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Supplemental Information

**Global Landscape and Dynamics of Parkin
and USP30-Dependent Ubiquitylomes
in iNeurons during Mitophagic Signaling**

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TITLES OF SUPPLEMENTAL MATERIALS

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Figure S1

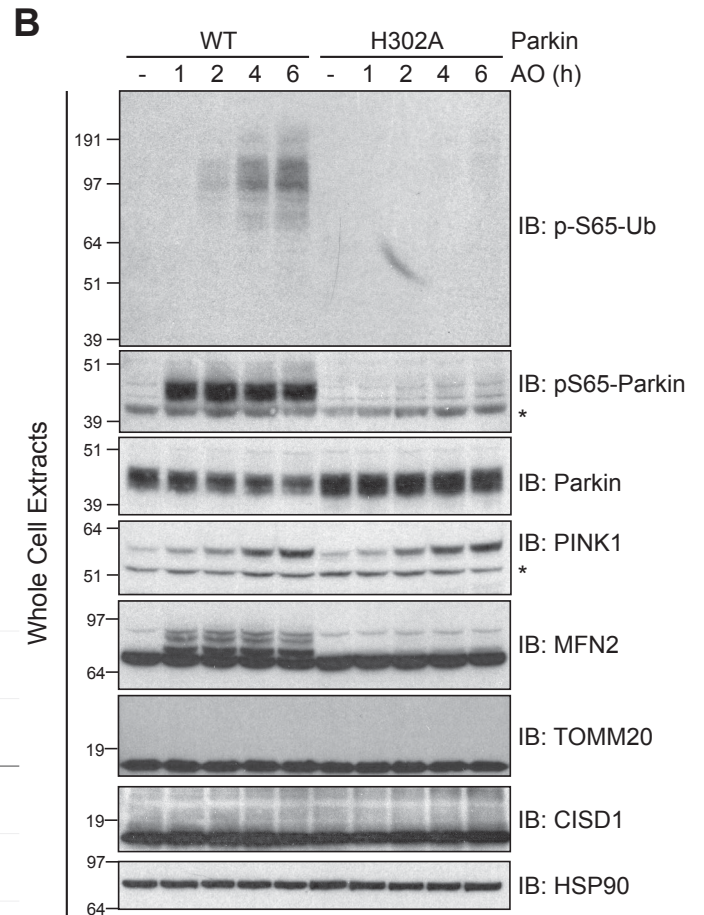
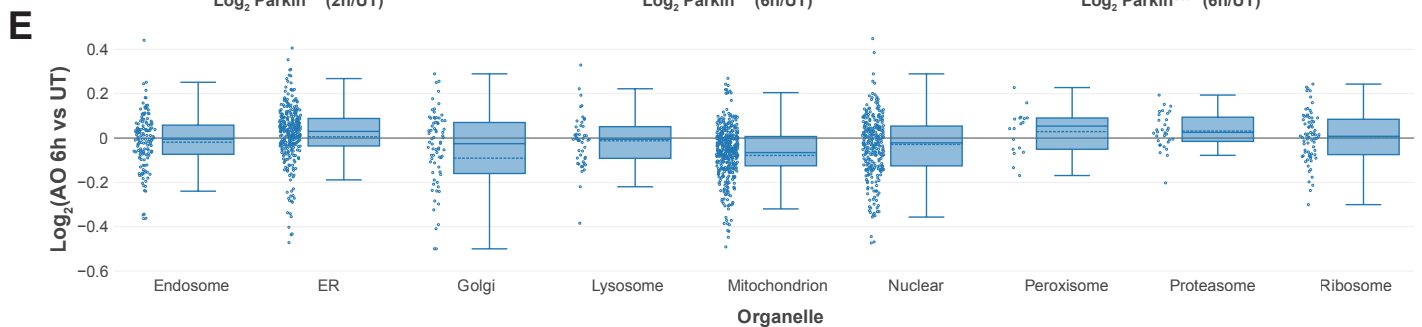
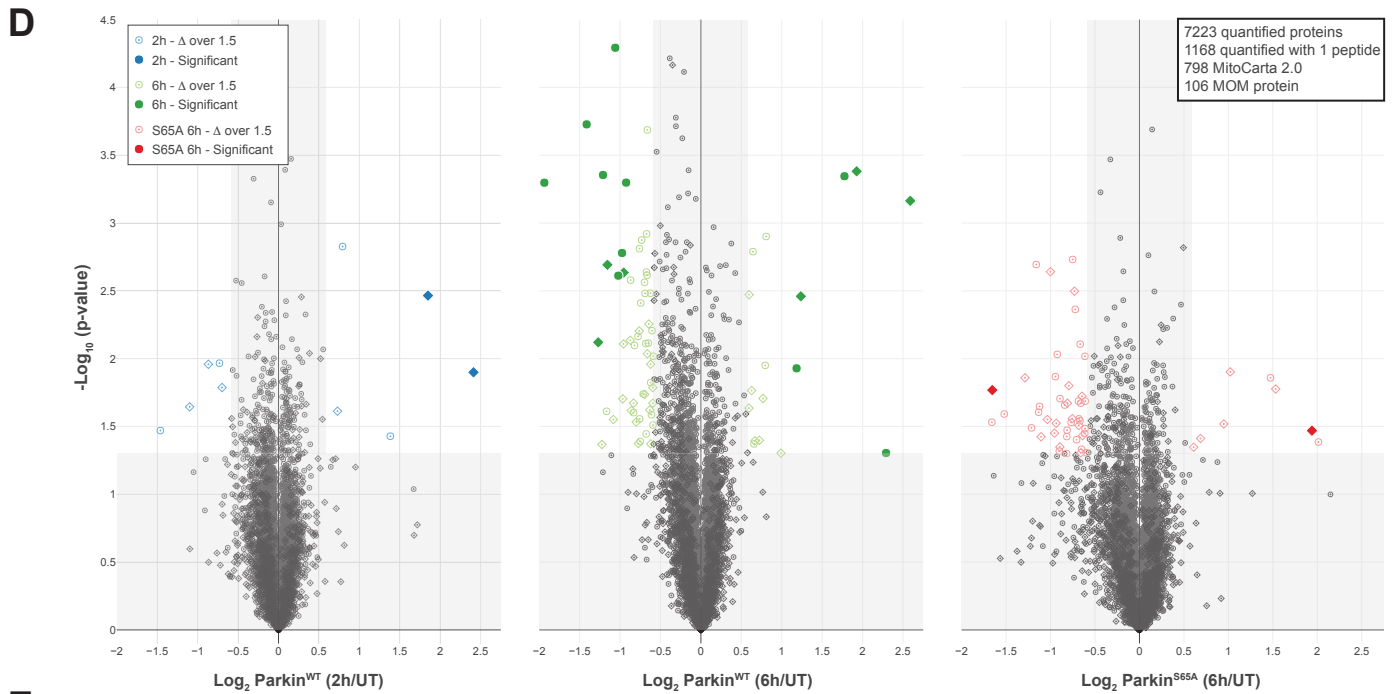
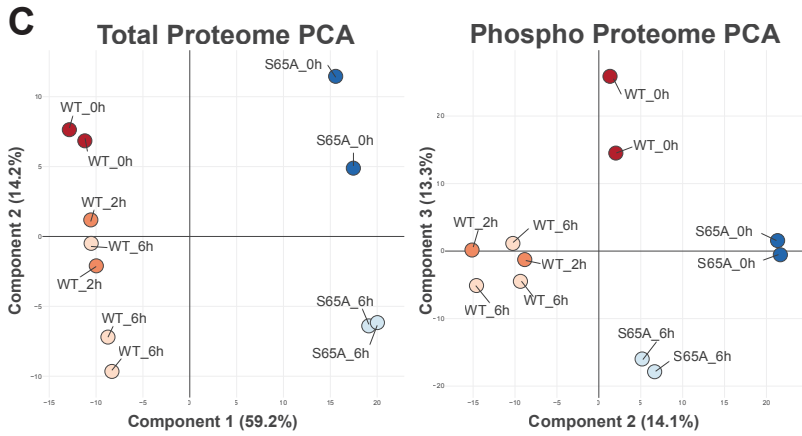
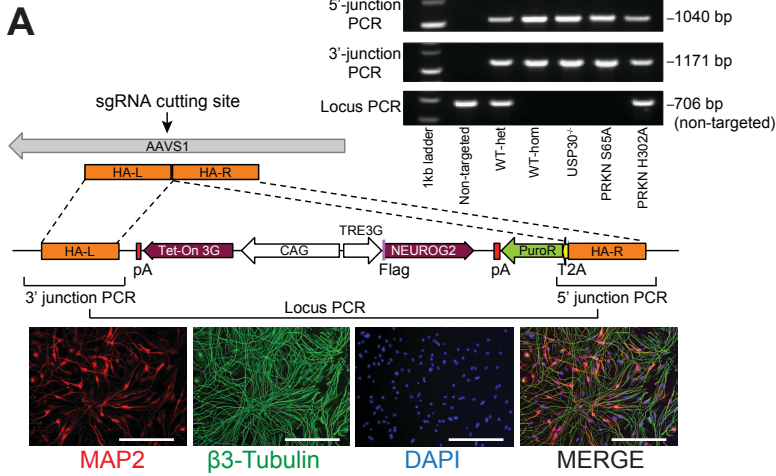


