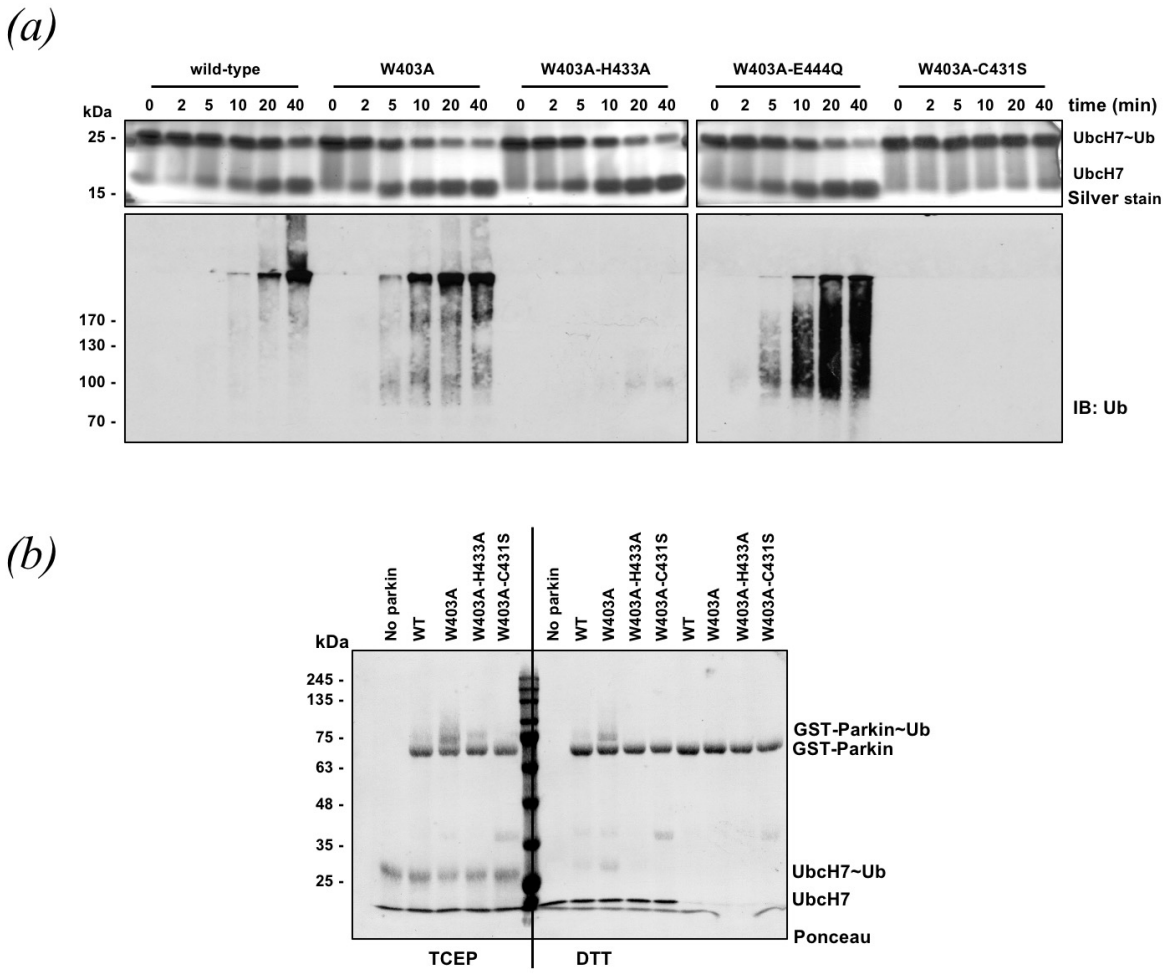

Figure S1. His433 is required for the E3 ubiquitin ligase activity of Parkin. (a) Immunoblot (bottom) and silver-stained (top) SDS-PAGE of UbcH7~Ub discharge assays with GST-Parkin mutants. Reactions were stopped with sample buffer containing tris(2-carboxyethyl)phosphine (TCEP) to reduce disulphide bonds but keep thioester bonds intact. Data for WT, W403A and C431S were originally reported in Trempe et al. (2013) **(b)** Ponceau-stained transferred membrane showing Ub-linkage after mixing non-labelled Ub, E1, ATP and UbcH7 with GST-Parkin. Incubation mixes stopped with TCEP or DTT sample buffer. The last four lanes are reaction controls without UbcH7.

