Expanded View Figures

Figure EV1. Mitophagy activation stimulates mitochondrial PA and DAG production.

A Hela cells were transfected with the Spo20p-GFP PA reporter and mCherry-Parkin followed by CCCP treatment for 5.5 h. Note that CCCP treatment led to the recruitment of Spo20p-GFP to Parkin-positive mitochondria. Scale bar = 10 μM.

B, C HeLa cells were transfected with Raf1-PABD-mCherry PA reporter or YFP-DAGR with Parkin-FLAG followed by antimycin (4 μM) and oligomycin (10 μM; O + A) treatment to activate mitophagy. Note that Raf1-PABD-mCherry PA reporter (B) and YFP-DAGR (C) both translocated to mitochondria (TOM20) after the treatment. Scale bar = 10 μM.

D Hela cells expressing KR-dMito and EBFP2-Parkin (cyan) were illuminated at 559 nm to induce mitophagy. Images were acquired at indicated time points after illumination. Note that PA accumulation on Parkin-positive mitochondria is induced by photodamage (t = 94 min and 112 min). Arrowheads point to the co-accumulation of Parkin and the PA reporter on damaged mitochondria. Scale bar = 10 μM.

E, F SH-SY5Y cells were transfected with (E) the Raf1-PABD-GFP PA reporter or (F) YFP-DAGR followed by antimycin (4 μM) and oligomycin (10 μM) or DMSO treatment, as indicated. Both reporters became enriched in the mitochondria (TOM20). Line scan analysis (Image J software) under the images indicates colocalization between the lipid reporters (green) and mitochondria (red) corresponding to the lines drawn in the images. Scale bar = 10 μm.

G Sh-Sy5Y cells were transfected with control (siNC) or Parkin siRNA (siParkin) followed by the PA-GFP reporter and treated with CCCP (5.5 h). Note that Parkin KD prevented mitochondrial (COX IV) aggregation and PA concentration. Scale bar = 10 μM.

H Immunoblotting confirmed Parkin KD's efficiency.

I, J Hela cells were transfected with PA reporter and DAGR-YFP without Parkin, by CCCP treatment, as indicated. Note that there is no accumulation of PA and DAGR reporters on mitochondria after CCCP treatment (by a Tom20 antibody). Scale bar = 10 μM.