FIGURE S1. Global proteomic and phospho-proteomic analysis of iNeurons in response to mitochondrial depolarization and Parkin activation. Related to Figure 1.

(A) Creation and validation of iNeurons generation. Scheme of the strategy used to target NGN2 to the AAVS1 locus and resulting PCR products for the various cell line used. Parental H9 hESCs gene edited to express NGN2 under the TRE3G promoter from the AAVS1 locus were induced to express NGN2 for 12 days and the extent of differentiation determined by co-immunostaining for MAP2, β 3-Tubulin and DAPI (scale bar = 200 microns).

(B) iNeurons expressing WT or H302A-Parkin were subjected to depolarization and total cell extracts subjected to immunoblotting with the indicated antibodies. *, non-specific band.

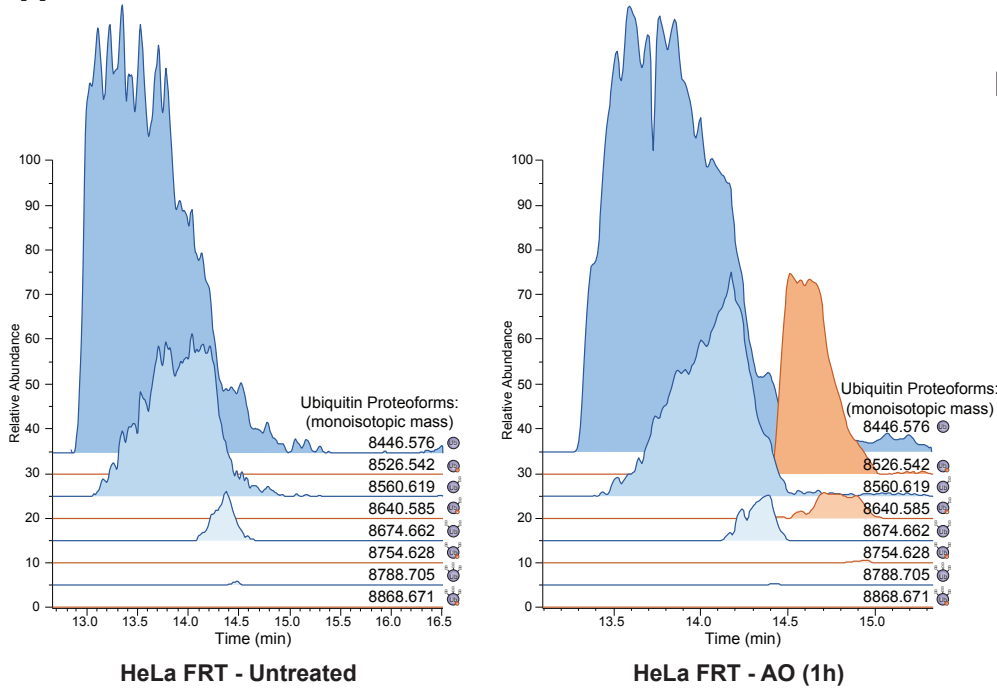
(C) Principal component analysis of proteomics data obtained for the 11-plex experiment described in **Figure 1A**.

(D) Volcano plots for total proteomes of WT or S65A-Parkin iNeurons depolarized for 2 or 6h compared with untreated cells. Protein quantified with more than one peptide are represented by a circle, while quantification based on a single peptide are represented by a square. Proteins with $\text{Log}_2\text{-Ratio} < -0.585$ or > 0.585 ($p\text{-value} < 0.05$) are indicated as colored empty dotted circles/squares, and filled colored circles/squares indicate statistically significant hits (Welch's t-test ($S_0=0.585$), corrected for multiple comparison by permutation-based FDR (5%)).

