SUPPLEMENTAL MATERIAL

Supplemental Figure Legends

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Figure S2. Recruitment of the mitophagy adaptor SQSTM1, but not TAX1BP1, to depolarized mitochondria requires TBK1.

Figure S3. Phosphorylation of OPTN, NDP52, and SQSTM1 in response to mitochondrial depolarization is abrogated upon depletion of TBK1 by siRNAs.

Figure S4. Analysis of OPTN phosphorylation and binding to linear (M1) poly-UB chains.

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Figure S6. Analysis of mitophagy in cells lacking NDP52 and OPTN, or TBK1.

Supplemental Table

Table S1. Peptides used for Parallel Reaction Monitoring.

Supplemental Experimental Procedures

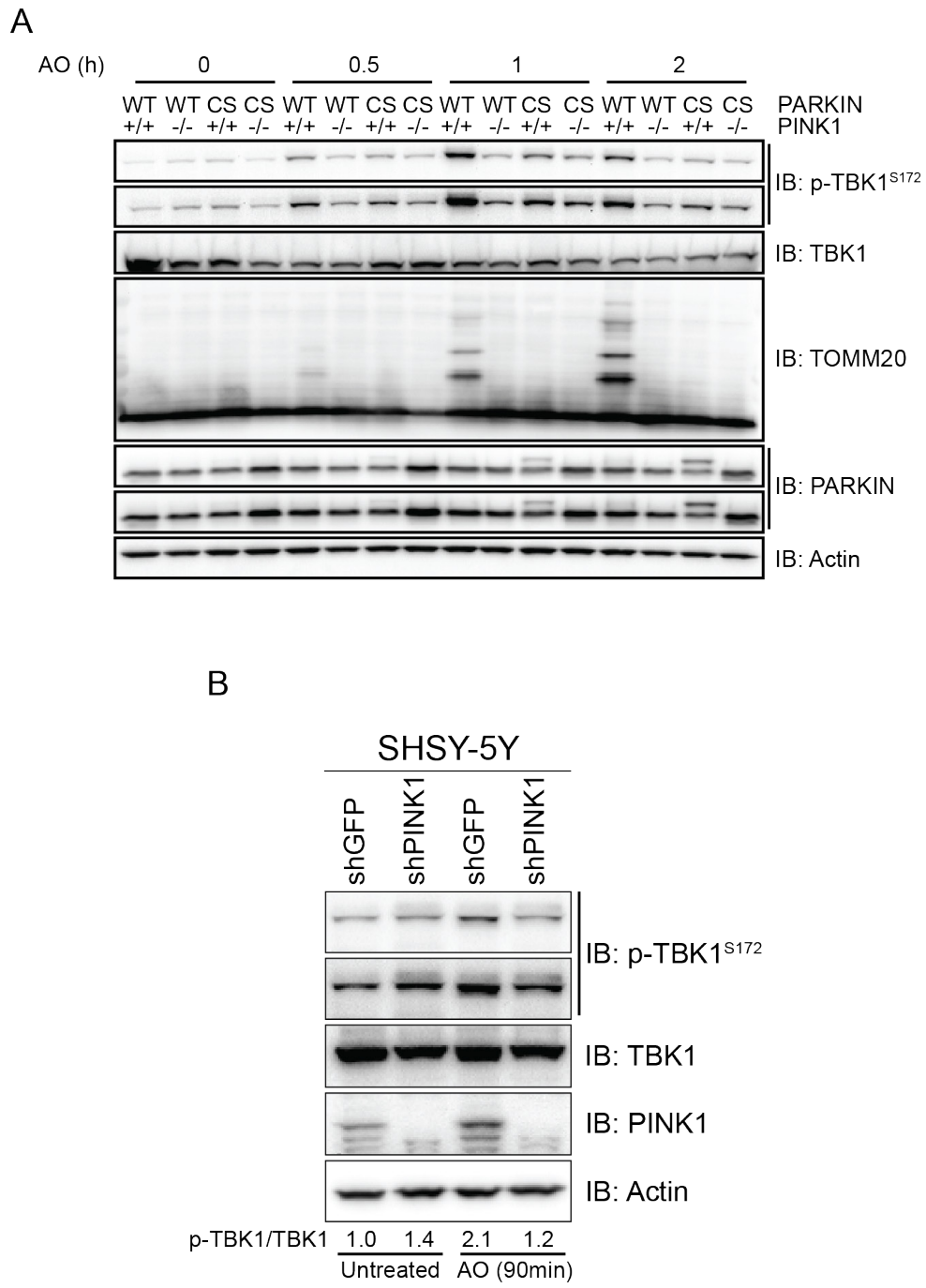

Figure S1. Time dependence of TBK1 phosphorylation in response to mitochondrial depolarization. Related to Figure 1.

(A) The indicated HFT cell lines were treated with AO for the indicated times and extracts subjected to immunoblotting with the indicated antibodies.

(B) SH-SY5Y cells expressing either shGFP or shPINK1 were left untreated or depolarizaed with AO for 90 min and extracts subjected to immunoblotting with the indicated antibodies. The ratio of p-TBK1 to total TBK1 signal was determined as described under SUPPLEMENTAL METHODS.

