Supplementary File for accompanying manuscript:

Ubiquitination and receptor-mediated mitophagy converge to eliminate oxidation-damaged mitochondria during hypoxia

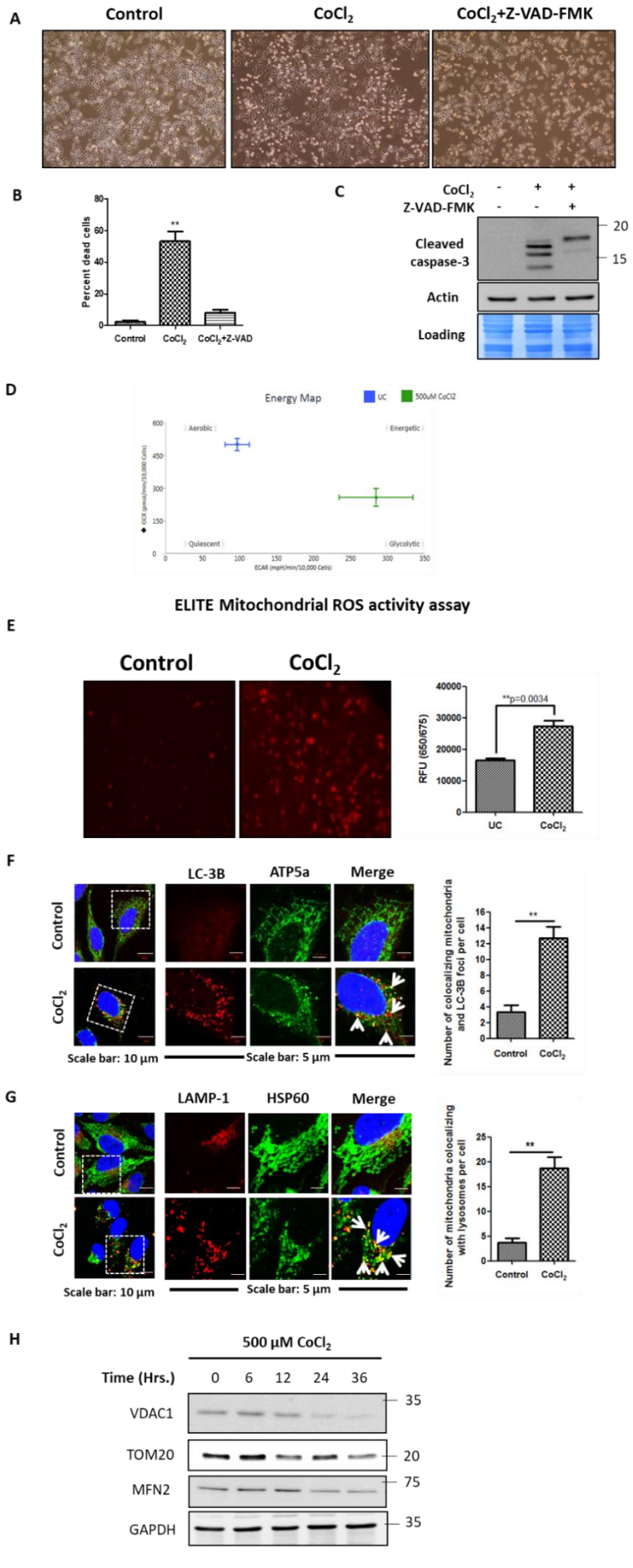
Prasad Sulkshane[a], Jonathan Ram[a], Anita Thakur[a], Noa Reis[a], Oded Kleifeld[a], and Michael H Glickman[a,*]

[a]The Faculty of Biology, Technion Israel Institute of Technology, Haifa 32000 Israel