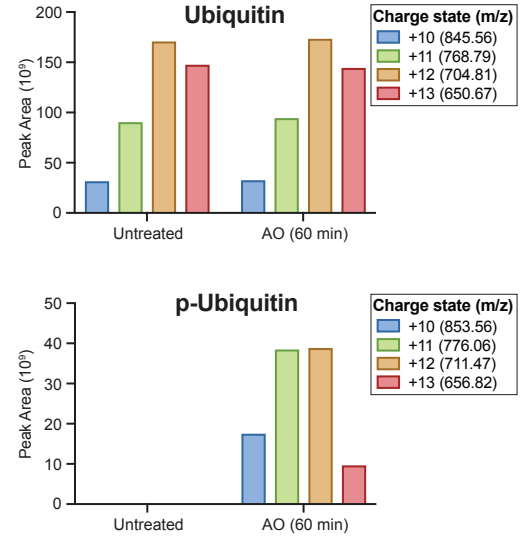
(E) Plot of $\text{Log}_2(6h\text{ AO}/\text{Untreated})$ for proteins localized in the indicated organelles or protein complexes. Proteins present in each organelle/protein complex were derived from ([ltzhak et al., 2016](#)).

Figure S2

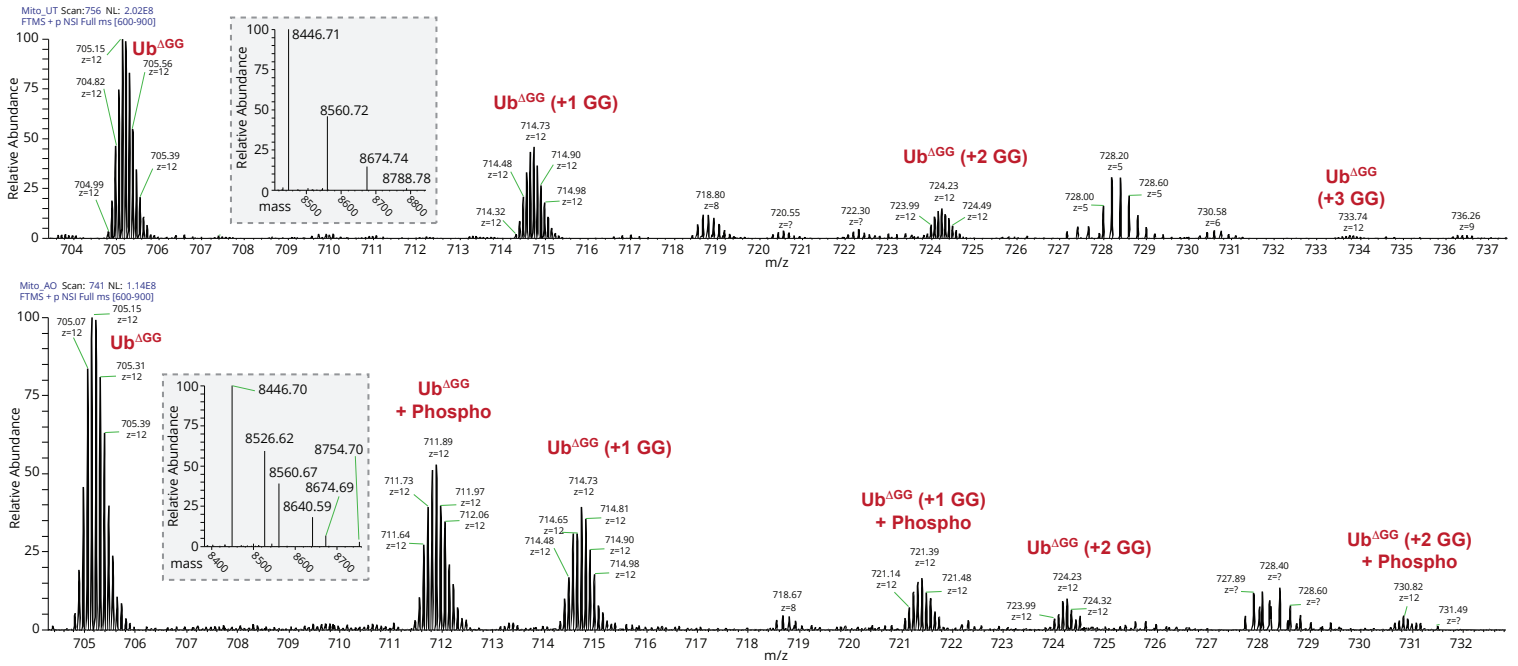
A



B



C



D

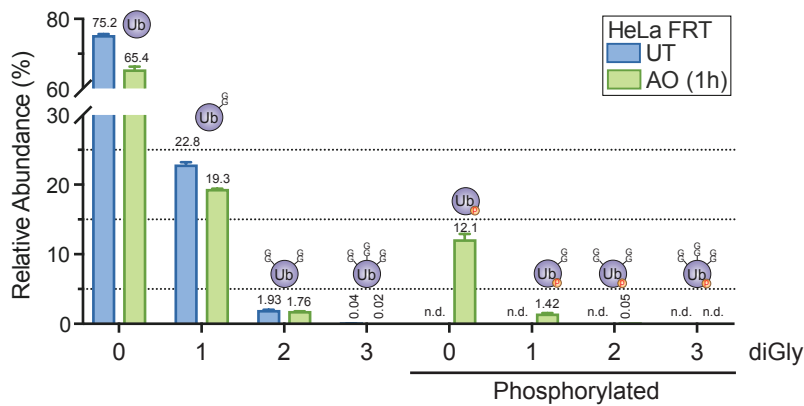


FIGURE S2. Quantitative analysis of pS65-Ub proteoforms on the MOM in iNeurons using Ub^{ΔGG} amputation and intact mass spectrometry. Related to Figure 2.

(A) Deconvoluted extracted ion chromatograms for Ub^{ΔGG} proteoforms from HeLa Flip-In Parkin cell derived mitochondria with or without depolarization (1h) with AO. Distinct Ub^{ΔGG} branched diGLY proteoforms in untreated cells largely co-migrate on reversed-phase (C4) LC while phospho-Ub^{ΔGG} proteoforms elute at a distinct position.