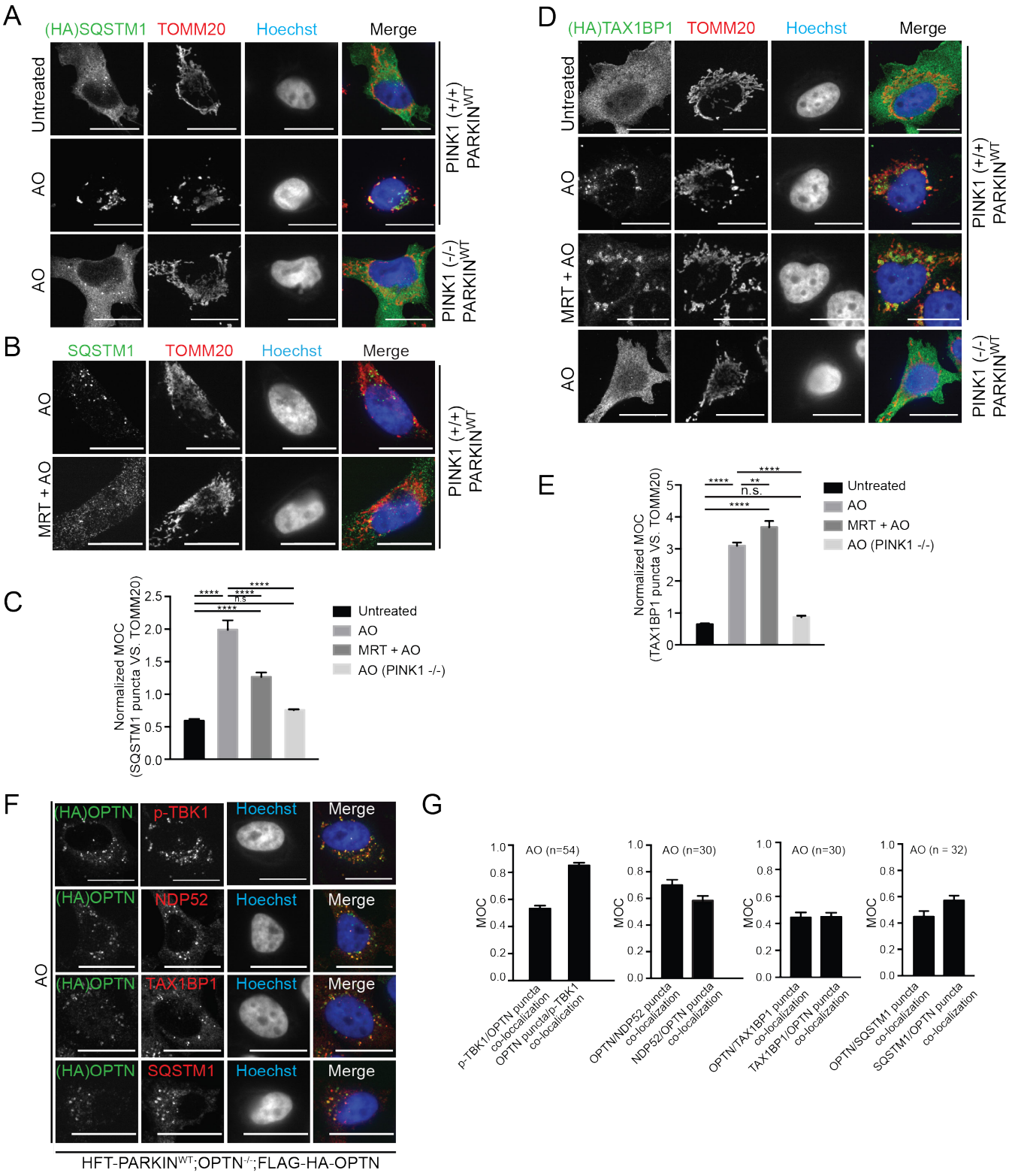

Figure S2. Efficient recruitment of the mitophagy adaptor SQSTM1, but not TAX1BP1, to depolarized mitochondria requires TBK1. Related to Figure 2.

(A-C) Efficient SQSTM1 localization to depolarized mitochondria requires TBK1 and PINK1. In Panel A, HFT-PARKIN^{WT} or HFT-PARKIN^{WT};PINK1$^{-/-}$ cells stably expressing FLAG-HA-SQSTM1 were either left untreated or treated with AO for 1hr to examine AO-dependent co-localization of SQSTM1 with mitochondria. Cells were imaged by confocal microscopy after staining with α-HA (SQSTM1) (green), α-TOMM20 (red), and Hoechst to detect DNA (blue) (scale bar, 20 microns). In Panel B, HFT-PARKIN^{WT} cells were depolarized for 1h with AO in the presence or absence of the TBK1 inhibitor MRT (2μM, pre-treatment time 1h). Cells were imaged by confocal microscopy after staining with α-SQSTM1 (green), α-TOMM20 (red), and Hoechst to detect DNA (blue) (scale bar, 20 microns). In Panel C, normalized MOC for panels A and B were analyzed by one-way ANOVA with Dunnett's multiple comparisons test (>50 cells). ****p<0.0001, n.s., not significant.

(D-E) TAX1BP1 localization to damaged mitochondria requires PINK1 but not TBK1 activity. The indicated HFT cells stably expressing HA-TAX1BP1 were left untreated or depolarized for 1h with AO in the presence or absence of either the TBK1 inhibitor MRT (2μM, pre-treatment time 1h) or PINK1. Cells were imaged by confocal microscopy after staining with α-HA (green), α-TOMM20 (red), and Hoechst to detect DNA (blue). Scale bar, 20 microns. In Panel E, normalized MOC for panel D was analyzed by one-way ANOVA with Dunnett's multiple comparisons test (> 40 cells). **p<0.01, ****p<0.0001. n.s., not significant.

(F,G) In Panel F, the co-localization of FLAG-HA-OPTN (α-HA, green) in HFT-PARKIN^{WT};OPTN$^{-/-}$;FLAG-HA-OPTN cells with p-TBK1^{S172}, NDP52, TAX1BP1, or SQSTM1 (red) was determined by immunofluorescence and confocal microscopy (scale bar, 20 microns) prior to image analysis to determine the MOC for co-localization with FLAG-HA-OPTN (panel G). Error bars represent SEM from triplicate experiments.