*correspondence: glickman@technion.ac.il

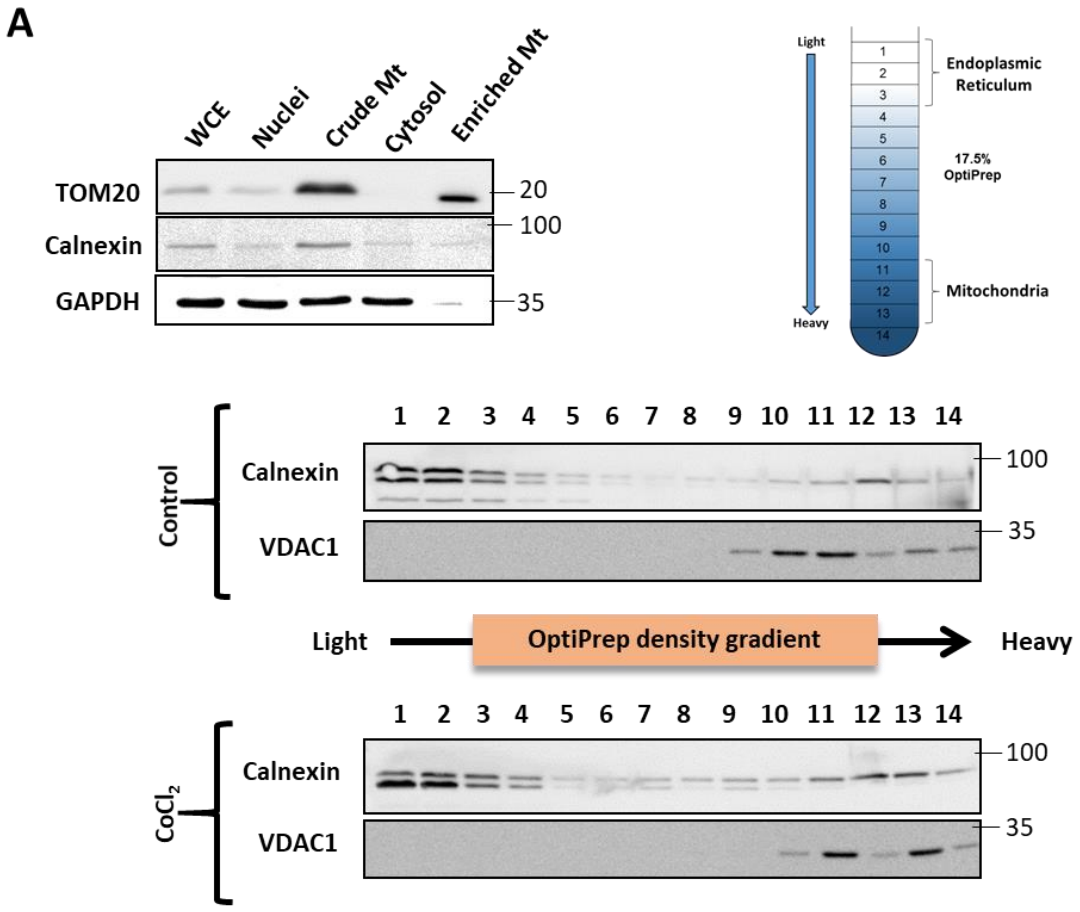
This file contains Ssupplementary Figures S1-S6 on pages 2-12