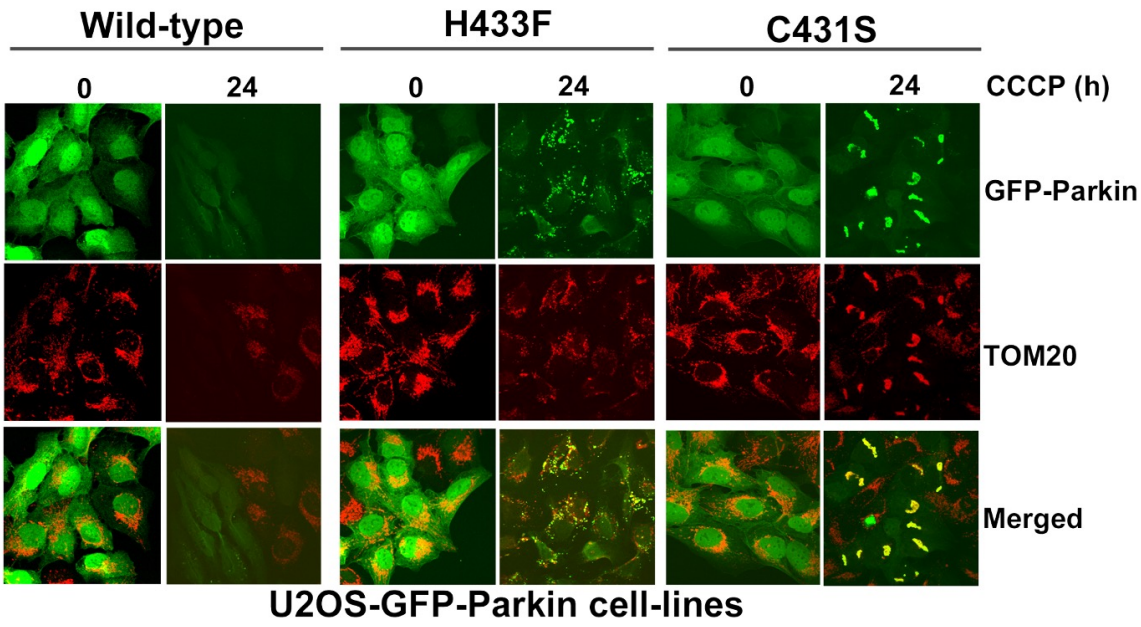

Figure S2. Representative images of CCCP-treated cells undergoing Parkin-dependent mitophagy. Confocal microscopy images taken 0 and 24 hours after addition of CCCP to U2OS cells stably expressing GFP-Parkin WT, H433F or C431S (green). Tom20 antibody was used to detect mitochondria (red). Cell transfected with WT GFP-Parkin are primarily devoid of mitochondria. H433F induces primarily puncta of mitochondria that colocalize with Parkin, while C431S induces aggregation of mitochondria and Parkin. Images were quantified and the results are showed in Figures 3(c) and 3(d).

