Expanded View Figures

Figure EV1. Purification of mitochondria from mammalian cells and validation of the PINK1 antibody.

A Workflow for the preparation and purification of mitochondrial fractions.

B Quality control for the purity of the extracted mitochondria proteins. Purified mitochondrial fractions (M) and whole cell lysates (WCL) were tested for purity by immunoblotting using the antibodies indicated. For both the purified mitochondria and the whole cell lysate 10% of the total amount of cells was used for the analysis.

C PINK1 is proteolytically processed by the mitochondrial processing peptidase (MPP) and the presenilin-associated rhomboid-like protein (PARL). Domain structure of PINK1 (left panel) and representation of PINK1 species in immunoblots (right panel). MTS: mitochondrial targeting sequence; TMD: transmembrane domain.

D Validation of the PINK1 antibody in cells silenced for PINK1 expression. HEK 293T cells were transfected with control or PINK1 siRNA. 48 h after transfection, the cells were treated with CCCP (10 μM, 90 min) before harvesting. The cell lysates were analyzed by immunoblotting using the PINK1 antibody D8G3 (rabbit mAb #6946) from Cell Signaling Technology. β-Actin was used as a loading control.

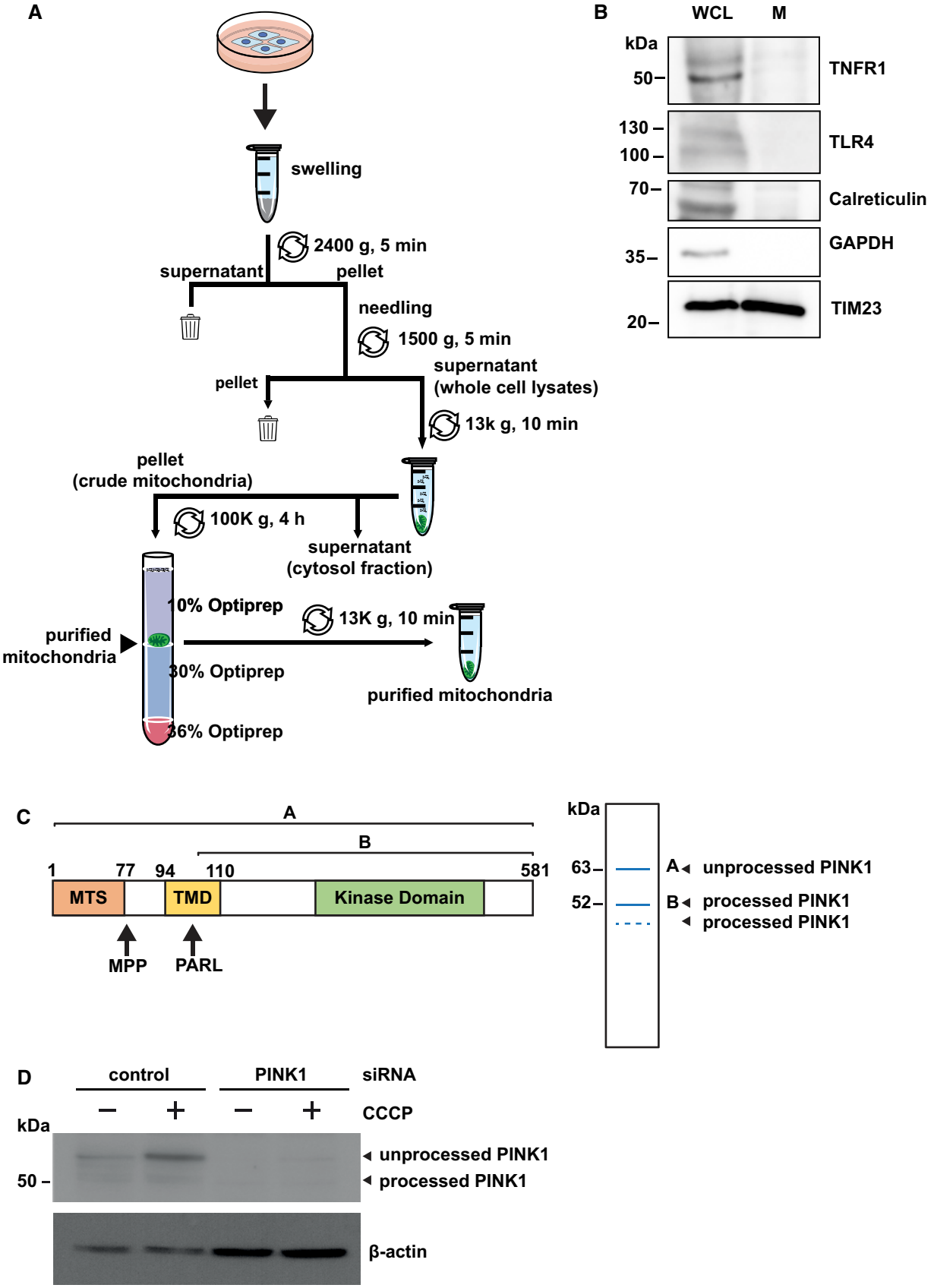

Figure EV1.

Figure EV2. TNF-induced mitochondrial membrane remodeling effect is not linked to mitophagy.

A TNF-induced PINK1 stabilization is not associated with a decrease in the mitochondrial membrane potential. HeLa cells were treated with TNF (25 ng/ml, 15 min) and the mitochondrial membrane potential was assessed by the fluorescence signal intensity ratio of TMRE and MitoTracker™ Green (MTG), One-way ANOVA, $n = 2$ (technical replicates). Each point presents the data from one cell (biological replicate). Box-and Whiskers plots: The error bars denote the standard deviation (SD), and the boxes represent the 25^{th} to 75^{th} percentiles. Whiskers: outliers not included. The vertical lines in the boxes represent the median values, whereas the square symbols in the boxes denote the respective mean values. Significance: $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