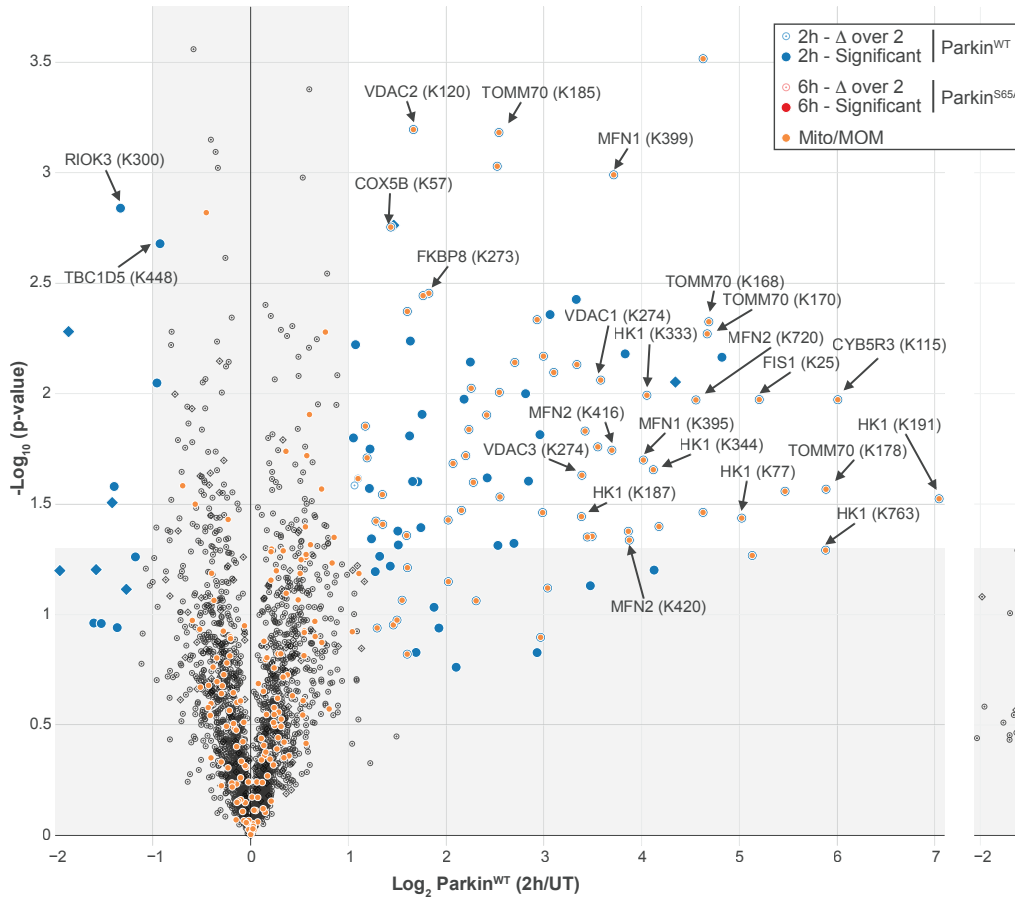
(B) Relative abundance of charge state distributions are different for Ub^{ΔGG} and pS65-Ub^{ΔGG}, and therefore integration across all charge states is required for quantification.

(C) Examples of mass spectrometry raw spectra showing detection of individual Ub^{ΔGG} proteoforms in untreated (top) or in response to depolarization (bottom) of HeLa cells expressing Parkin^{WT}. Shown are spectra of the isotopic cluster for the z=12 precursors. Deconvoluted spectra and mass of the various proteoforms are indicated in grey boxes.

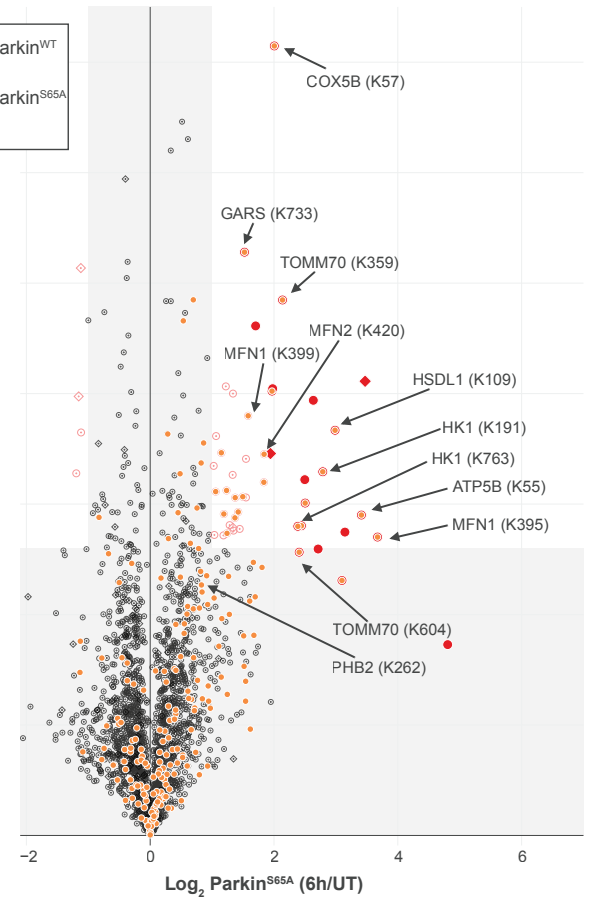
(D) Relative abundance of Ub^{ΔGG} proteoforms using the workflow described in **FIGURE 2B**, but applied to HeLa cells. Error bars represent SEM, n=3. n.d., not determined.