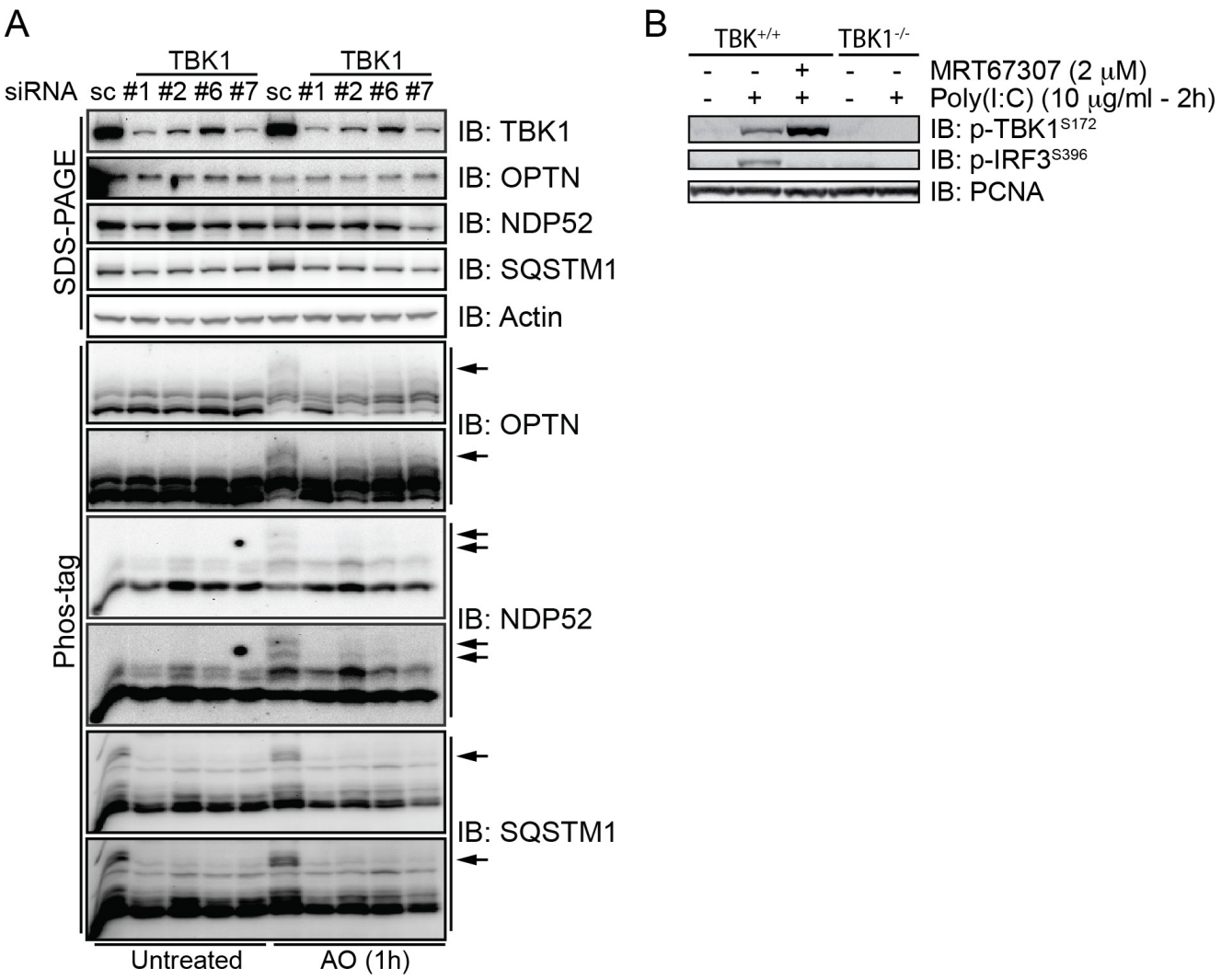

Figure S3. Phosphorylation of OPTN, NDP52, and SQSTM1 in response to mitochondrial depolarization is abrogated upon depletion of TBK1 by siRNAs. Related to Figure 4.

(A) HFT-PARKIN^{WT} cells were transfected with scrambled control and four independent siRNAs targeting TBK1. After 72h, cells were depolarized with AO (60 min) and extracts subjected to SDS-PAGE or electrophoresis on Phos-tag gels prior to immunoblotting with the indicated antibodies. The position of depolarization dependent phosphorylated forms of mitophagy adaptors are indicated by arrows. For each Phos-tag blot, short and long exposures are shown.

(B) HFT or HFT;TBK1$^{-/-}$ cells when indicated were pretreated with the TBK1 inhibitor MRT67307 for an hour, prior to transfection with Poly(I:C) to activated the RIG-I/MDA5/MAVS pathway. After 2 hours cells were harvested and extracts subjected to SDS-PAGE prior to immunoblotting with the indicated antibodies.