B TNF does not affect FCCP-induced translocation of Parkin to mitochondria. HeLa cells transiently expressing Parkin were treated with FCCP (10 μM) for the indicated time with or without a TNF pretreatment (25 ng/ml for 15 min). Then, the cells were fixed and analyzed by immunocytochemistry and fluorescence microscopy using Parkin and Hsp60 antibodies. Shown is the mean ± standard deviation (student's *t*-test) based on three independent experiments; at least 300 cells per condition were assessed.

C Neither TNF treatment nor OTULIN silencing increases mitophagy. SH-SY5Y cells were treated as indicated: Parkin overexpression plus FCCP 10 μM (red), FCCP 10 μM only (green), TNF 25 ng/ml (purple), or OTULIN silencing for 48 h (blue). A ratiometric analysis of fluorescence intensities was performed at the time indicated by live-cell microscopy in cells transiently expressing mt-mKeima. Shown are the mean normalized fluorescence intensities ± standard deviation measured every 5 min over 500 min. At least five cells were monitored for each condition.

D OTULIN does not affect CCCP-induced translocation of Parkin to mitochondria. HeLa cells transiently expressing Parkin or Parkin and OTULIN were treated with CCCP (10 μM) for 90 min. Then, untreated and CCCP-treated cells were fixed and analyzed by immunocytochemistry and fluorescence microscopy using Parkin and Hsp60 antibodies. Middle panel: Quantification of mitochondrial Parkin translocation. Shown is the mean ± standard deviation (student's *t*-test) based on three independent experiments. Right panel: Expression controls. Scale bar: 10 μm.

E OTULIN does not affect CCCP-induced PINK1/Parkin-dependent mitophagy. HeLa cells transiently expressing Parkin or Parkin and OTULIN were treated with CCCP (10 μM) for 24 h. Then, the cells were fixed and analyzed by immunocytochemistry and fluorescence microscopy using Parkin and Hsp60 antibodies. Middle panel: Quantification of mitochondrial clearance. Shown is the mean ± standard deviation (student's *t*-test) based on three independent experiments; at least 300 cells per condition were assessed. Right panel: Expression controls. Scale bar: 10 μm.