Figure S3

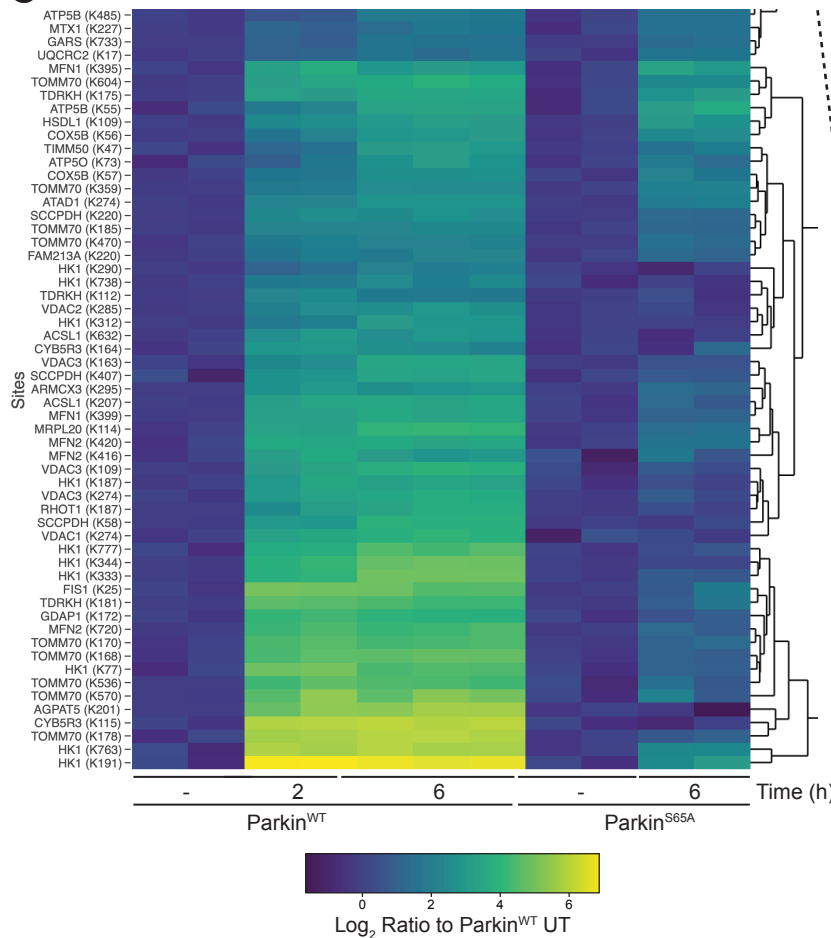
A



B



C



D

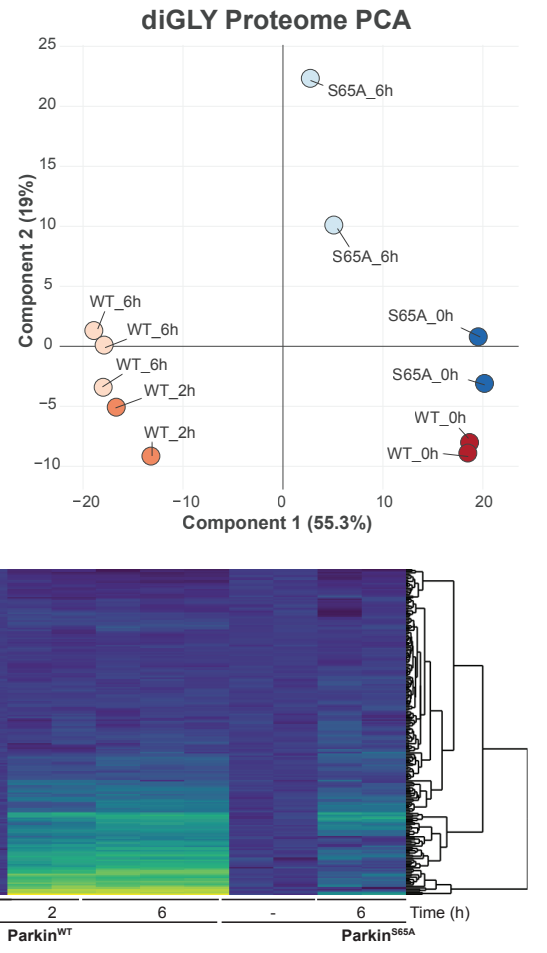


FIGURE S3. Quantitative discovery of the Parkin-dependent ubiquitylome in iNeurons during mitophagic signaling. Related to Figure 3.

(A,B) Volcano plot of diGLY-containing peptides identified by TMT-MS³ from whole-cell lysates derived from (A) WT iNeurons with or without depolarization for 2h, or (B) Parkin^{S65A} iNeurons with or without depolarization for 6h. The abundance of diGLY-peptides was normalized against the total protein abundance when available (circle) or not (square). Filled colored circles/squares indicate statistically significant hits (Welch's t-test (S0=1), corrected for multiple comparison by permutation-based FDR (1%)). diGLY-modified residue is indicated in bracket after the protein name. diGLY-peptide of proteins associated with mitochondria (MitoCarta 2.0 ([Calvo et al., 2016](#))) or mitochondrial outer membrane identified by proximity biotinylation ([Hung et al., 2017](#))) are overlaid with an orange circle.

(C) Heat map of mitochondrial-diGLY peptides across the WT and S65A-Parkin cells without or with depolarization for 2 or 6h. Only diGLY-peptides of proteins associated with mitochondria (MitoCarta 2.0 ([Calvo et al., 2016](#))) or mitochondrial outer membrane identified by proximity biotinylation ([Hung et al., 2017](#))) are represented.

(D) Principal component analysis of diGLY data across the WT and S65A-Parkin cells without or with depolarization for 2 or 6h.

Figure S4

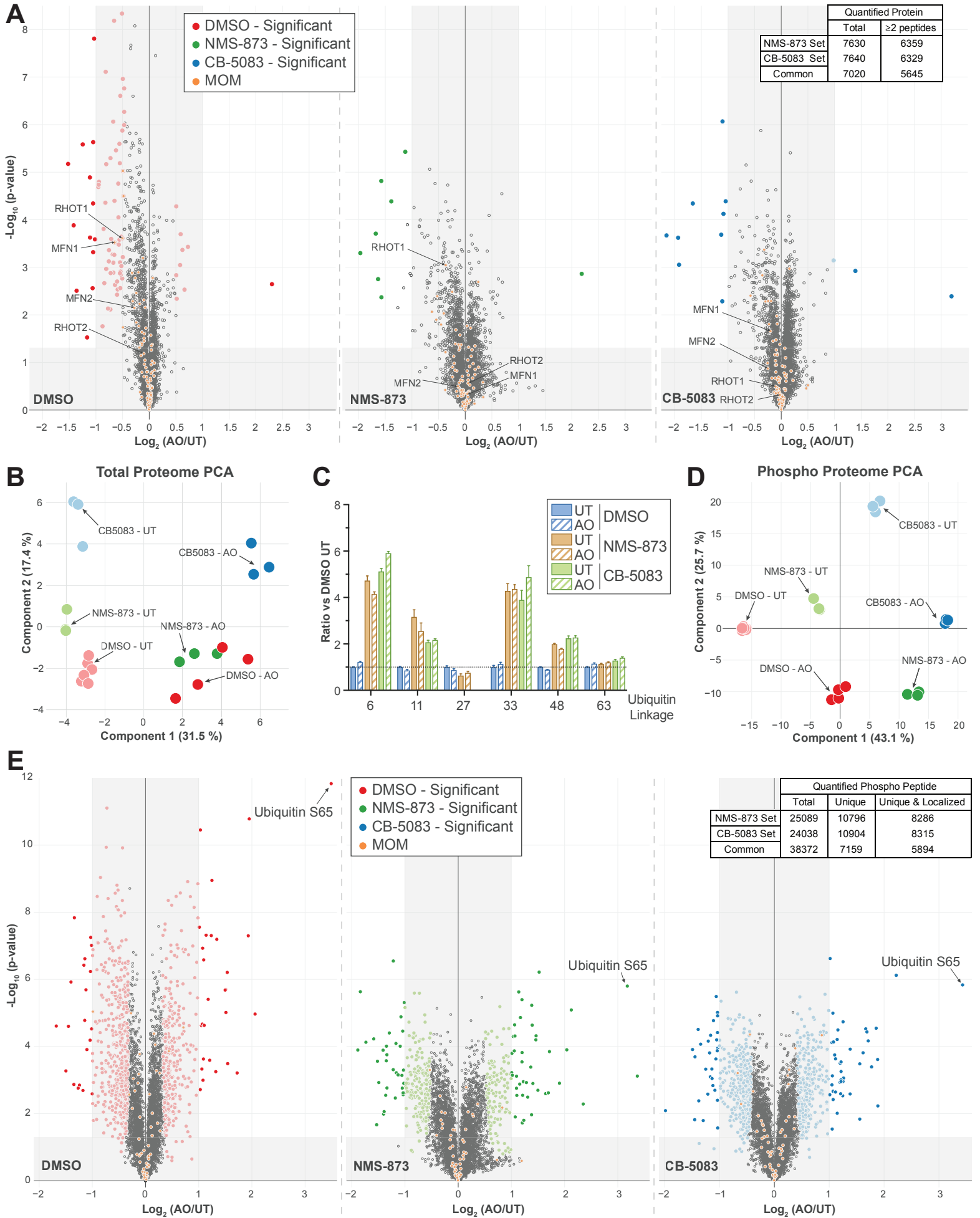


FIGURE S4. Quantitative analysis of p97-dependent mitochondrial proteome remodeling and PINK1-Parkin pathway activity in iNeurons. Related to Figure 4.