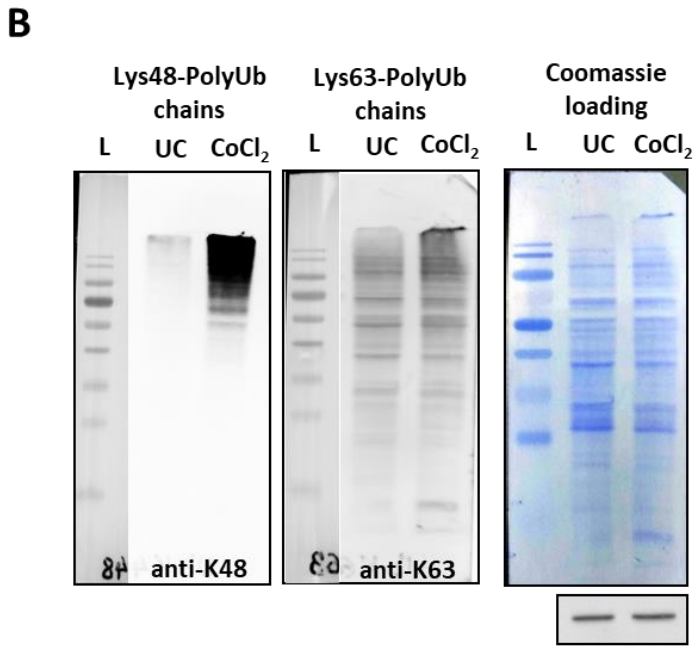
Supplementary Figure S1

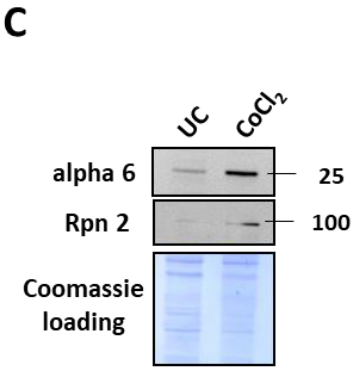


Supplementary figure S1: $CoCl_2$ limits mitochondrial respiration and can induce apoptotic cell death in HeLa cells. A. HeLa cells were treated for 24 hours with either 500 µM $CoCl_2$ alone, in combination with 25 µM pan-caspase inhibitor Z-VAD-FMK, or with vehicle control (0.05% DMSO). **B.** The percentage of cell death after 24 hours of treatment was quantified by Trypan Blue dye exclusion assay. **C.** Caspase-3 cleavage was evaluated by western blotting along with actin (loading control). The cells were treated with 500 µM $CoCl_2$ alone or in combination with 25 µM Z-VAD-FMK for 24 hours and compared to vehicle control. **D.** Mitochondrial energy production of $CoCl_2$ treated cells. A plot of Oxygen Consumption Rate (OCR) versus Extracellular Acidification Rate (ECAR) generates the energy map, representative of the metabolic status (Aerobic Vs Anaerobic). The absolute values of OCR and ECAR were normalized to the protein concentration obtained by Bradford assay for the respective wells. **E.** The mitochondrial oxidative stress was measured using the ELITE mitochondrial ROS activity assay. A representative fluorescence image shows the ROS levels in the control and $CoCl_2$ treated cells. The levels of ROS were measured on a microplate reader with 650 nm excitation and 675 nm emission wavelengths and represented by relative fluorescence units (RFU). The induction of mitophagy by $CoCl_2$ was confirmed by immunostaining and analyzing the colocalization of the mitochondrial marker proteins ATP5a and HSP60 with the **F.** autophagosomal (LC-3B) and **G.** lysosomal (LAMP-1) markers. **H.** The protein levels of the mitochondrial marker proteins VDAC1, TOM20 and MFN2 were evaluated in the lysates of HeLa cells treated with 500 µM $CoCl_2$ for the indicated time points.