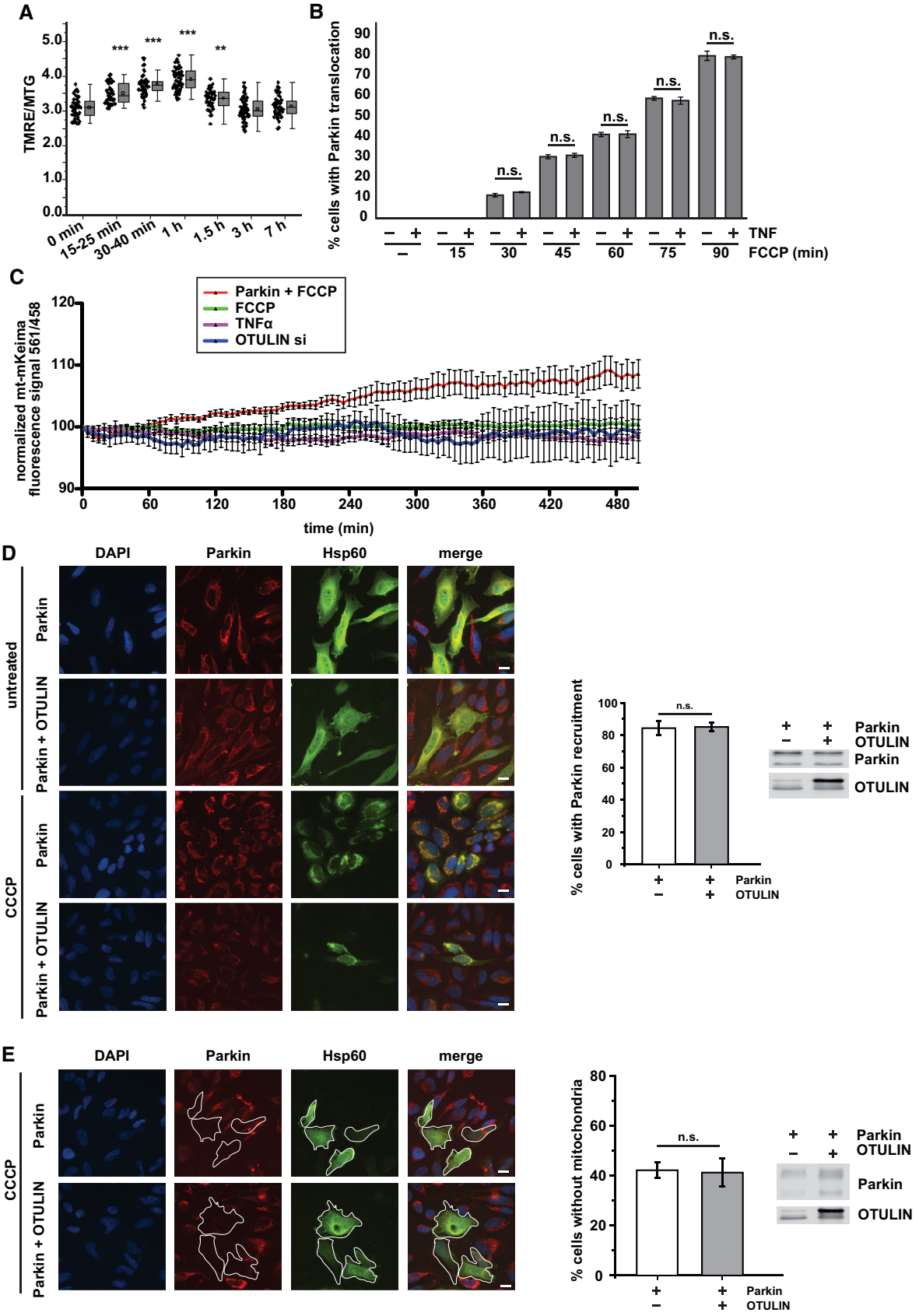

Figure EV2.

Figure EV3. PINK1 co-immunoprecipitates with HOIP and phosphorylates M1-linked tetra-ubiquitin.

A, C Schematic representation of the HOIP constructs used to map the interaction between HOIP and PINK1. All constructs are equipped with an N-terminal HA tag. IBR, in-between RING domain; LDD, linear ubiquitin chain determining domain; NZF, nuclear protein localization 4-type zinc finger domain; PUB, peptide N-glycosidase/ubiquitin-associated domain; RING, really interesting new gene; UBA, ubiquitin-associated domain; ZF, zinc finger domain.