(A) Volcano plots for total proteomes of iNeurons either left untreated or depolarized for 6h in the presence or absence of the p97 inhibitors NMS-873 or CB-5083. Only proteins quantified in both p97 inhibitor dataset and also quantified with more than one peptide are represented (circle). Filled colored circles/squares indicate statistically significant hits (Welch's t-test ($S_0=1$), corrected for multiple comparison by permutation-based FDR (1%)). Proteins associated with mitochondrial outer membrane identified by proximity biotinylation ([Hung et al., 2017](#)) are overlaid with an orange circle.

(B) PCA analysis of whole proteome data from panel A.

(C) Ub chain linkage types were determined as ratio versus untreated for iNeurons either left untreated or depolarized for 6h in the presence or absence of the p97 inhibitors NMS-873 or CB-5083. Error bars represent SEM, $n=3$.

(D) PCA analysis of phospho-proteome data from panel E.

(E) Volcano plots for phospho-proteomes of iNeurons either left untreated or depolarized for 6h in the presence or absence of the p97 inhibitors NMS-873 or CB-5083. Only peptides quantified in both p97 inhibitor datasets are represented (circle). Filled colored circles indicate statistically significant hits (Welch's t-test ($S_0=1$), corrected for multiple comparison by permutation-based FDR (1%)). Phospho-peptide of proteins associated with mitochondrial outer membrane identified by proximity biotinylation ([Hung et al., 2017](#)) are overlaid with an orange circle.

Figure S5

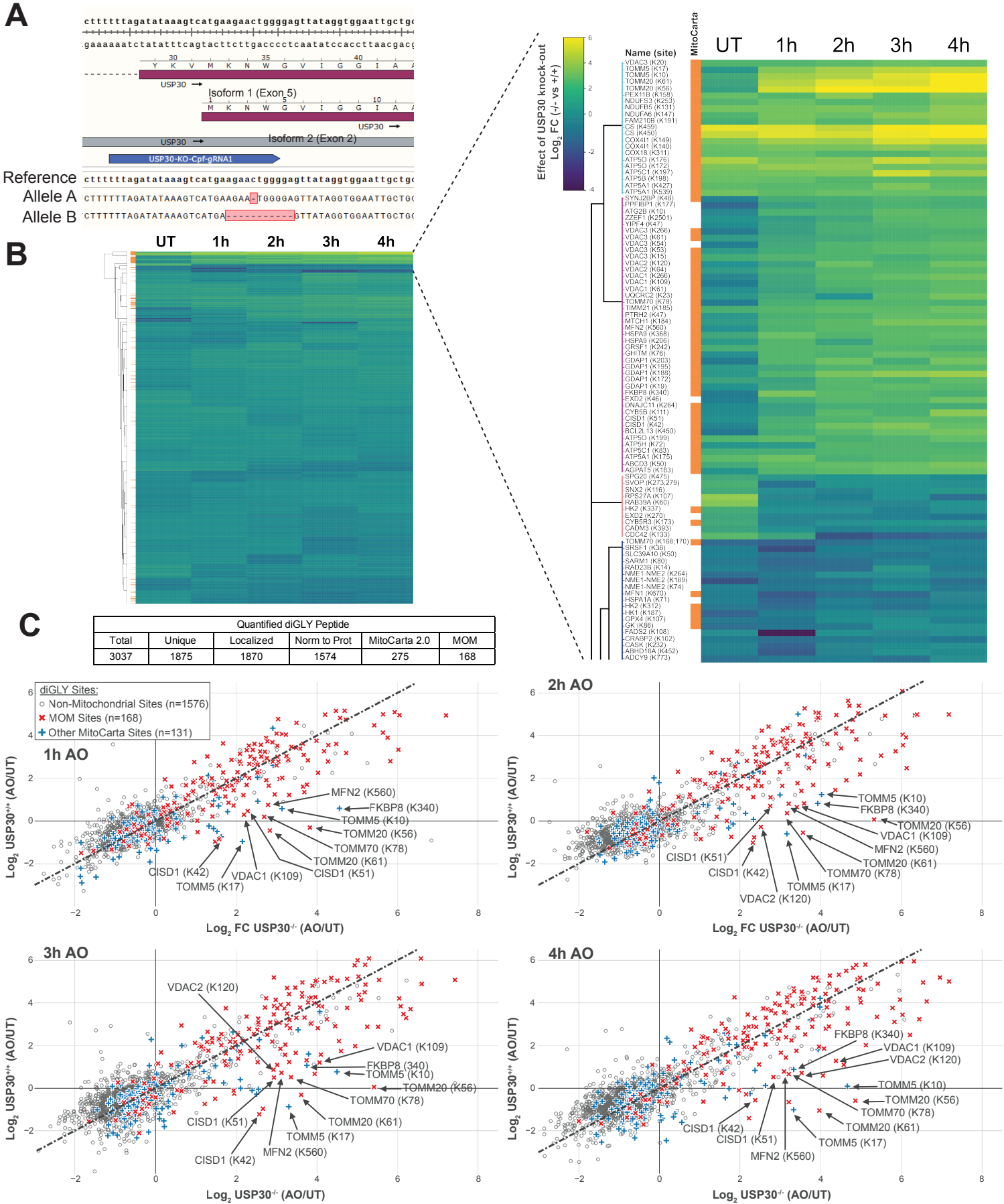


FIGURE S5. Selectivity of USP30-dependent buffering of MOM ubiquitylation by Parkin in iNeurons. Related to Figure 5.

(A) Validation of USP30 deletion in hESCs. The indicated gRNA, which targets both isoform 1 and 2 of USP30 (exon 5 and exon 2, respectively) was used to produce a frameshift in both alleles of USP30 using Cpdf1 (Cas12) nuclease, as determined by sequence analysis of the isolated USP30 clone. Loss of USP30 protein was verified by immunoblotting of iNeuron extracts (**Figure 5B**).