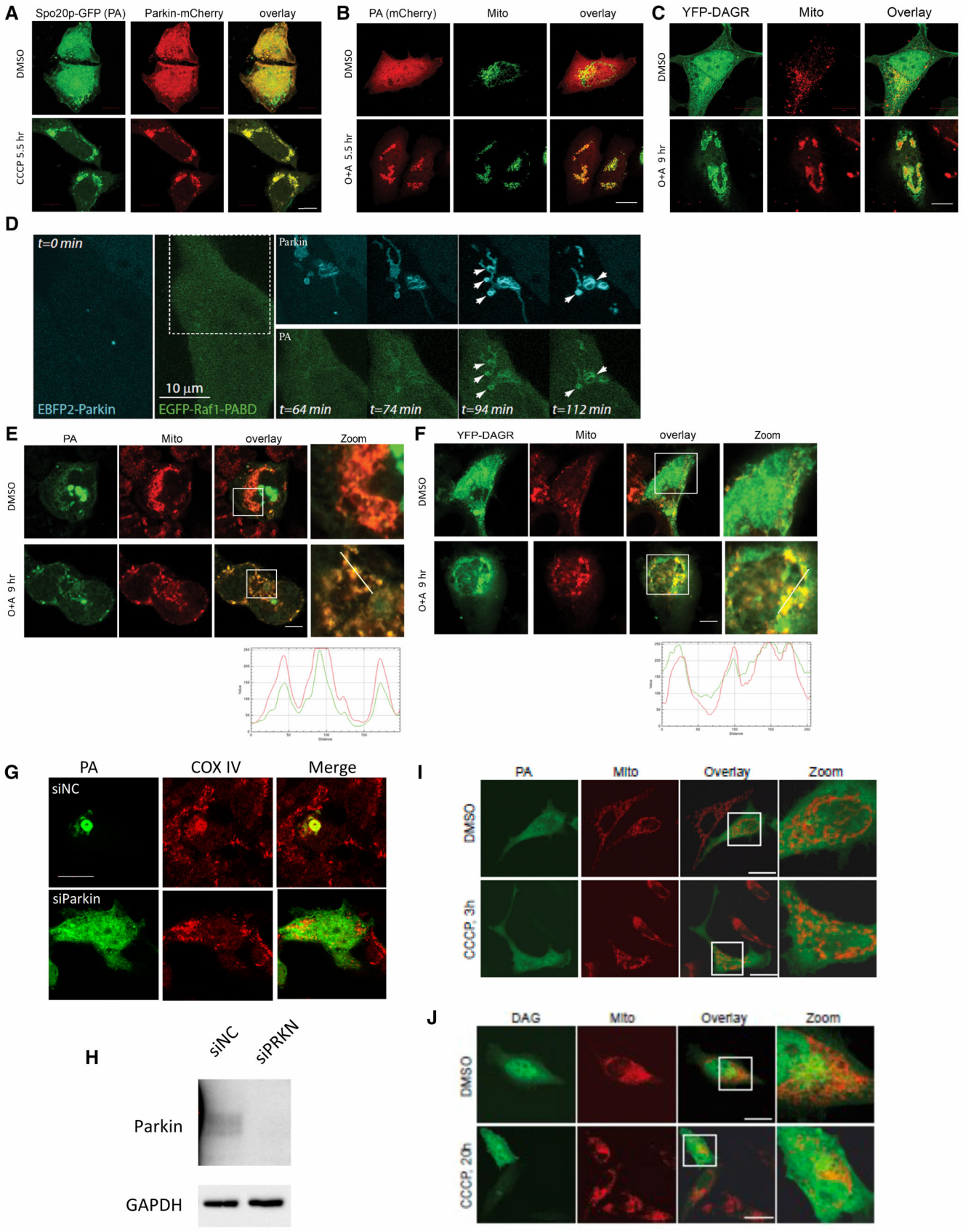

Figure EV1.

Figure EV2. PLD1 is not translocated to mitochondria, and Lipin-1 is dispensable for PA production.

A Hela cells were transfected with YFP-PLD1 and mCherry-Parkin followed by CCCP (10 μM) treatment at indicated time points. Note that PLD1 is not translocated to mitochondria upon CCCP treatment. Scale bar = 10 μM.

B Hela cells were transfected with YFP-PLD2 and mCherry-Parkin, followed by CCCP or oligomycin/antimycin treatment for the indicated time. Note that PLD2 became colocalized with Parkin and mitochondria (TOM20) under both treatment conditions. Scale bar = 10 μM.

C HEK-293T cells expressing PLD2-YFP and FLAG-Parkin cDNA were treated with CCCP for 5.5 h. Parkin was then pulled down by a FLAG antibody from cell lysates and blotted with a PLD2 antibody. Note that PLD2 interaction with Parkin was enhanced by CCCP.

D–G Hela cells were transfected with siRNA for PLD2 followed by expression plasmids for mCherry-Parkin and the PA or GFP reporter, and subject to DMSO or CCCP treatment, as indicated. PLD2 knockdown inhibited both mitochondrial PA reporter (D-E) and DAG reporter accumulation (F). $n = 3$ biological replicated. The bars indicate mean ± SEM. PLD2 knockdown efficiency was confirmed by immunoblotting (G).

H Hela cells were transfected with Lipin-1 siRNA followed by plasmids for FLAG-Parkin and the PA reporter and treated with DMSO or CCCP, as indicated. Note that PA accumulated on mitochondria (TOM20) in Lipin-1 knockdown cells. Scale bar is 25 μM, and zoom is 3×.

I Hela cells stably expressing mCherry-Parkin were treated with PLD2 inhibitor (VU0364739, 3 μM) and subjected to CCCP (10 μM) and Bafilomycin A1 (0.1 μM, lysosomal inhibitor) for 18 h. Note that PLD2 inhibition rescued mitochondrial protein degradation. The band intensity of mitochondrial proteins relative to control untreated conditions was determined by the Image J. software.