B PINK1 co-immunoprecipitates with the N-terminal part of HOIP. HEK293T cells were transfected with V5-tagged PINK1 and the indicated HA-tagged HOIP constructs. Twenty-four hours after transfection cells were lysed under native conditions and subjected to immunoprecipitation using HA antibodies. Immunopurified proteins were analyzed by immunoblotting using V5 antibodies to detect PINK1.

D The UBA domain enhances the interaction between HOIP and PINK1. HEK293T cells were analyzed as described in B.

E PINK1 co-elutes with HOIP in cellular lysates. HEK293T cells expressing V5-tagged PINK1 were lysed, and soluble proteins were separated by size-exclusion chromatography. Fractions were collected and analyzed by immunoblotting using antibodies against HOIP and V5.

F PINK1 phosphorylates M1-linked tetra-ubiquitin *in vitro*. M1-linked tetra-ubiquitin was incubated with or without recombinant tcPINK1 in kinase buffer for 48 h. The samples were analyzed by Phos-tag™ SDS–PAGE and immunoblotting using ubiquitin antibodies.

G The efficiency of OTULIN to hydrolyze M1-linked ubiquitin is decreased in the presence of PINK1. Recombinant M1-linked tetra-ubiquitin was incubated with or without recombinant *Tribolium castaneum* tcPINK1 for 48 h. Then, recombinant OTULIN was added for the indicated time. The reaction was stopped by adding Laemmli sample buffer and the samples were analyzed by immunoblotting using ubiquitin antibodies.