(B) Heat map of the effect of USP30 deletion on the abundance of individual diGLY-containing peptides at each time point from the TMT-MS³ data. diGLY-peptide of proteins associated with mitochondria (MitoCarta 2.0 ([Calvo et al., 2016](#))) are with an orange box.

(C) Correlation plots of diGLY peptide TMT values for USP30^{+/+} iNeurons versus USP30^{-/-} iNeurons at the indicated time points post depolarization.

Figure S6

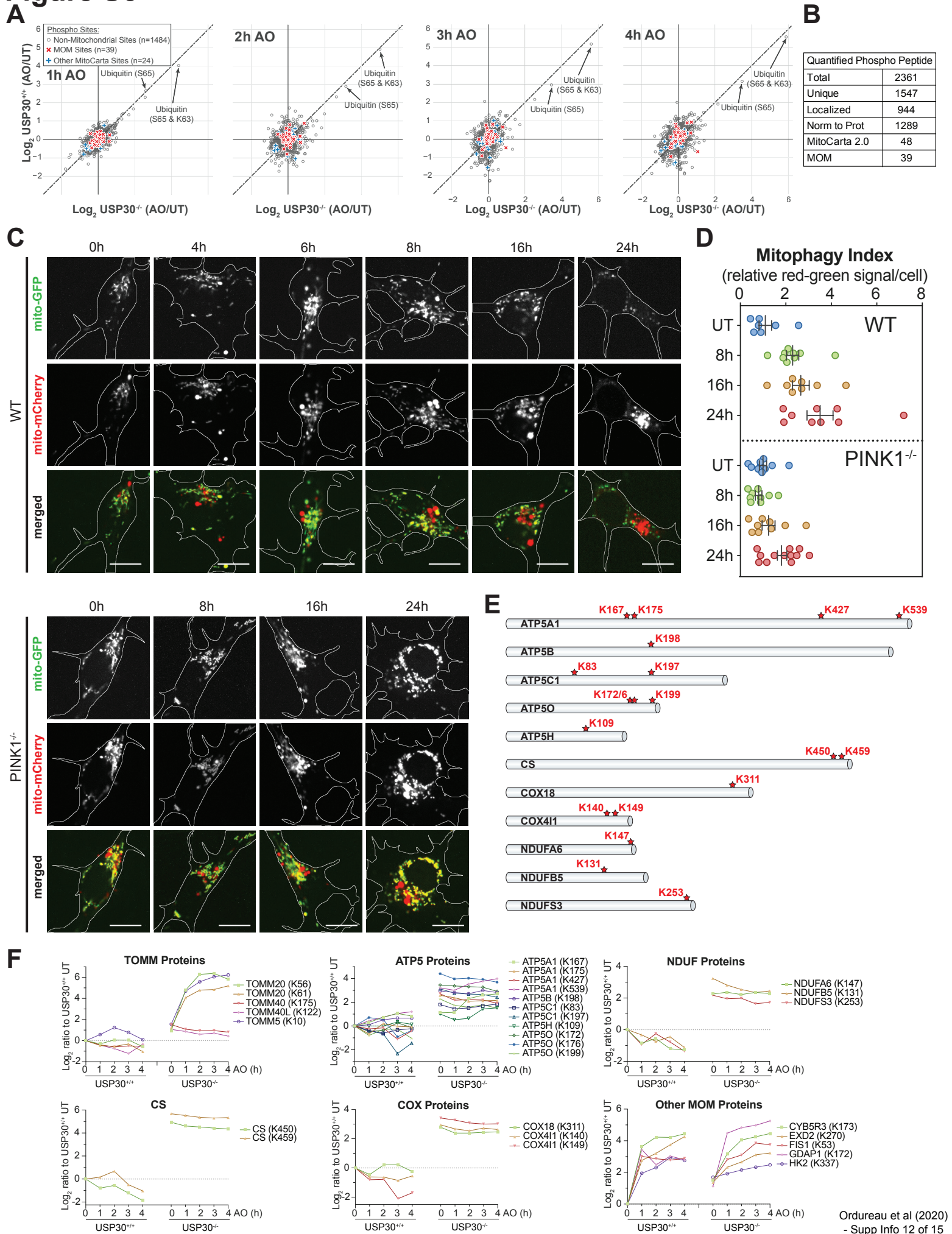


FIGURE S6. Analysis of the effect of USP30 deficiency on mitochondria in response to depolarization. Related to Figure 6.

(A) Correlation plots of phospho-peptide TMT values for USP30^{+/+} iNeurons versus USP30^{-/-} iNeurons at the indicated time points post depolarization.

(B) Summary of phospho-peptides quantified in the experiment described in **Figure 5A**.

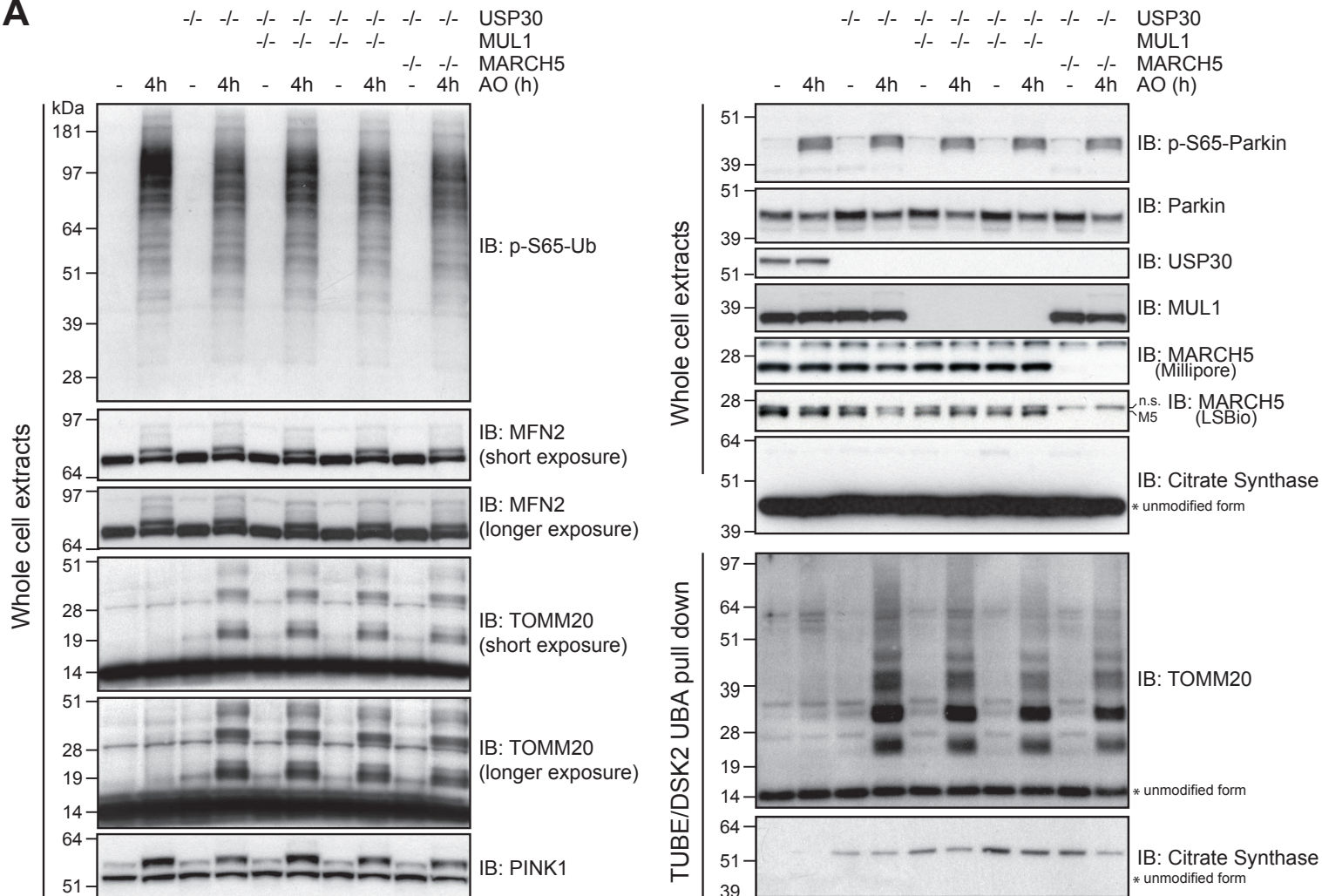
(C, D) Live-cell imaging time course in iNeurons expressing mtx-QC^{XL}. Cells were depolarized with 0.5 μ M Antimycin A/0.5 μ M Oligomycin A and cells imaged (panel C) and the mitophagic flux index (panel D) as described in **STAR METHODS**.