Supplementary Figure S2

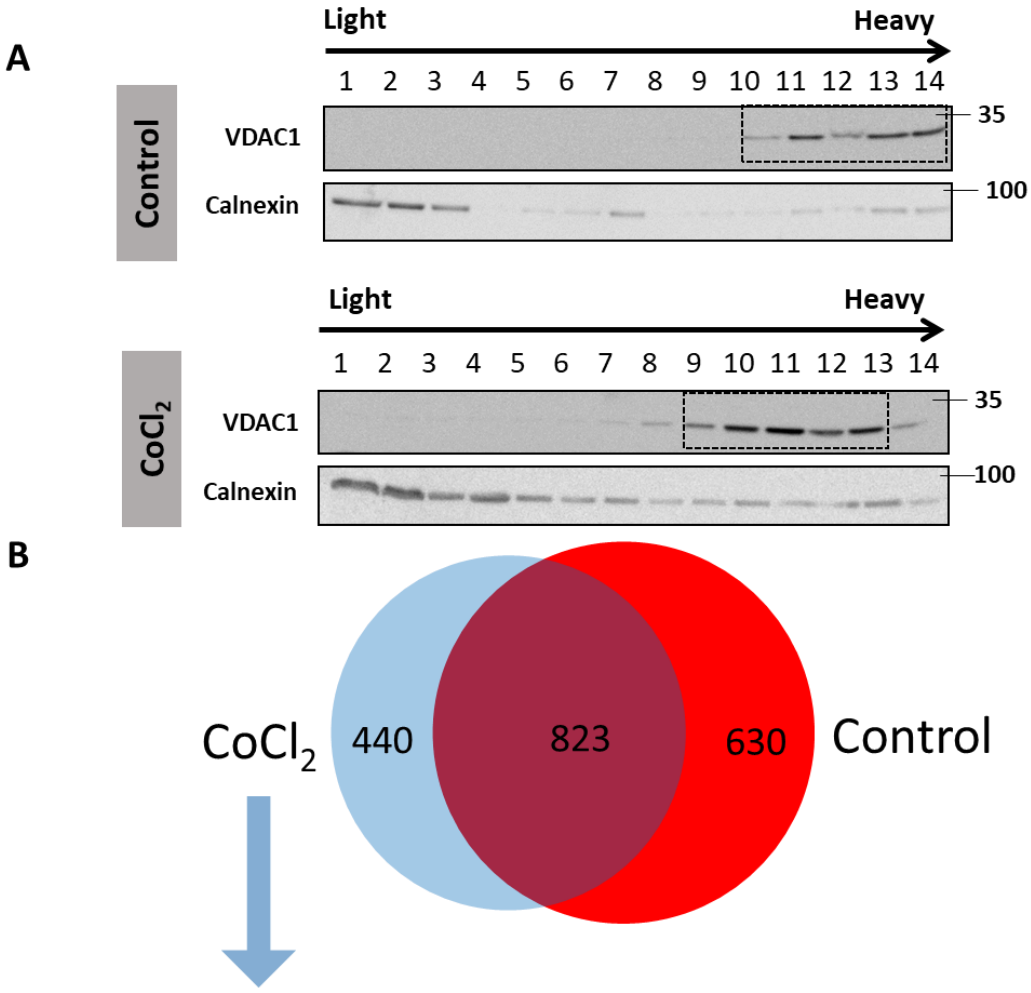






Supplementary figure S2: $CoCl_2$ treatment leads to extensive mitochondrial ubiquitination. A. Whole cell extracts of HeLa cells were fractionated by differential centrifugation and the various subcellular fractions obtained during the mitochondria isolation were subjected to western blotting for TOM20 (Mitochondrial marker), Calnexin (Endoplasmic Reticulum marker) and GAPDH (Cytosolic marker). The Crude mitochondrial fraction obtained from the differential centrifugation was subjected to OptiPrep density gradient ultracentrifugation. The individual fractions obtained after the ultracentrifugation were evaluated for presence of mitochondria by western blotting for Calnexin and VDAC1 (Mitochondrial marker). **B.** Equal amounts of the enriched mitochondrial fractions from control and $CoCl_2$-treated cells were subjected to western blotting for the detection of total ubiquitin, Lysine 48 (K48)-linked ubiquitin and Lysine 63 (K63)-linked ubiquitin. ATP5a served as the loading control for the enriched mitochondrial fractions. **C.** The mitochondrial fractions were probed with antibodies against the proteasomal subunits alpha 6 and Rpn 2.