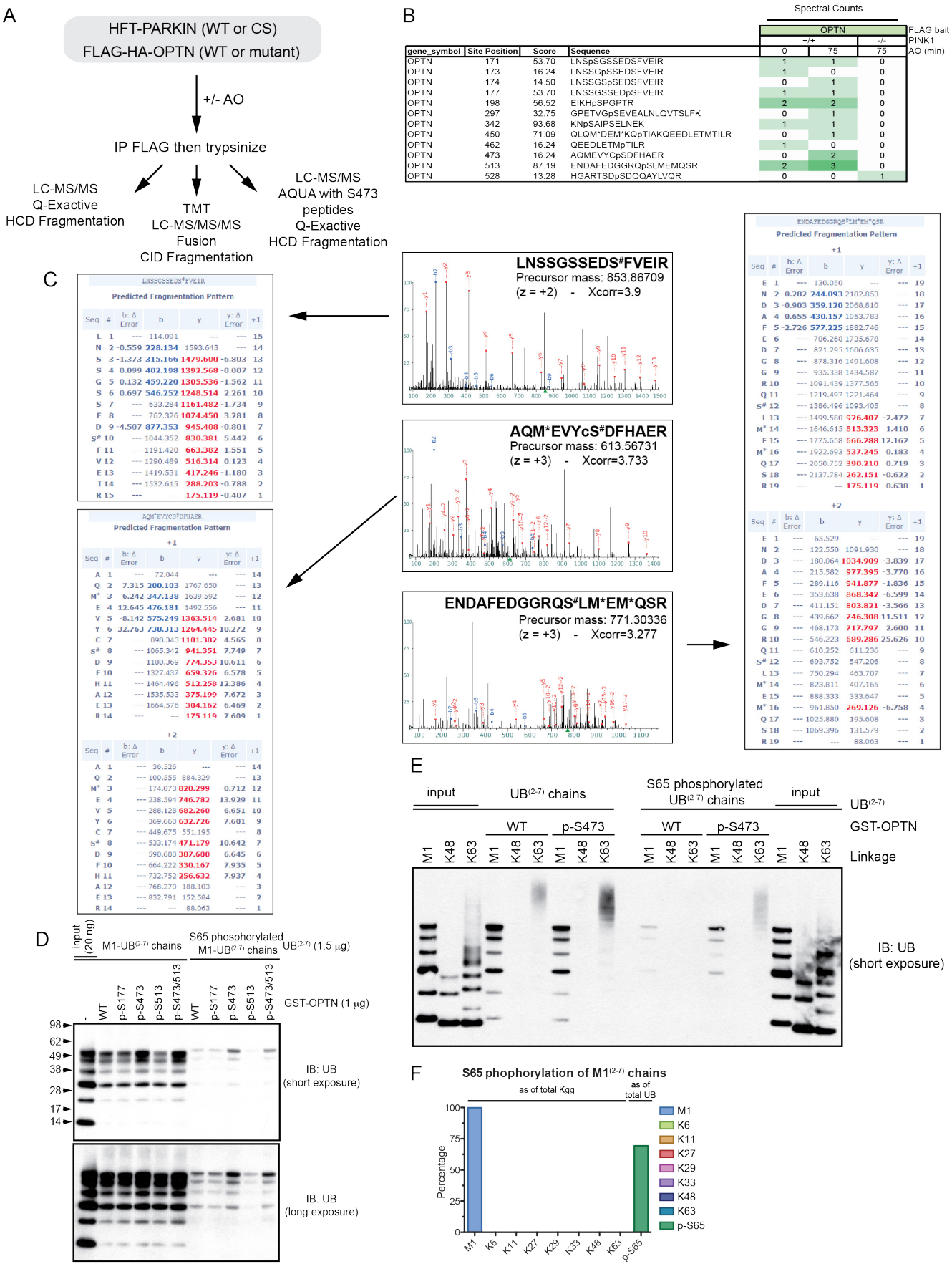

Figure S4. Analysis of OPTN phosphorylation and binding to linear (M1) poly-UB chains. Related to Figure 5.

(A) Scheme depicting the three-pronged approach for examining OPTN phosphorylation in response to mitochondrial depolarization using mass spectrometry.
(B) OPTN phosphorylation sites identified by mass spectrometry using a Q-Exactive instrument and spectral counting. The score indicated is the Modification (Mod)-score used to establish the site of phosphorylation. The number of spectral counts is shown with and without AO treatment or PINK1.
(C) In vitro phosphorylation of OPTN by TBK1. OPTN was incubated with TBK1 for 30 min prior to trypsinization and analyzed by mass spectrometry. This led to the identification of S177, S473, and S513 phosphopeptides, as indicated.
(D-E) The indicated unphosphorylated or p-S65 phosphorylated $UB^{(2-7)}$ chains were incubated with the indicated phosphorylated forms of GST-OPTN, and after washing, bound proteins were released and subjected to SDS-PAGE and immunoblotting with α-UB.
(F) M1 chains were phosphorylated in vitro with TcPINK1 and the stoichiometry of phosphorylation determined by AQUA proteomics as described (Ordureau et al., 2014).

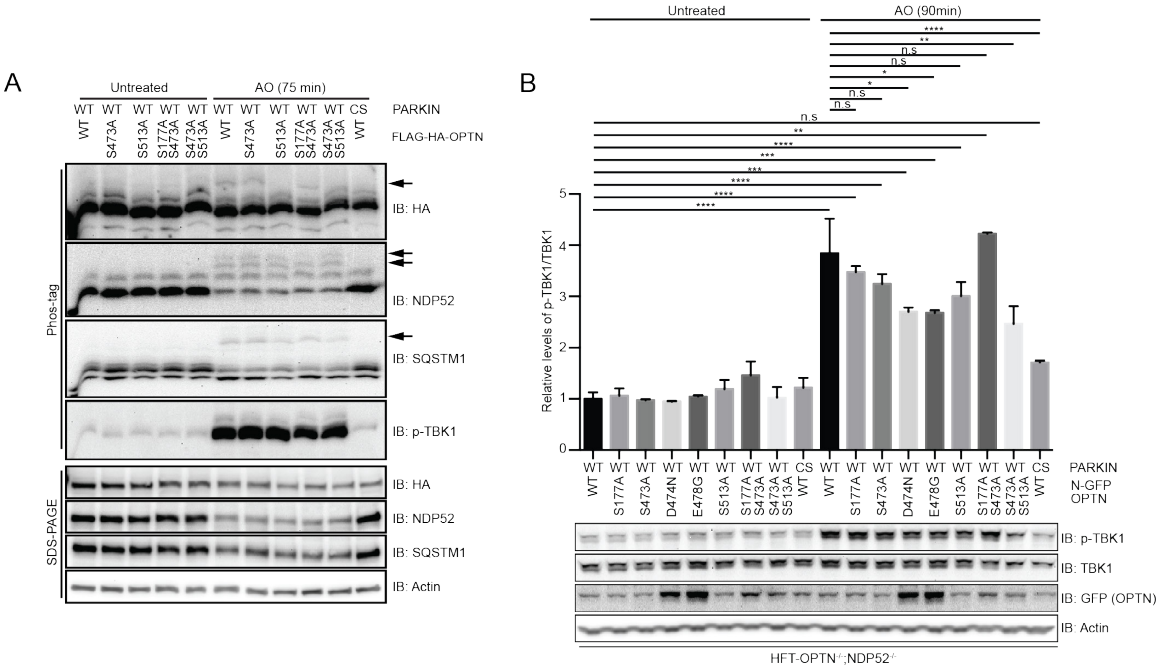

Figure S5. Analysis of phosphorylation of OPTN phospho-site mutants in response to mitochondrial depolarization. Related to Figure 6.

(A) The indicated OPTN mutants were stably expressed in HFT-PARKIN^{WT} or PARKIN^{CS} cells and cells treated with AO for 75 min. Extracts were subjected to electrophoresis using either SDS-PAGE or Phos-Tag gels and immunoblotted with the indicated antibodies. The position of depoloarization-dependent forms of OPTN, NDP52, and SQSTM1 are indicated by the arrow.

(B) Phosphorylation of S473 and S513 is required for efficient TBK1 activation in response to mitochondrial depolarization. The indicated GFP-OPTN mutants were expressed in HFT-PARKIN (WT or CS mutants) cells engineered to lack both OPTN and NDP52 using CRISPR-Cas9. Cells were either left untreated or treated with AO for 90 min. The lysates were then subjected to immunoblotting with the indicated antibodies. The relative levels of p-TBK1^{S172}/TBK1 were analyzed by one-way ANOVA with Dunnett's multiple comparisons test (n=3 biological replicates). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n.s., not significant.