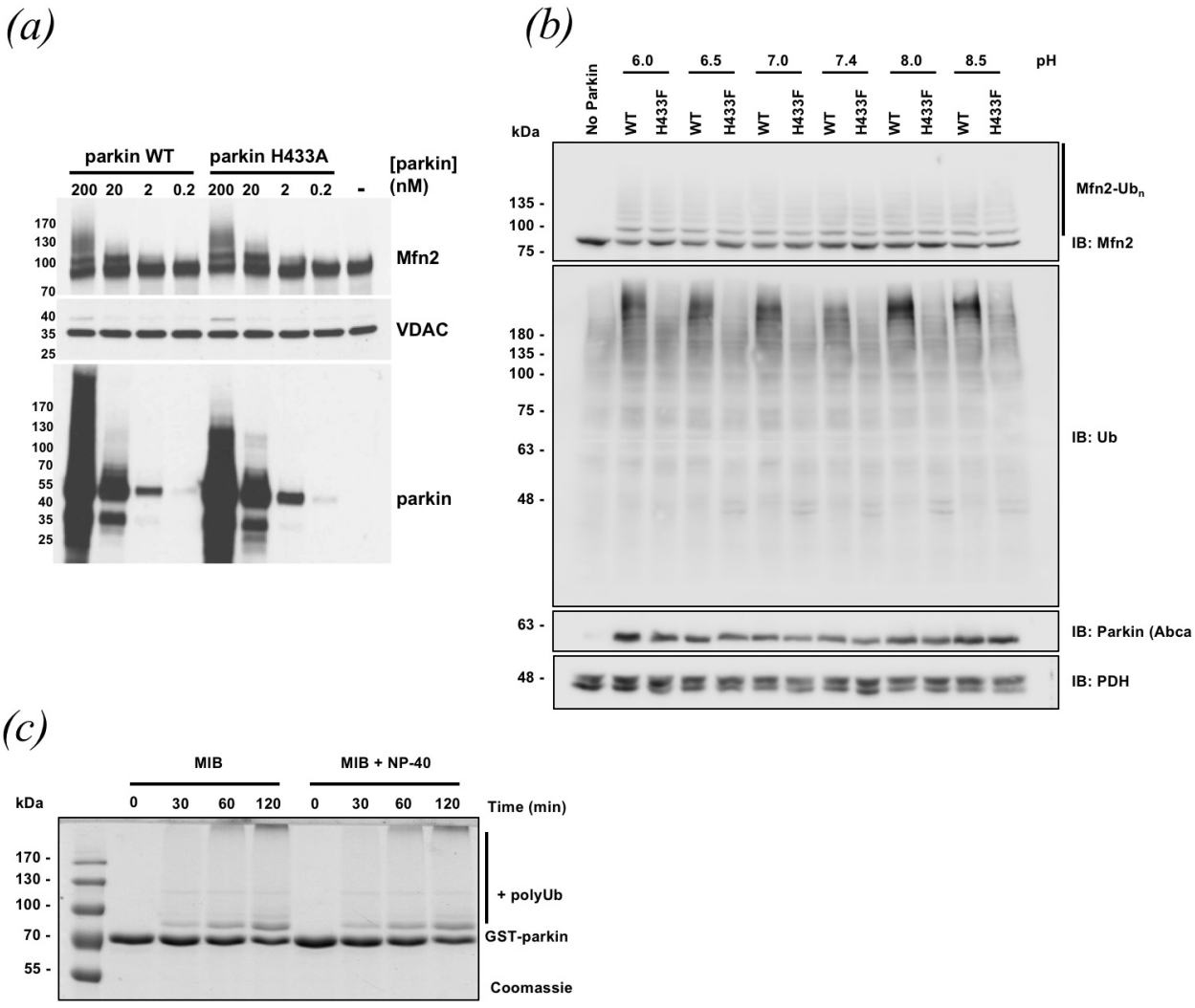

Figure S3. Mutation of Parkin His433 has differential effects on substrate ubiquitination. (a) Immunoblot of *in organello* ubiquitination assay showing that ubiquitination of Mfn2 is not affected by H433A mutation and is proportional to the amount of Parkin in the reaction. Positive ubiquitination is detected by high-molecular weight bands above the unmodified protein bands. **(b)** Immunoblot of *in organello* ubiquitination assay showing that the overall ubiquitination levels detected at high molecular weight protein bands is significantly affected when His433 is mutated, whereas Mfn2 ubiquitination is not. **(c)** CBB-stained SDS-PAGE of Parkin auto-ubiquitination assay in the presence of the non-ionic detergent NP40.