Supplementary Figure S3

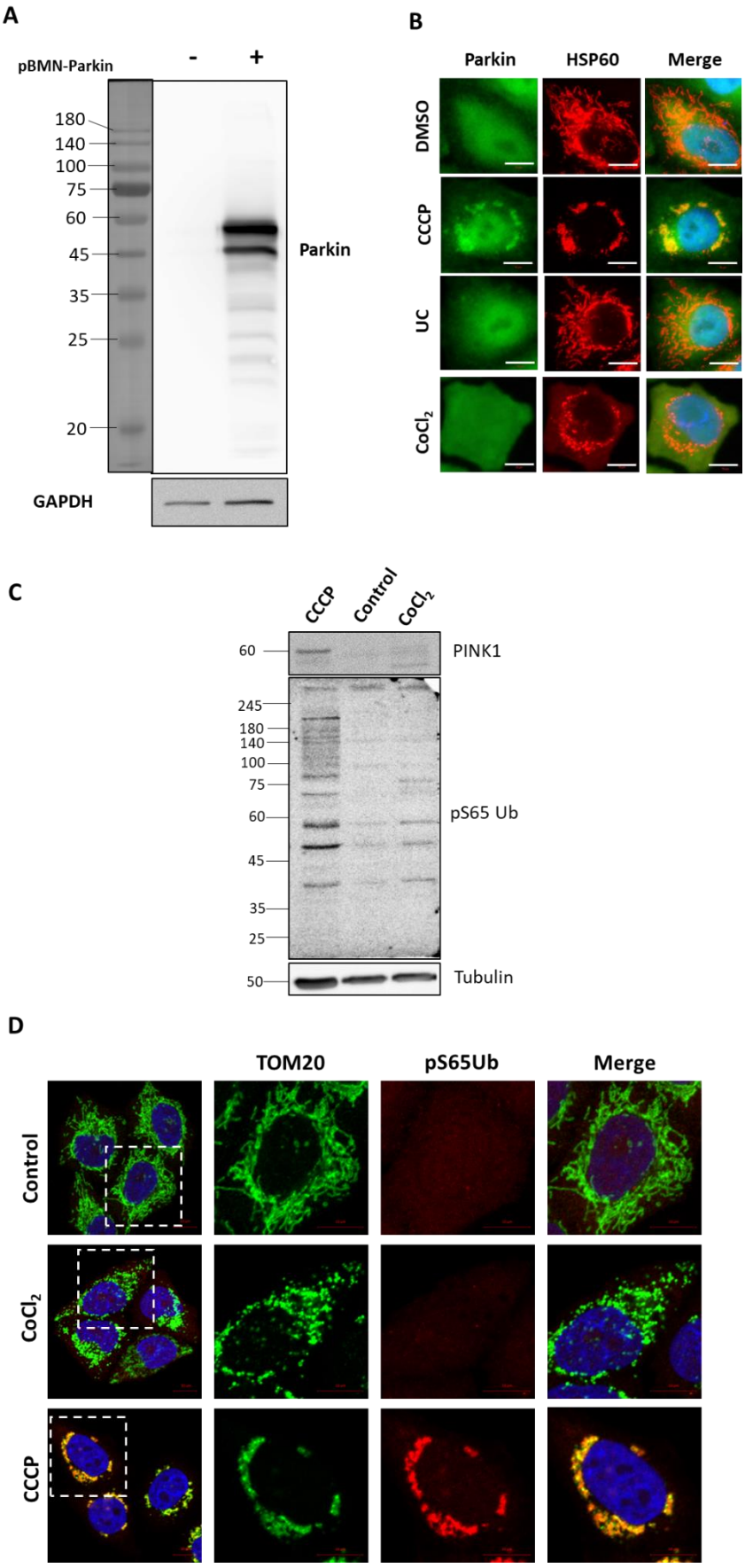


Unique UPS components identified only with mitochondria from $CoCl_2$ treated cells

Protein	Significance
HUWE1	E3 Ubiquitin ligase
UBR4	E3 Ubiquitin ligase
TRIM25	E3 Ubiquitin ligase
MARCH5	E3 Ubiquitin ligase
ZNF598	E3 Ubiquitin ligase
UBA1	Ubiquitin activating enzyme
UBE2S	Ubiquitin conjugating enzyme
SQSTM1	Ubiquitin-binding protein p62
ZFAND5	Stimulates 26S Proteasome to degrade Ubiquitinated proteins
USP33	Deubiquitinating enzyme
Proteasome subunits	Proteolysis
DDB1/XAP1	Integral component of a Cullin-RING E3 ligase
DMAC2/ATP5SL	Involved in SCF-dependent target protein Ubiquitination
VCP/p97	Required for extraction of membrane-associated proteins
UBXN4/UBXD2	Interacts with p97