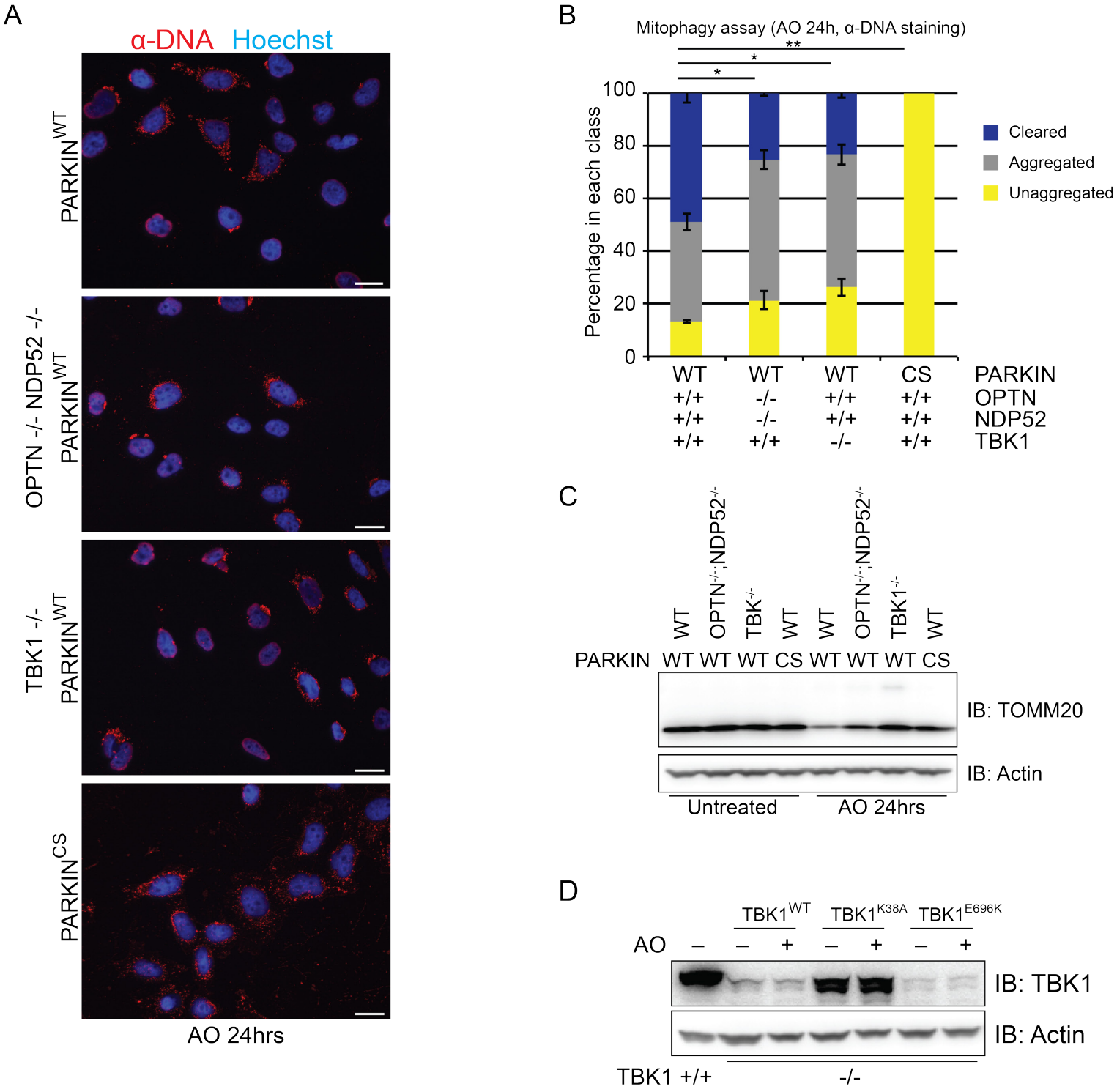

Figure S6. Analysis of mitophagy in cells lacking NDP52 and OPTN, or TBK1. Related to Figure 7.

(A) HFT-PARKIN^{WT}, HFT-PARKIN^{WT}; NDP52$^{-/-}$;OPTN$^{-/-}$, HFT-PARKIN^{WT};TBK1$^{-/-}$, or HFT-PARKIN^{CS} cells were subjected to AO treatment (24h) and stained for mitochondrial DNA using α-DNA (red). Nuclei were stained with Hoechst (blue).

(B) Cells from panel A were monitored for mitochondrial clearance and quantified using α-DNA staining and image analysis. Cells with cleared mitochondria were analyzed by one-way ANOVA with Dunnett's multiple comparisons test. *$p<0.05$, **$p<0.01$.
(C) Immunoblot of cells as described in panel B using anti-TOMM20 to examine mitochondrial abundance and actin as a loading control.
(D) $TBK1^{-/-}$ HFT-$PARKIN^{WT}$ cells were infected with lentiviruses expressing $TBK1^{WT}$, $TBK1^{K38A}$, or $TBK1^{E696K}$ and the expression examined by immunoblotting with and without AO treatment.

Table S1. Peptides used for Parallel Reaction Monitoring. Related to Figure 5.
Peptides used for AQUA-PRM quantitation. Internal standards were isotopically labelled (^{13}C, ^{15}N) and the corresponding residue is bold and underlined. Modified residues with oxidized methionine (m), carboxymethylation (c) or Serine phosphorylation (s) are indicated by lower case.

Name$^{\text{residue number}}$	Peptide Sequence	Charge (z)	Light (m/z)	Heavy (m/z)
OPTN$^{466\text{-}479}$	AQmEVYcSD**F**HAER	3	586.9119	590.2543
p-S473-OPTN$^{466\text{-}479}$	AQmEVYcsD**F**HAER	3	613.5673	616.9097
OPTN$^{512\text{-}520}$	QS**L**mEmQSR	2	571.2524	574.7610
p-S513-OPTN$^{502\text{-}520}$	ENDAFEDGGRQs**L**mEmQSR	3	771.3034	773.6424