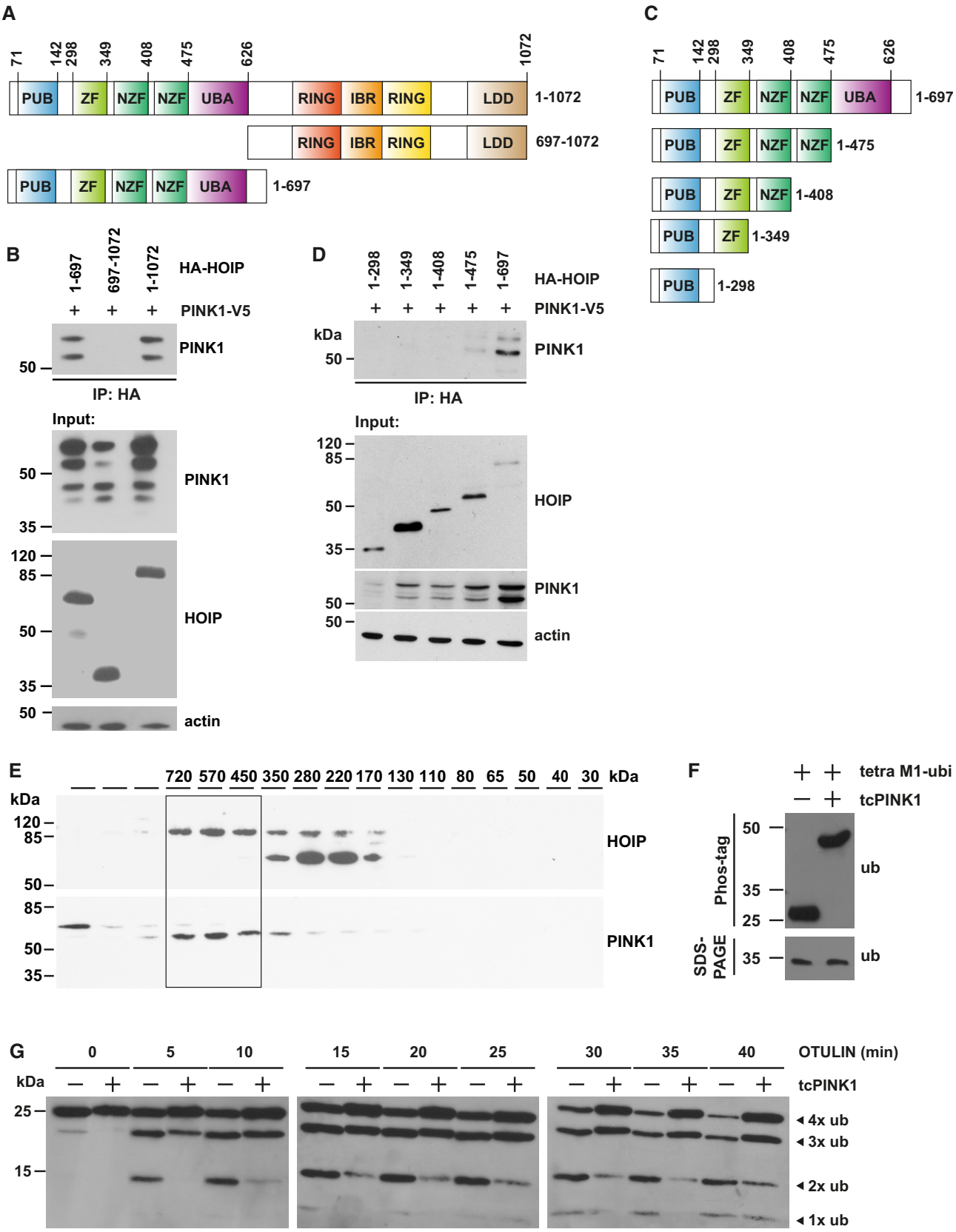

Figure EV3.