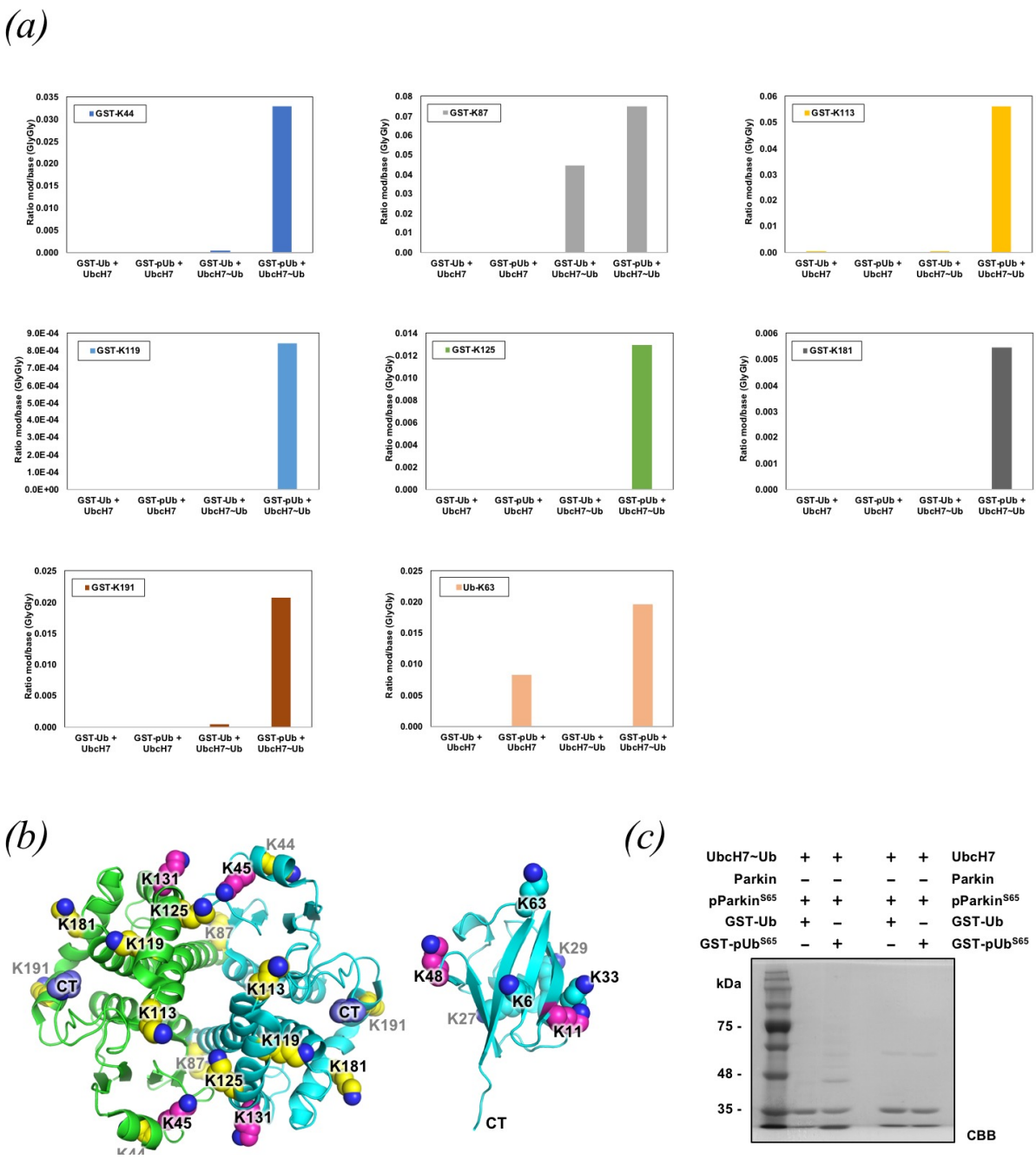

Figure S4. Parkin preferably ubiquitinates substrates tethered to phospho-ubiquitin. (a) Mass spectrometry analysis of the ubiquitination sites found after ubiquitination assay reactions where Parkin (phospho or non phospho) was incubated with UbcH7 or UbcH7~Ub and GST-Ub or GST-pUb^{S65}. **(b)** 3D structure of glutathione S-transferase (GST; PDB 6RWD, left) and ubiquitin (PDB 1UBQ, right). Target lysine side-chains are shown as spheres; major sites in magenta, minor sites in yellow. **(c)** CBB-stained SDS-PAGE of the ubiquitination assay where purified Parkin or phosphorylated Parkin (pParkin^{S65}) was incubated with UbcH7~Ub as a ubiquitin source and GST-Ub or GST-pUb^{S65} as the ubiquitin acceptor. Right lanes with UbcH7 only are reaction controls.