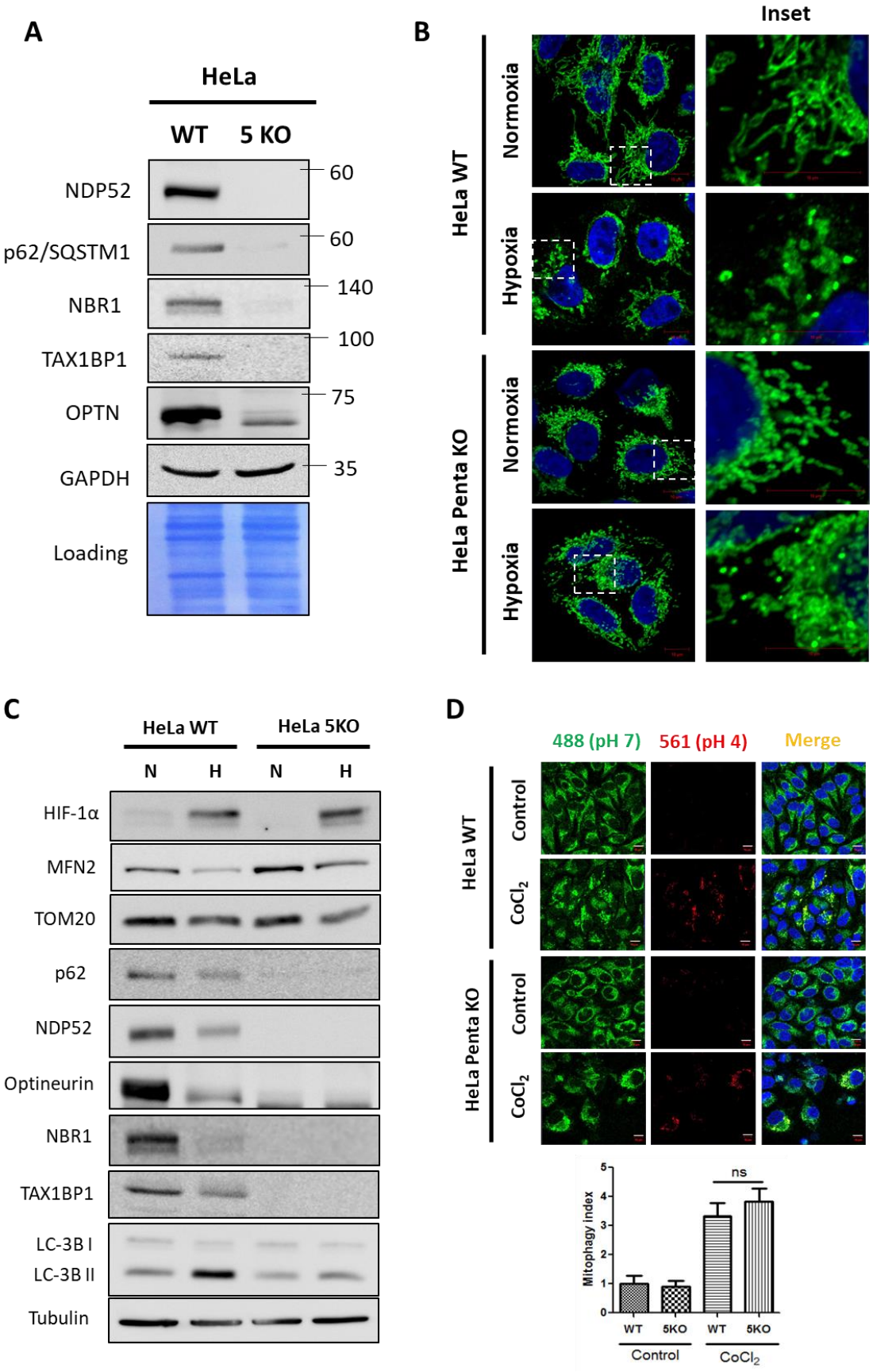
Supplementary figure S3: Proteins selectively enriched in the mitochondrial fractions of $CoCl_2$-treated HeLa cells. **A.** Isolation of mitochondria from control and $CoCl_2$-treated HeLa cells. Equal amounts of the mitochondria-enriched fractions from control and $CoCl_2$-treated HeLa cells were subjected to SDS PAGE followed by Mass spectrometry. **B.** List of select proteins closely associated with UPS specifically identified only in the enriched mitochondrial fractions of $CoCl_2$-treated HeLa cells. Only unique hits related to the ubiquitin system identified only in isolated mitochondria following $CoCl_2$ (undetected in mitochondria isolated from untreated control cells) are displayed.