J Hela cells were transfected with Lipin-1 siRNA followed by plasmids for FLAG-Parkin, DAGR-YFP, and Lipin-1-WT or Lipin-1-catalytic dead (CD) mutant and treated with CCCP (5.5 h), as indicated. Note that Lipin-1-WT, but not the CD mutant, restored mitochondrial DAGR-YFP accumulation induced by CCCP. Scale bar: 10 mm.

K Lipin1 knockdown and overexpression efficiency was confirmed by immunoblotting (G).

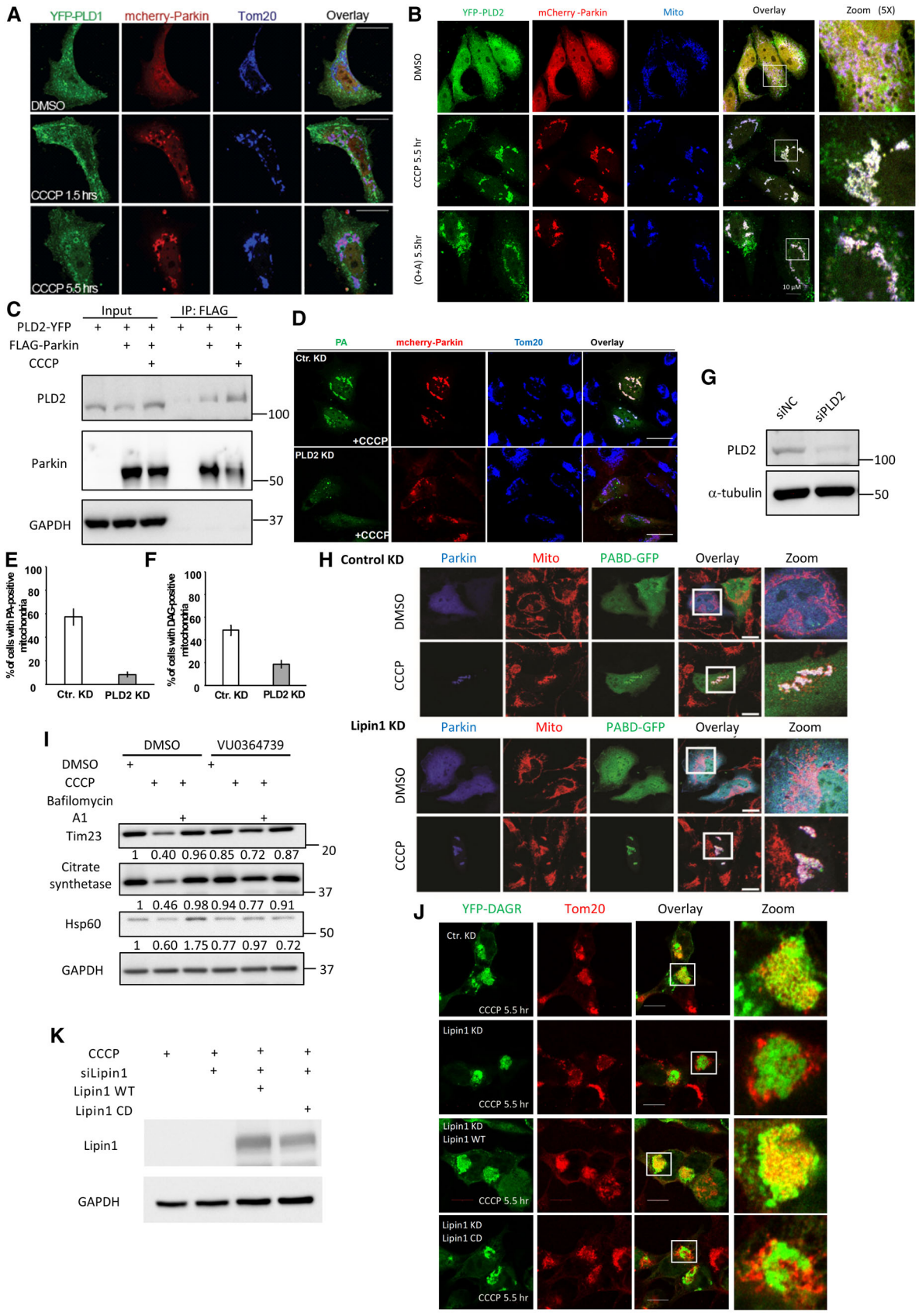

Figure EV2.