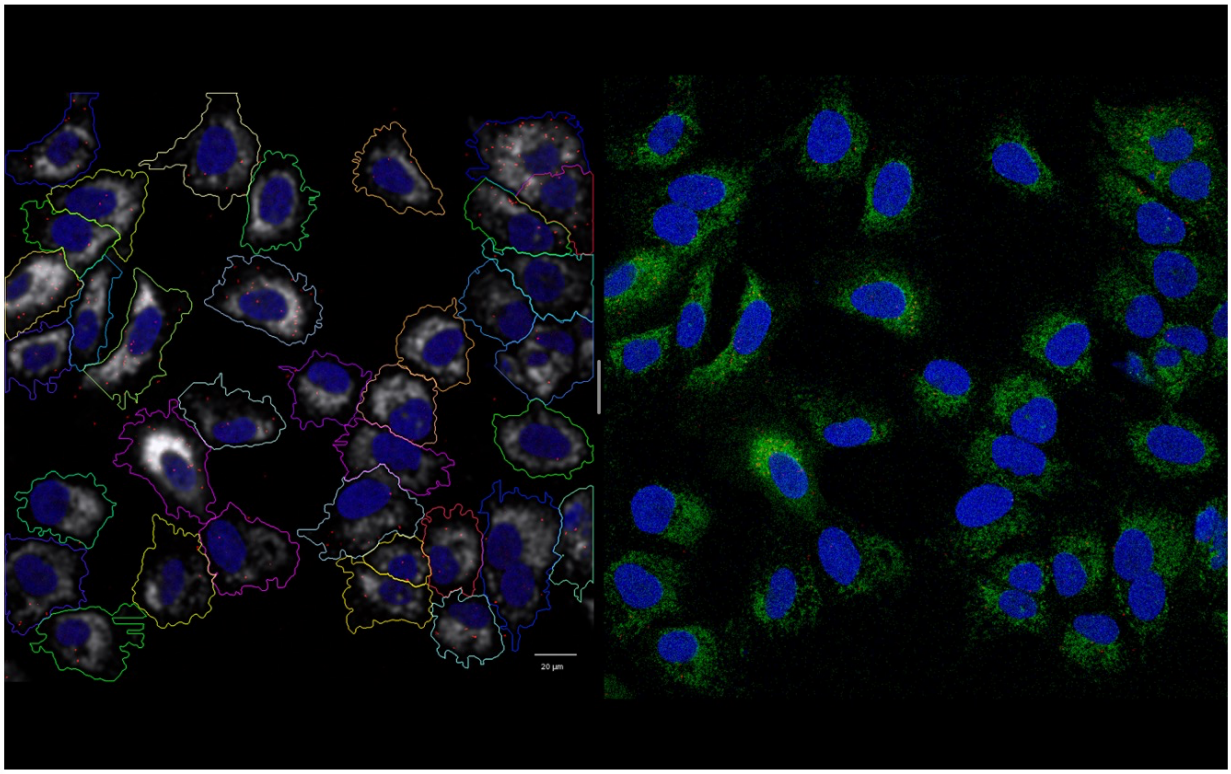

Figure S5: Defining the region of interest for quantification of proximity ligation assay (PLA) spots using confocal microscopy images. Right, original images showing the PLA signals resulting from anti-HA and and anti-Mfn2 antibodies in PINK1-HA expressing PINK1-KO cells treated with CCCP (red), anti-calnexin staining for the ER (green) and DAPI staining for the nucleus (blue). Left, images processed by the Columbus Image Analysis System (PerkinElmer), showing the region of interest (ROI) defined by the ER staining (white). PLA spots within each ROI are shown in red.