Supplemental Experimental Procedures

Cell culture and gene-editing

HeLa Flp-In T-REx (HFT) cells (generously provided by Brian Raught, Ontario Cancer Institute) were grown in DMEM with 10% fetal bovine serum, 15 µg/ml Blasticidin and 100 µg/ml Zeocin. To generate HFT cells conditionally expressing the gene(s) of interest, the indicated gene(s) were transferred from pDNR221-based plasmids into pcDNA5-FRT/TO-FLAG-HA-Gateway-FRT-Hygromycin or pcDNA5-FRT/TO-GFP-Gateway-FRT-Hygromycin based vectors using λ recombinase in vitro and these plasmids transfected into HeLa Flp-In T-REx cells followed by selection with Hygromycin (200 µg/ml). To induce lower protein expression for each protein of interest, cells were treated with 0.5 µM doxycycline (DOX) for 16 h to induce low levels of expression of the proteins of interest. Cells were either left untreated or depolarized with a mixture of Antimycin A (10 µM) and Oligomycin A (5 µM) (Sigma Chemical Company) (referred to as AO) for the indicated time period. For MRT67307 treatment, cells were pretreated with 2 µM MRT67307 (EMD Millipore) for 1 hr and subjected to AO treatment as indicated. 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, 1mM sodium orthovanadate, 1% (v/v) NP-40, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM benzamidine and 1mM AEBSF), to produce whole-cell extracts. Mitochondria were purified as described previously (Ordureau et al., 2014). In some experiments, we created dual expression constructs by introducing IRES sequences between each gene within the pcDNA5-FRT/TO-FLAG-HA-Gateway-FRT-Hygromycin vector. Gene-editing for creation of TBK1, OPTN, and NDP52 null alleles were performed as described previously (Ran et al., 2013) using the following guide sequences: TBK1 (Exon 1; 5'-AGACATTTGCAGTAGCTCCT); OPTN (Exon 1, 5'- AAACCTGGACACGTTTACCC), and NDP52 (Exon 1, 5'- GGATCACTGTCATTTCTCTC). PINK1$^{-/-}$ HFT cells were described previously (Ordureau et al., 2014). To generate TBK1 and OPTN mutants, quick change mutagenesis was performed on the wild type constructs by using the KOD hot polymerase (EMD Millipore) followed by Dpn1 digestion. For expression of TBK1 and OPTN variants, lentiviral constructs containing the indicated open reading frames were packaged and virus used to infect the indicated cells, and stable cell lines selected in puromycin as described previously (Ordureau et al., 2014). Poly(I:C) was transfected as described previously (Ordureau et al., 2013). Briefly, Lipofectamine 2000 was used to transfect cells with Poly(I:C) (#tlrl-pic, InvivoGen) at a final concentration of 10 µg/ml in the culture medium and cells were harvested at the indicated time point. For siRNA transfection, cells were transfected with either scRNA (D-001210-01-05, GE Dharmacon) or siRNAs against TBK1 (MU-003788-02-0002, GE Dharmacon) by using Lipofectamine RNAiMax reagent (13778075, Life Technology) at the final concentration of 10nM. Cells were then incubated for 3 days and were either left untreated or treated with AO as indicated.

Antibodies, immunofluorescence and image analysis

The following antibodies were used in this study: α-MFN2 ([NIAR164], Abcam), α-TOMM20 (sc-11415, Santa-Cruz), α-PARKIN (sc-32282, Santa-Cruz, [EPR5024(N)], Abcam), α-UB (Dako), α-HA (11867423001, Roche), α-TBK1 (3013S, CST), α-p-S172 TBK1 (5483P, CST), α-GST (2625P, CST) α-Actin (sc-69879, Santa-Cruz), α-SQSTM1 (5114S, CST), α-NDP52 (9036S, CST), α-TAX1BP1 (HPA024432-100UL, Sigma), α-DNA (CBL186, EMD Millipore), α-MAVS (ab25084, Abcam), α-p-S396 IRF3 (4947S,

CST), α-IRF3 (4302S, CST) α-p-S65 UB (ABS1513, EMD Millipore), α-LC3B (2775S, CST).

For immunofluorescence, the indicated cells were left untreated or incubated with AO for the indicated time period. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X 100, blocked with 1% BSA, and subjected to immunostaining using either α-HA, α-OPTN, α-TAX1BP1, α-NDP52, α-SQSTM1, α-p-TBK1^{S172}, α-S65 UB, or α-LC3B in combination of α-TOMM20 to mark mitochondria. Nuclei were identified with Hoechst staining. Cells were then imaged using a Yokogawa CSUX1 spinning disk confocal lens on a Nikon Ti-E inverted microscope equipped with a 100Å~ Plan Apo N.A. 1.4 objective lens in the Nikon Imaging Center at Harvard Medical School. Image analysis was performed using the Fiji coloc2 plug-in, which provides Mander's Overlap Correlation. To obtain normalized MOC, the obtained MOC was further divided by the fraction of mitochondria in the whole cell. For quantifying LC3B recruitment to mitochondria, N-GFP-LC3B-IRES-PARKIN was integrated into the Flp-in locus in both HFT and HFT;OPTN $^{-/-}$;NDP52$^{-/-}$ cells as described above. To perform high content imaging, each cell line was first plated in 384-well plate (3712, Corning) and treated with DOX for 16 hrs to induce N-GFP-LC3B and PARKIN expression. Cells were then treated with AO for 75 min and fixed with 4% paraformaldehyde. Cells were then permeabilized with 0.2% Triton-X and subjected to Hoechst and Cellmask (H32721, Invitrogen) staining. After fixation, cells were permeabilized with 0.2% Triton-X and subjected to Hoechst and Cellmask (H32721, Invitrogen) staining. Those cells were eventually imaged by the ImageXpress® Micro XLS System (Molecular Device) in the ICCB Longwood and *per cent* cells with GFP-LC3B puncta were analyzed with the script developed by the IDAC at HMS by using the Matlab software.

For mitophagy assays, each cell line was treated with DOX (0.5 μM to induce PARKIN expression) for 16h and then treated with both AO as indicated (Ordureau et al., 2015a). At the indicated time, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 1% BSA, and immunostained with either α-TOMM20 or α-DNA antibody followed by Hoechst staining. Images were acquired as described above. For quantification, ~100 cells were counted based on the classification of unaggregated mitochondria, aggregated mitochondria, and cleared mitochondria in biological triplicate.

Phospho-proteomics and analysis of phosphoproteins

To identify phosphorylation sites in OPTN, cells stably expressing FLAG-HA-OPTN were left untreated or treated with AO (75 min), the cells lysed in lysis buffer (see previous section) supplemented with phosphatase inhibitors (PhosStop, Roche), and FLAG-OPTN purified using α-FLAG magnetic beads from 20 mg of cell extract. Immune complexes were eluted twice with 3xFlag peptide (5 time the volume of packed beads (125 ug.ml^{-1} in 50 mM Tris pH 7.4, 150 mM NaCl) and 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 TEAB, 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 when required methionine were oxidized with the addition of H_2O_2 (0.005% final) for 15 min prior to C18 StageTip (packed with Empore C18; 3M Corporation) desalting.

