and UB^F was detected. (*F*) For single-turnover reactions to examine PARKIN auto-ubiquitylation, UBCH7 or $UBCH7^{F63A}$, which has a mutation in the canonical RING-binding residue (13), was charged with E1, UB^F, and ATP, and purified by gel filtration. UBCH7~UB^F or the indicated mutant was mixed with p-WT PARKIN protein in the presence of p-S65-UB, and reactions were quenched at the indicated times postmixing. Reaction mixtures were separated by SDS/PAGE, and UB^F was detected. (*G*) Enhancement of UBCH7~UB^F discharge by increasing p-WT PARKIN. The indicated concentrations of p-WT PARKIN were incubated with UBCH7~UB^F for the indicated periods of time. Reaction mixtures were separated by SDS/PAGE, and UB^F was detected. (*H*) p-S65-UB promotes transfer of Lys-free UB from UBCH7 to GST-MIRO in a single-turnover assay. As in *D*, with or without inclusion of GST-$MIRO^{180–581}$. (*I*) Binding of p-S65-UB to phosphorylated $PARKIN^{C431S}$ promotes association with UBCH7-UB^{iso} (isopeptide-linked). The indicated proteins were mixed and subjected to gel filtration. The levels of free UBCH7-UB^{iso} are reduced in the presence of p-S65-UB. mAu, milli-absorbance units. (*J*) Analysis of the abundance of p-S65-UB in the steady-state pool of free (monomeric) UB within $PINK1^{+/+}$ or $PINK1^{-/-}$ HeLa cells expressing WT or C431S (C/S) PARKIN 1 h after depolarization. Activation of the PINK1/PARKIN pathway was measured by immunoblotting for MFN2 and CISD1. (*Left*) PCNA was used as a loading control. (*Right*) Monomeric pool of UB was isolated as described in *SI Materials and Methods*, and the abundance of total UB and p-S65-UB was measured using UB-AQUA/PRM (*Materials and Methods*). Error bars represent triplicate measurements (±SEM).

Dataset S1. List of primary di-GLY sites found on mitochondrial proteins in U2OS replacement cells

Dataset S1