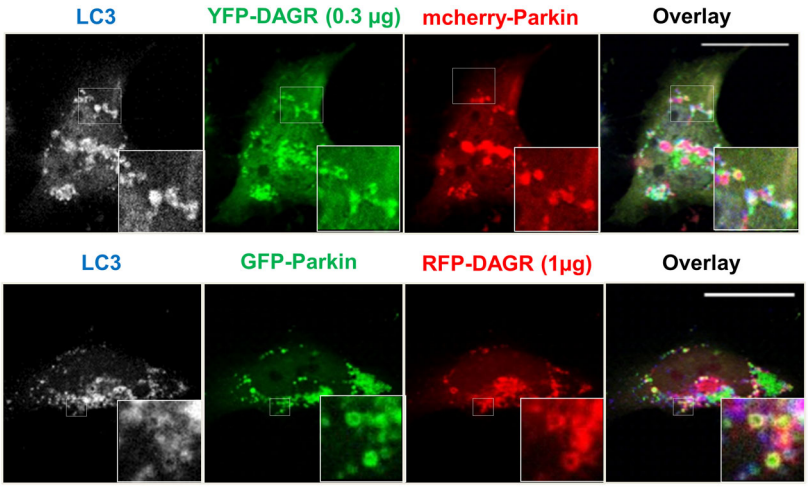

Figure EV3. RFP-DAGR and YFP-DAGR can label similar Golgi and mitophagosome structures.

Hela cells were transfected with mCherry-Parkin and 0.3 µg of YFP-DAGR (top panels) or GFP-Parkin and 1 µg of RFP-DAGR (bottom panels), followed by CCCP treatment. The formation of autophagosomes was assessed by immunostaining with an LC3 antibody. Note that, similar to RFP-DAGR, under lower expression levels, YFP-DAGR also surrounded LC3-positive and Parkin-tagged mitochondria. Scale bar is 25 µM, and zoom is 5× (top panel), 10× (bottom panel).