For figure 5B, eluted peptide were resuspended in 5% FA and mass spectrometry data were collected using a Qexactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with a Famos Autosampler (LC Packings) and an Accela600

liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were separated on a 100 µm inner diameter 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 50 min gradient of 3 to 20% acetonitrile in 0.125% formic acid with a flow rate of ~300 nL/min. The scan sequence began with an MS^1 spectrum (Orbitrap analysis; resolution 70,000; mass range 300−1500 m/z; automatic gain control (AGC) target 1×10^6; maximum injection time 250 ms). In addition, unassigned and singly charged species were excluded from MS^2 analysis and dynamic exclusion was set to automatic. Precursors for MS^2 analysis were selected using a Top20 most abundant peptides. MS^2 analysis consisted of high-energy collision-induced dissociation (quadrupole ion trap analysis; AGC 1×10^5; normalized collision energy (NCE) 25; maximum injection time 60 ms; resolution 17,500). 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 (Huttlin et al., 2010). Parameters used for database searching include: 50 p.p.m. Precursor mass tolerance; 0.03 Da (or 0.9 for TMT-MS3 exeriment) product ion mass tolerance; tryptic digestion with up to three missed cleavages; Carboxyamidomethylation of Cys was set as a fixed modification, while oxidation of Met and phosphorylation modification of Ser, Thr, Tyr were set as variable modifications. Localization of phosphrylation sites used a modified version of the A-score algorithm (Beausoleil et al., 2006) as described (Kim et al., 2011). A-scores of 13 were considered localized. Quantification based on spectral counts was done as described (Kim et al., 2011).

For Figure 5C, eluted peptides were resuspended in 200 mM Tris pH 8.5 and labeled using 9-plex tandem mass tag (TMT) reagents (Thermo Fisher Scientific, Rockford, IL). TMT reagents (0.8 mg) were dissolved in 42 µl dry acetonitrile (ACN) and 10 µl was added to 100 µg of phosphopeptides dissolved in 100 µl of 200 mM HEPES, pH 8.5. After 1hr (RT), the reaction was quenched by adding 4 µl of 5% hydroxylamine. Labeled peptides were combined, acidified with FA (pH ~2), dried down, resuspended in 5% (vol/vol) FA, and subjected to C18 StageTip (packed with Empore C18; 3M Corporation) desalting. Mass spectrometry data were collected using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to a Proxeon EASY-nLC II liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were fractionated on a 100 µm inner diameter 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, Newark, DE). Peptides were separated using a 60 min gradient of 4 to 27% acetonitrile in 0.125% formic acid at a flow rate of ~425 nL/min. The scan sequence began with an MS^1 spectrum (Orbitrap analysis; resolution 120,000; mass range 400−1400 m/z; AGC target 2×10^5; maximum injection time 100 ms, monoisotopic peak). In addition, unassigned and singly charged species were excluded from MS^2 analysis and dynamic exclusion (1 min) was set to 10 after 30 sec. A list of targeted mass (m/z (7 ppm mass tolerance), z, 10 min schedule window) for OPTN phosphorylation sites of interest (p-S177, p-S473 and p-S513) was defined. Each targeted mass was assigned a specific MS^2 dependent scan event and data dependent analysis was performed if no target species were found. By default precursors for MS^2 analysis were selected using the Top10 most abundant peptides. MS^2 analysis consisted of high energy collision-induced dissociation (quadrupole ion trap analysis; AGC 4×10^3; CID normalized collision energy (NCE) 35; maximum injection time 150 ms; isolation window 0.5-0.7; with injection of all available parallelizable time). We performed multinotch MS^3 (McAlister et al., 2014) with synchronous-precursor-selection (SPS) where precursor ions (n=10) were collected (Orbitrap analysis; resolution 60,000; mass

range 100−1000 m/z; HCD normalized collision energy 55; AGC target 5×10^4; maximum injection time 150 ms (or 300 ms for ions in the targeted list); with injection of all available parallelizable time).

For figure 5D-E immune complexes (from 20 mg cell extract starting material) were eluted from the magnetic beads with 2% LDS sample buffer prior to SDS-PAGE. SDS-PAGE gels were then stained with colloidal Coomassie Blue, bands of interest excised and subject to reduction (5 mM TCEP) and alkylation (20 mM chloroacetamide) followed by in-gel digestion with trypsin at 37 °C with trypsin (in 100 mM TEAB). 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 when required methionine were oxidized with the addition of H_2O_2 (0.0005% final) for 15 min prior to C18 StageTip (packed with Empore C18; 3M Corporation) desalting. 80% of eluted peptides were then processed for AQUA/PRM analysis (see below) and 20% left labeled with TMT (10-plex) and processed as described above for Figure 5C.

For figure 5H, purified proteins were separated by SDS/PAGE. SDS-PAGE gels were then stained with colloidal Coomassie Blue, bands of interest excised and subject to reduction (5 mM TCEP) and alkylation (20 mM chloroacetamide) followed by in-gel digestion with trypsin at 37 °C with trypsin (in 100 mM TEAB). 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 when required methionine were oxidized with the addition of H_2O_2 (0.005% final) for 15 min prior to C18 StageTip (packed with Empore C18; 3M Corporation) desalting. Eluted peptides were labeled with TMT (5-plex) and processed as described above for Figure 5C