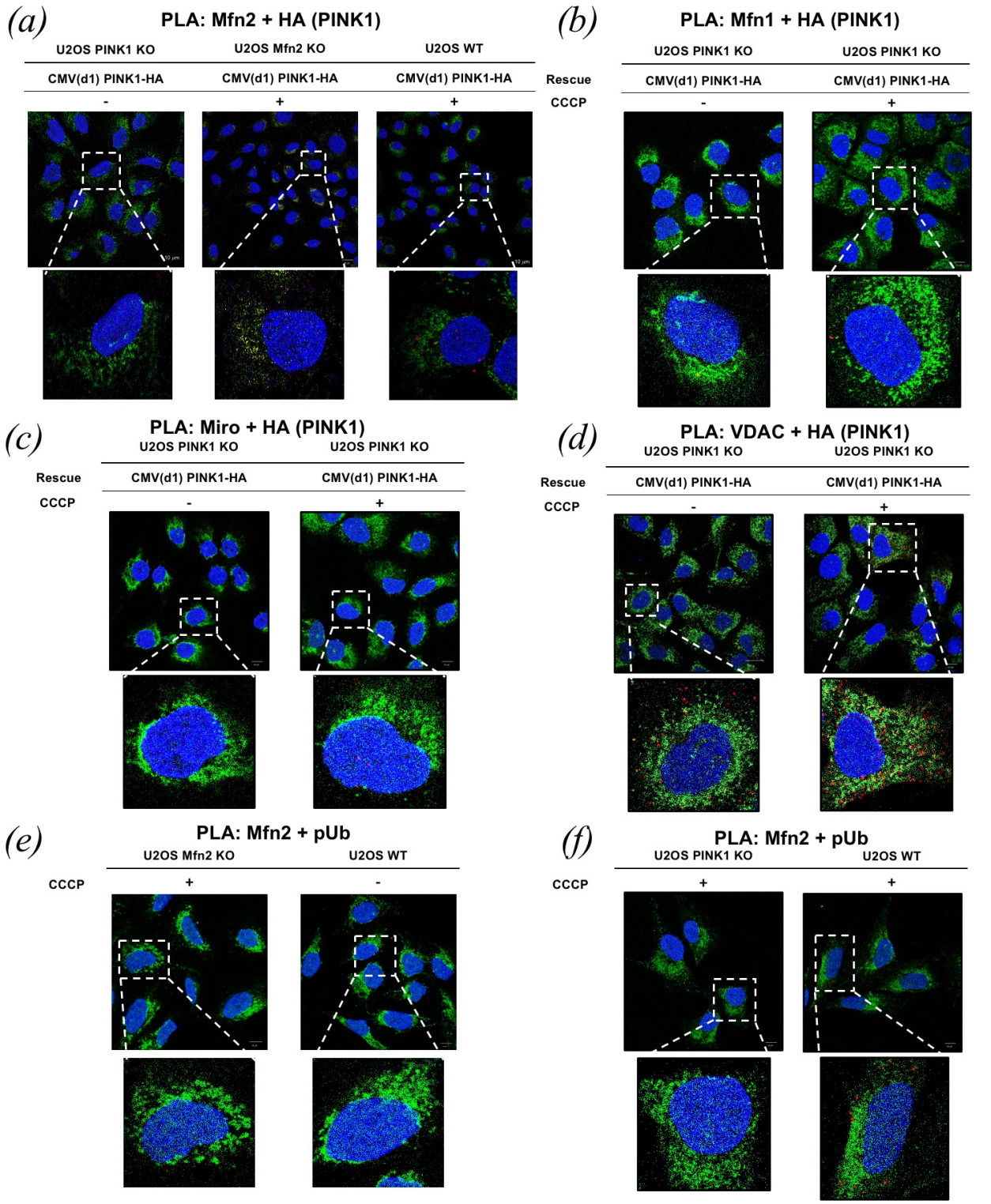

Figure S6: Proximity ligation assay confocal microscopy images to assess the co-localization of PINK1 and outer mitochondria membrane proteins on damaged mitochondria. (a) Experiment controls for PLA targeting Mfn2 and PINK1 including U2OS WT, PINK1 and Mfn2 KO cell lines expressing recombinant PINK1-HA. **(b)** PLA targeting Mfn1 and PINK1. **(c)** PLA targeting Miro and PINK1. **(d)** PLA targeting VDAC and PINK1. **(e,f)** PLA targeting targeting Mfn2 and pUb in U2OS WT and knockout cell line backgrounds.