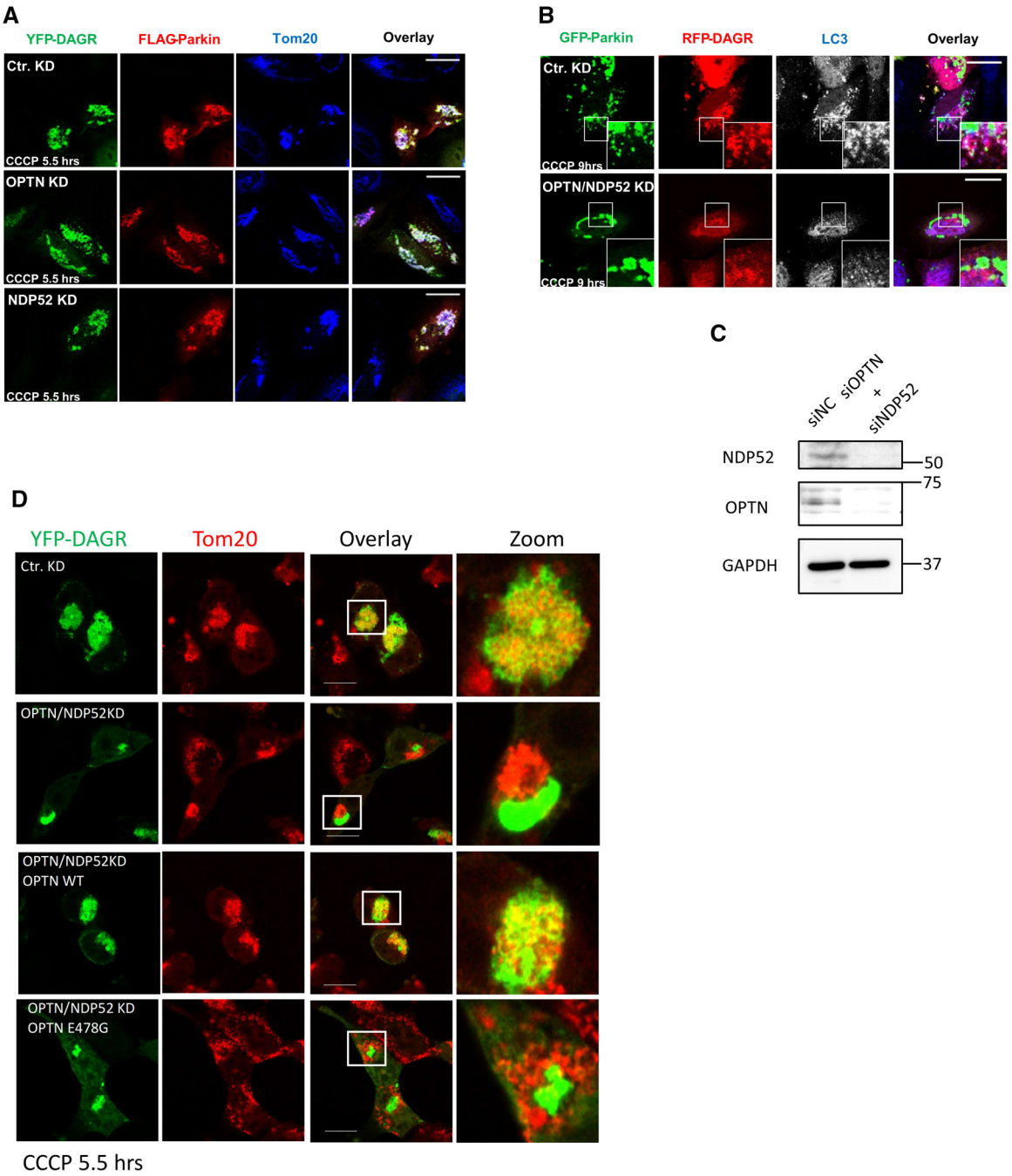

Figure EV4. The effect of optineurin (OPTN) or NDP52 single knockdown on mitochondrial DAG production and double knockdown on mito-autophagosome (mitophagosome) formation.

A Hela cells were transfected with control siRNA, OPTN-siRNA, or NDP52-siRNA. Knockdown cells were subsequently transfected with the DAGR-YFP reporter and FLAG-Parkin, followed by CCCP treatment. Note that DAGR accumulated on mitochondria (Tom20) in OPTN and NDP52 single knockdown cells. Scale bar = 25 μM.

B OPTN and NDP52 double knockdown cells were transfected with GFP-Parkin and RFP-DAGR, followed by CCCP treatment (10 μM) for 9 h. Autophagosomes were assessed by immunostaining with an LC3 antibody. Note that DAG-positive LC3 vesicle production was reduced in OPTN and NDP52 double knockdown cells. Scale bar = 25 μM, and zoom is 5×.

C Knockdown efficiency for OPTN and NDP52 was confirmed by immunoblotting.

D Hela cells were transfected with OPTN-siRNA and NDP52-siRNA followed by plasmids for FLAG-Parkin, DAGR-YFP, and OPTN-WT or OPTN-E478G mutant and treated with CCCP (5.5 h), as indicated. Note that OPTN-WT, but not the ubiquitin-binding deficient E478G mutant, restored mitochondrial DAGR-YFP accumulation induced by CCCP. Scale bar: 10 mm.