In-vitro kinase assay and OPTN phosphorylation site mapping

OPTN was expressed in Escherichia coli as a GST fusion protein and purified by affinity chromatography on glutathione-Sepharose. GST-OPTN (2 µM) was incubated with 0.25 µM TBK1 (~1.12 Unit) (purified from Sf21 insect cells, ubiquigent #66-0016-050) in reaction buffer (50 mM Tris-HCl, pH 7.5, 0.1% (v/v) TCEP, 0.1 mM EGTA, 10 mM magnesium chloride). Assay was initiated by adding ATP to a final concentration of 0.1 mM. The reactions was terminated after 30 min at 30°C by the addition of 1% LDS containing 20 mM EDTA, pH 7.0, heated for 5 min at 75 °C and separated by SDS-PAGE, followed by Commassie blue staining. The gel band corresponding to GST-OPTN was excised and subject to reduction (5 mM TCEP) and alkylation (20 mM chloroacetamide) followed by in-gel digestion with trypsin at 37°C with trypsin (in 50 mM TEAB). Digests were acidified with an equal volume of 5% (vol/vol) formic acid (FA) to a pH of ~2, dried down, resuspended in 5% (vol/vol) FA and methionine were oxidized with the addition of H_2O_2 (0.005% final) for 15 min prior to C18 StageTip (packed with Empore C18; 3M Corporation) desalting. Eluted peptides were resuspended in 5% FA/5%ACN and data were collected using a targeted Single Ion Monitoring (tSIM) approach on a Qexactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) with a Famos Autosampler (LC Packings) and an Accela600 liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were separated on a 100 µm inner diameter 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 50 min gradient of 3 to 32% acetonitrile in 0.125% formic acid with a flow rate of ~300 nL/min. The scan sequence began with tSIM sequence where retention time, m/z and charge information for the peptide of interest (p-S177 (853.86709 Th ;z=2), p-S473 (613.56731 Th ;z=3) and p-S513 (771.30336 Th ;z=3)) of OPTN were added to an inclusion list (Orbitrap analysis; resolution 70,000; mass range 200−2000 m/z; isolation window of 2 m/z; automatic gain control (AGC)

target 1×10^5; maximum injection time 150 ms, loop=1). In addition, unassigned and singly charged species were excluded from MS^2 analysis. MS^2 analysis consisted of high-energy collision-induced dissociation (Orbitrap resolution 17,500, AGC 2×10^5; normalized collision energy (NCE) 27; maximum injection time 250 ms; loop count 5, isolation windows of 2 m/z and fixed first mass of 100 m/z).

AQUA/PRM of p-S473-OPTN and p-S513-OPTN

For AQUA proteomics, heavy reference peptides of human OPTN, containing a single 13C/15N-labeled amino acid (see TableS1) was provided by Cell Signaling Technologies and quantified by amino acid analysis. The AQUA peptide (50 fmol) were added to samples after trypsin digestion and samples examined by Parallel Reaction Monitoring on a Q-Exactive Mass Spectrometer as described previously (Ordureau et al., 2015a). Intensities were measured to determine the total area of each peptides using Skyline software (3.1.07382) (MacLean et al., 2010). Total area measured for the oxidized and non oxidized form of each peptide were summed together, prior to calculation of ratio light/heavy. Data generated from Skyline was exported into a Microsoft Excel spread sheet and GraphPad Prism for further analysis as previously describe (Ordureau et al., 2014).

Expression and purification of phosphorylated proteins.

In order to prepare site-specific phosphorylated OPTN, we used a suppressor tRNA system in bacteria, allowing for direct incorporation of phosphoserine at a desired residue position (Pirman et al., 2015). Briefly, human OPTN DNA fused to His6-GST at its N-terminus was mutated by PCR to replace the Ser codon of interest (S473 and/or S513) with an Amber stop codon (TAG), which is used for phosphoserine incorporation. Mutations in OPTN phosphorylation sites were generated using following the QuikChange II site-directed mutagenesis method, but using KOD Hot Start DNA Polymerase. 6His-GST-OPTN constructs were then subcloned in the pCRT7-NT-Topo-tetR/pLtetO Amp vector (52053; Addgene) by Gibson assembly. A more advanced phosphoserine system was used and contained the following modifications (Pirman et al., 2015). C321.ΔA strains (derived from a previously described strain (Lajoie et al., 2013) with the following genotype modifications: ΔmutS:zeo, ΔtolC, Δbla:tolC, SerB-/ΔSerB) were co-transformed with the pCRT7 plasmid of interest and pKD-SepRS9-EFSep21-4x $tRNA^{Sep\text{-}A37}$ (SepOTS λ). Enhanced SepRS9 and EFSep21 have been described previously (Lee et al., 2013) and the $tRNA^{Sep\text{-}A37}$ mutation increases suppressor activity. 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 C321.ΔA 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 C321.ΔA + SepOTS λ + 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 (TB supplemented with carbenicillin, kanamycin, 2 mM phosphoserine, 0.08% glucose) to an OD600 of ~0.1 and grown at 30 °C with shaking until the OD600 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 21 °C for the duration of the protein expression procedure (~24 h). The cells were collected and lysed in 50 mM Hepes (pH 7.5), 250 mM NaCl, 50 mM NaF, 270 mM Sucrose, 0.03% Brij-35, 15 mM imidazole, 0.25 mM TCEP, and 1 mM AEBSF. 6His-GST-OPTN and specific phospho-

forms were purified by His-TALON as previously described (2) prior to gel filtration on a gel filtration Superdex 75 10/300 GL system (GE Healthcare).

In-vitro ubiquitin binding assay

In order to examine binding of OPTN and its phospho-forms with UB chains, GST-tagged proteins (1-2 µg) were immobilized on glutathione-Sepharose [10 µL of packed beads equilibrated in 25 mM Hepes (pH 7.5), 1 mM EGTA, 2 mM $MgCl_2$, 25 mM NaF, 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 buffer containing poly-UB chains (1-2 µg; $K48^{(2-7)}$ and $K63^{(2-7)}$ from Boston Biochem; $M1^{(2-7)}$ from Enzo Life Sciences) in the same buffer. The beads were washed five times with buffer plus 250 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 xg; the supernatants were heated for 5 min at 75 °C, subjected to SDS/PAGE, and transferred to PVDF membranes; and proteins were detected by immunoblotting. Analogous experiments were performed with p-S65 -M1, -K48 and -K63 UB chains prepared as described previously (Ordureau et al., 2015a) by incubating the indicated UB chains with recombinant TcPINK1 (Ordureau et al., 2014) and then removing TcPINK1.

SDS-PAGE, Phos-tag SDS-PAGE and western blotting

For SDS-PAGE, indicated cells were lysed in lysis buffer described above. Forty µg of lysate was then loaded onto the SDS-polyacrylamide gel and subjected to the electrophoresis. For Phos-tag SDS-PAGE, Phos-tag acrylamide (300-93523, Wako) was added to the 6% SDS-acrylamide gel mixture as suggested by the manufacturer. After SDS-PAGE, gels were transferred to PVDF membrane and subjected to immunoblotting with indicated antibodies.

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