Supplementary Figure S4



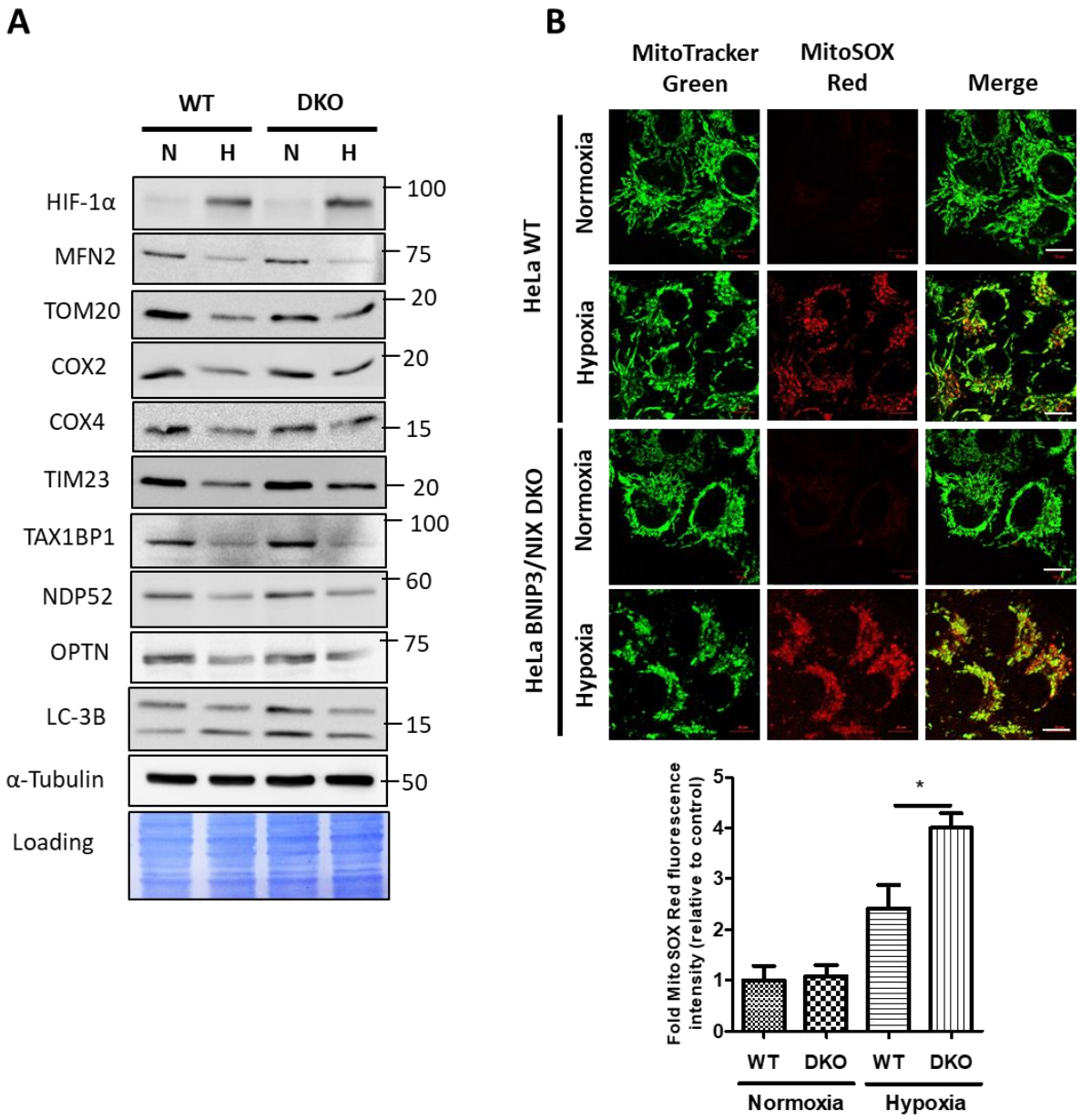
Supplementary figure S4: $CoCl_2$-induced mitophagy occurs independent of the PINK1-Parkin pathway. A. Wild type HeLa cells do not express Parkin protein. Whole cell extracts from HeLa cells (left lane) and from a HeLa line stably expressing untagged Parkin generated by viral transduction (right lane) were resolved by SDS-PAGE and immunoblotted with anti-Parkin antibody. **B.** HeLa cells stably expressing Parkin were treated either with 10 µM CCCP for 4 hours, 500 µM $CoCl_2$ and 25 µM Z-VAD-FMK for 24 hours with vehicle control (DMSO) for 24 hours or left untreated as control (UC). The cells were immunostained with anti-Parkin and anti-HSP60 antibodies and visualized using a confocal microscope to evaluate the extent of Parkin translocation to mitochondria. Scale bar: 10 µm. **C.** The whole cell lysates of wild type HeLa cells treated with either CCCP or $CoCl_2$ were subjected to western blotting for the detection of PINK1, phospho-Ubiquitin (pS65 Ub) and compared to untreated cells (control). Tubulin levels serve as a loading control (bottom). **D.** HeLa cells overexpressing Parkin were treated with either CCCP or $CoCl_2$ followed by immunostaining with TOM20 and phospho-Ubiquitin (pS65 Ub) antibodies. The images were acquired under a confocal microscope. Scale bar: 10 µm.