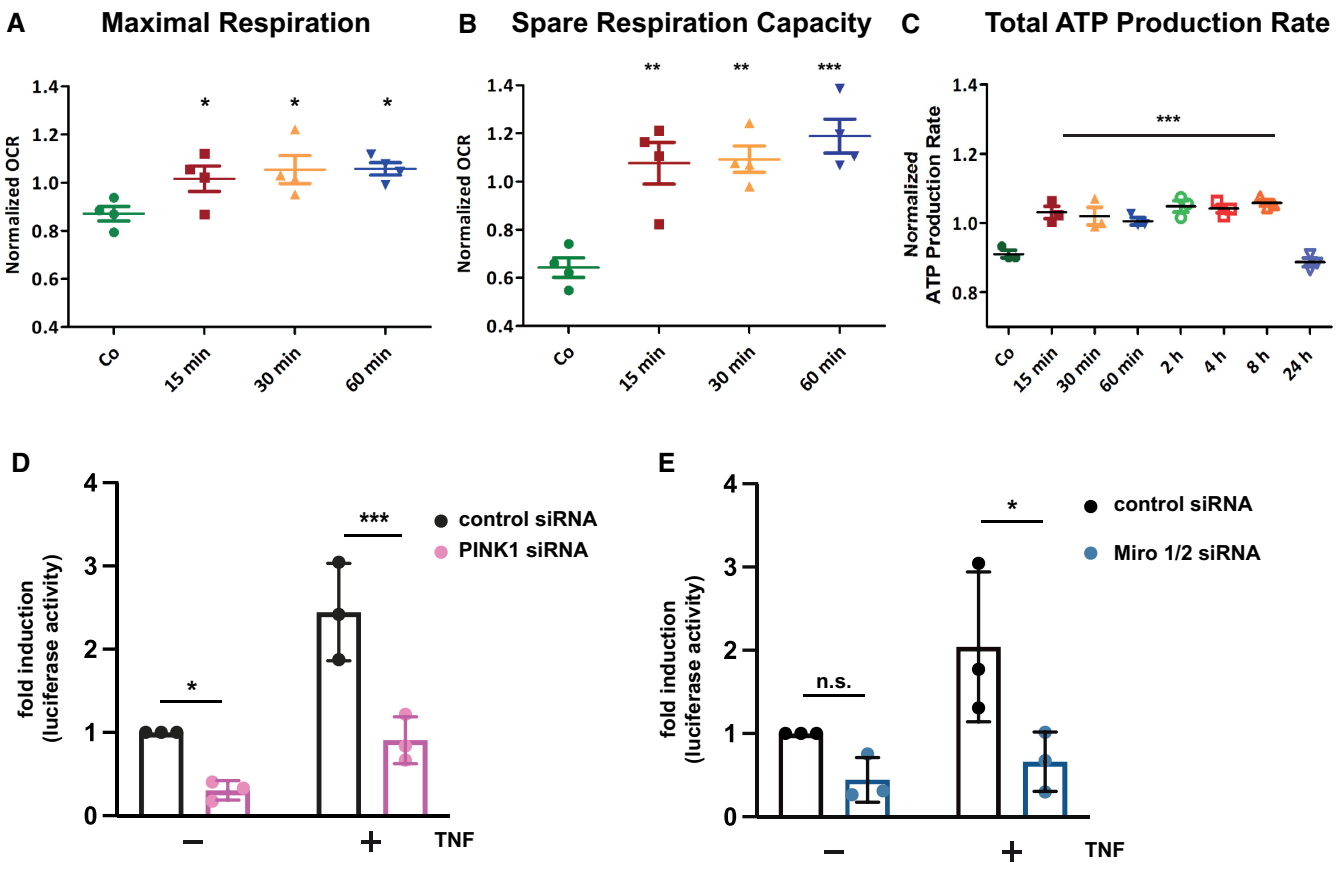

Figure EV4. TNF influences the bioenergetic profile and TNF-induced NF-κB transcriptional activity is impaired in PINK1- and Miro1/2-deficient cells.

A, B TNF increases maximal respiration (A) and spare respiratory capacity (B). HeLa cells were treated with TNF for the indicated time and analyzed by the Agilent Seahorse XF Cell Mito Stress Test. The spare respiratory capacity is the difference between maximal and basal respiration. One-way ANOVA with Tukey's multiple comparisons test, $n = 4$ independent experiments, bars represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

C TNF increases total ATP production. HeLa cells were treated with TNF for indicated time and analyzed by the Agilent Seahorse ATP Rate Assay. One-way ANOVA with Tukey's multiple comparisons test, $n = 3$ independent experiments, bars represent mean ± SEM. $^{***}P < 0.001$.

D PINK1 silencing decreases NF-κB transcriptional activity. Hela cells were treated with PINK1-specific or control siRNA. The next day, cells were transfected with an NF-κB luciferase reporter construct. Twenty-four hours after transfection, cells were treated with TNF (25 ng/ml, 3 h) harvesting. Luciferase activity was quantified in cell lysates with luciferase activity in untreated control cells set to 1. Data represent the mean with standard deviation. Sidak's multiple comparisons test, $n = 3$ independent experiments, $^{*}P < 0.05$, $^{***}P < 0.001$.

E Miro1/2 silencing decreases NF-κB transcriptional activity. Hela cells were treated with Miro 1/2-specific or control siRNA. The next day, cells were transfected with an NF-κB luciferase reporter construct and analyzed as described in (A). Data represent the mean with standard deviation. Sidak's multiple comparisons test, $n = 3$ independent experiments, n.s., not significant, $^{*}P < 0.05$.