(E) Position(s) of the ubiquitylation site, constitutively upregulated in USP30^{-/-}, in a cohort of substrates.

(F) Ubiquitylation kinetics for individual diGLY sites that are elevated 2-fold or more in USP30^{-/-} iNeurons relative to WT cells under basal (untreated) conditions.

Figure S7

A



B

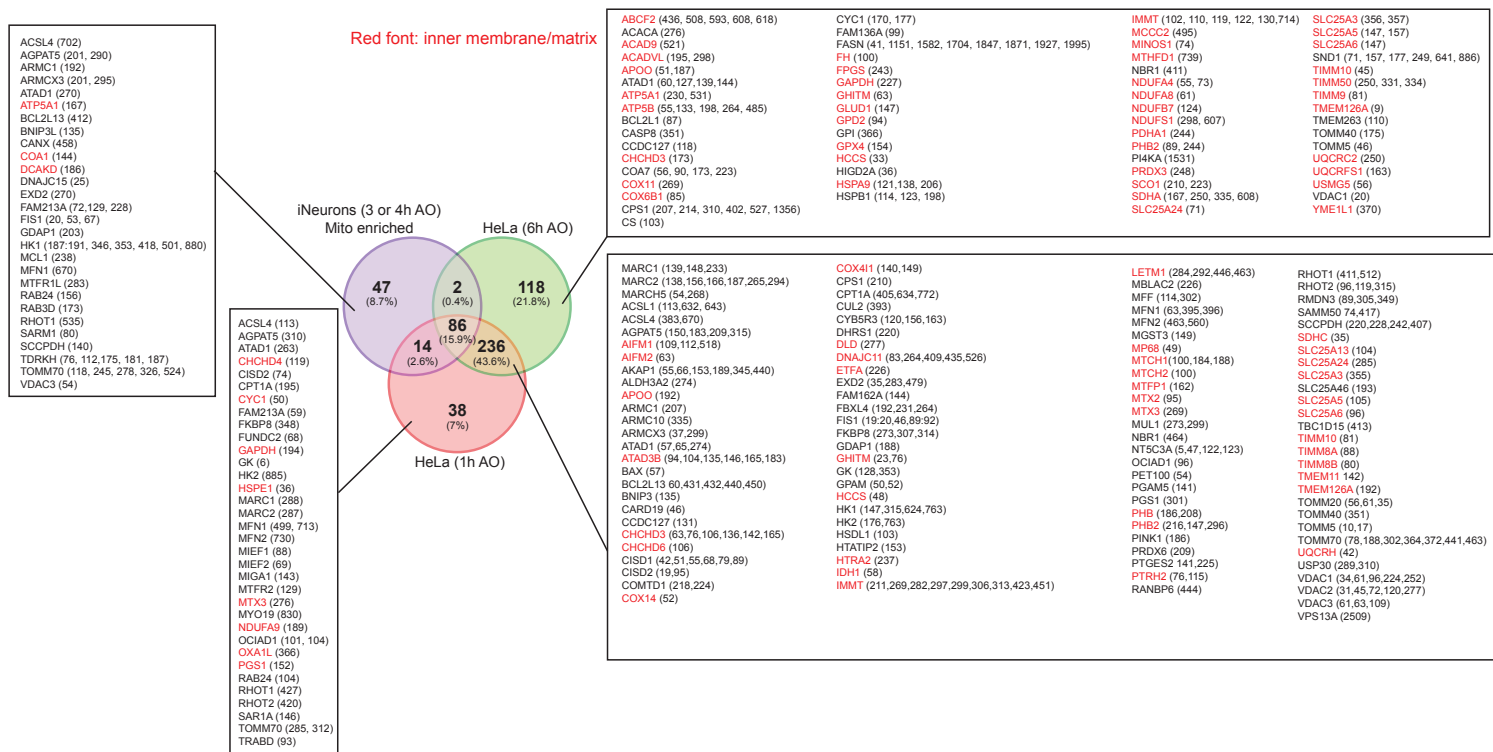


FIGURE S7. Mitochondria resident E3 ligases MUL1 and MARCH5 do not modify USP30^{-/-} phenotype and detailed landscape of Parkin-dependent ubiquitylation across cell lines. Related to Figure 7.

(A) The indicated iNeurons were either left untreated or depolarized (10 μ M Antimycin A/5 μ M Oligomycin A) for 4h prior to analysis of cell extracts by immunoblotting using the indicated antibodies. To analyze CS ubiquitylation, whole cell extracts were used for ubiquitin enrichment using immobilized ubiquitin binding domain (UBA) fusion proteins and bound proteins subjected to immunoblotting.

(B) Venn diagram (from **Figure 7B**) is expanded to show the identity of individual diGLY sites that were identified in each of the indicated experiments. The overlapping 86 proteins found in all 3 experiments are shown in **Figure 7C**. Sites in red indicate proteins located in either the inner mitochondrial membrane or the matrix.