Supplementary Figure S5



Supplementary Figure S5: The ubiquitin-binding autophagy receptors are dispensable for $CoCl_2$-induced mitophagy. A. Validation of the penta-knockout (5KO) cell line lacking five ubiquitin-binding autophagy recptors. Equal amounts of the whole cell lysates of HeLa wild type and penta knockout (5KO) cell lines were subjected to immunoblotting with antibodies specific for the five known ubiquitin binding autophagy receptor proteins autophagy (NDP52, SQSTM1, NBR1, TAX1BP1 and Optineurin). GAPDH and Coomassie stained blot serve as loading controls. **B.** The wild type (WT) and Penta knockout (5KO) cells were exposed to 1% O_2 (Hypoxia) for 24 hours or grown under routine conditions (normoxia). The cells were immunostained with HSP60 antibody. **C.** The whole cell lysates of WT and 5KO cells exposed to hypoxia (H) or normoxia (N) were immunoblotted for the indicated proteins. **D.** The WT and 5KO cells stably expressing Mitokeima were exposed to 500 µM $CoCl_2$ with 25 µM Z-VAD-FMK for 24 hours followed by live-cell imaging to evaluate the extent of active mitophagy. The mitophagy index was calculated as described in the methods section. ns: not significant. Scale bar: 10 µm.

Supplementary Figure S6



Supplementary Figure S6: BNIP3-NIX are critical for ROS-induced mitophagy. HeLa wild type (WT) and BNIP3-NIX double knockout (DKO) cells were either exposed to 1% O_2 for 24 hours (Hypoxia-H) or grown under normal conditions with 21% O_2 (Normoxia-N). **A.** The whole cell extracts were immunoblotted for the detection of the indicated proteins. **B.** At the end of the treatment period, the cells were stained with MitoSOX Red and MitoTracker Green dyes followed by imaging. The extent of mitochondrial ROS was evaluated as discussed earlier. Data represented as mean+SEM of 3 experiments. Scale bar: 10 μm.