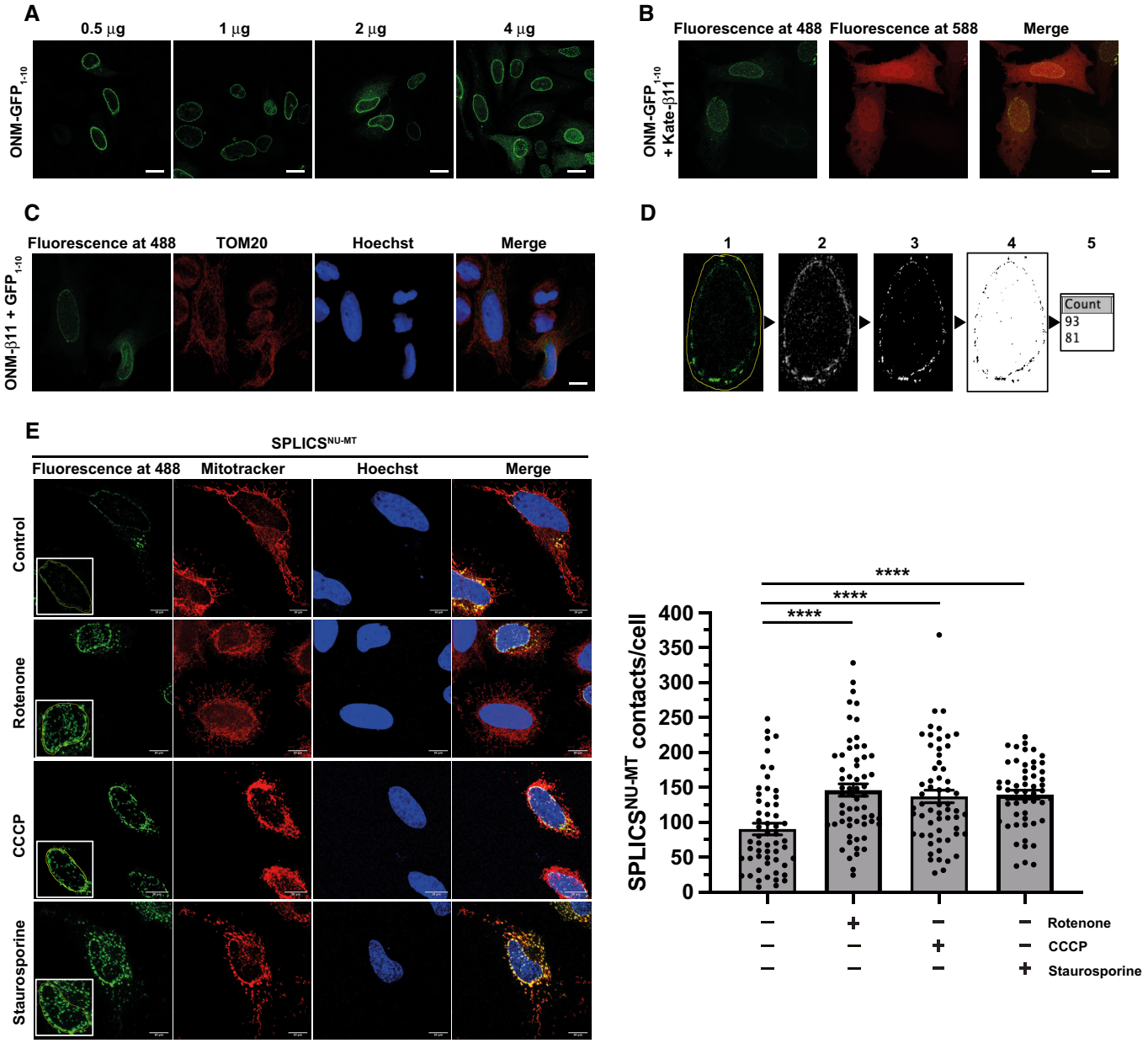

Figure EV5. Targeting, self-complementation, and ability to respond to the MRR activation of the SPLICS^{NU-MT} reporters.

A Assessment of the nuclear envelope localization of the ONM-targeted GFP_{1-10} fragment at different plasmid concentrations transfected into HeLa cells. The GFP_{1-10} fragment was stained with a mouse anti-GFP primary antibody (Santa Cruz, 1:100) followed by an anti-mouse AlexaFluor 488-conjugated secondary antibody (ThermoFisher, 1:100). Scale bar: 10 μm.

B Assessment of the self-complementation capability of the ONM-targeted GFP_{1-10} fragment with a cytosolic mKate-tagged β11. Scale bar: 10 μm.

C Nuclear envelope localization and self-complementation of the ONM-targeted β11 fragment co-transfected with an untargeted, cytosolic GFP1-10 fragment. Nuclei were stained with Hoechst33342 (ThermoFisher, 1 μg/ml). Scale bar: 10 μm.

D Schematic representation of the contact quantification process using ImageJ plugin VolumeJ and custom-made macros specifically developed for the analysis: (1) an ROI is traced around the nucleus of the cell; (2) the first macro is applied, which clears all the signal outside the ROI and convolves the signal inside; (3) VolumeJ is used to generate a rendering of the convolved signal; (4) the second custom macro is applied on the first and the last frame of the rendering; (5) the contacts per each frame are quantified and the overall number of contacts per cell is calculated by averaging the two results.

E Representative images and contact site quantification of HeLa cells after induction of the mitochondrial retrograde response (MRR) upon treatment with rotenone (5 μM for 3 h), CCCP (10 μM for 3 h) or staurosporine (1 μM for 2 h). Mitochondria were stained with MitoTracker Red CMXRos (ThermoFisher, 100 nM) in HBSS for 30 min. Nuclei were stained as described in (C). Scale bar: 10 μm. Kruskal–Wallis test followed by Dunn's multiple comparison test, $n = 54–60$ from three independent experiments, ****$P < 0.0001$. Bars represent mean ± SEM.