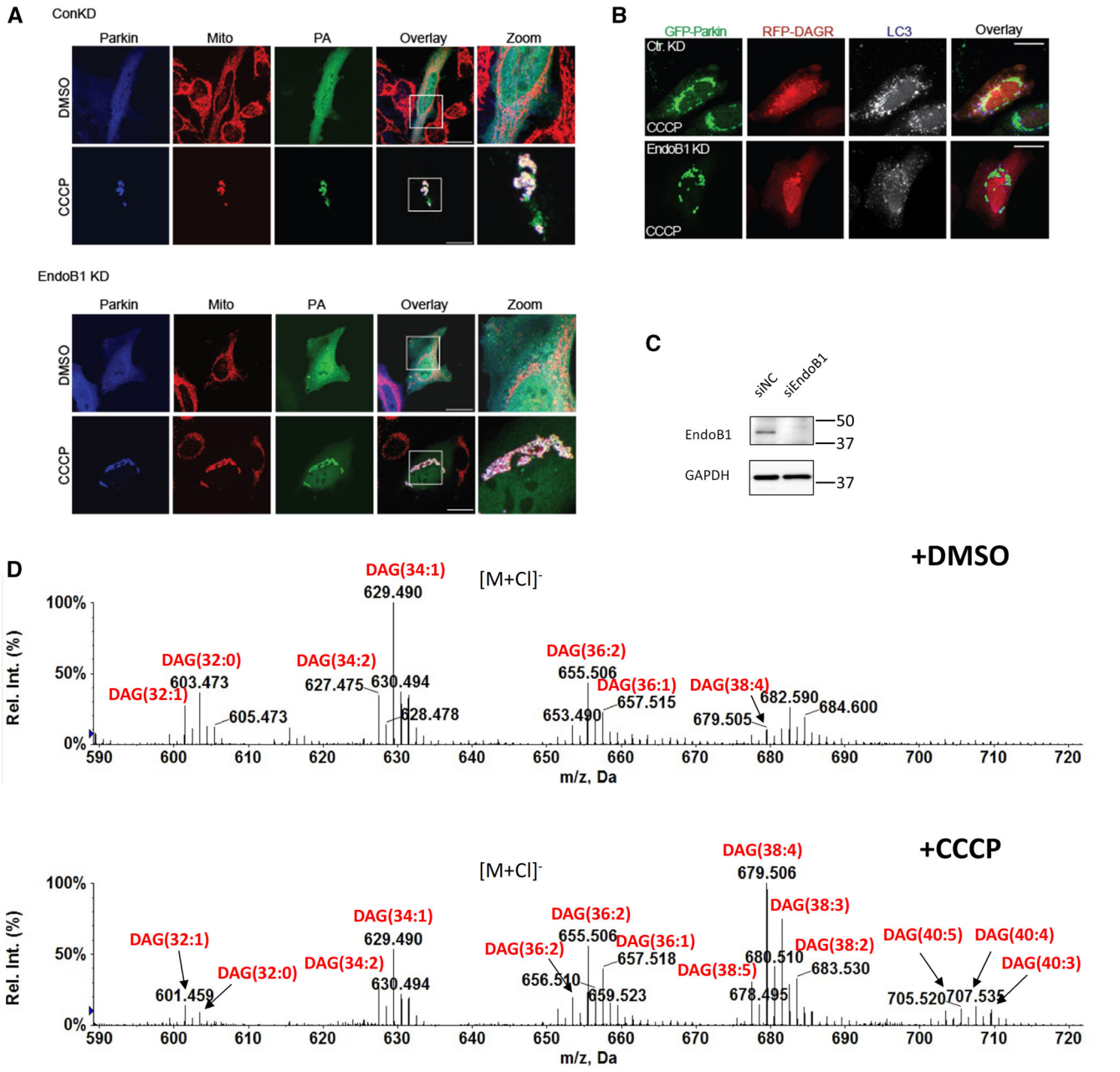

Figure EV5. Effect of EndoB1 knockdown on mitochondrial PA and mito-autophagosome (mitophagosome) production.

A EndoB1 knockdown cells were transfected with FLAG-Parkin and the PA reporter, followed by CCCP treatment. Mitochondria was assessed by immunostaining with a Tom20 antibody. Note the prominent accumulation of the PA reporter (PA) on mitochondria in EndoB1 knockdown cells. Scale bar = 25 μM and zoom is 3×.

B EndoB1 knockdown cells were transfected with GFP-Parkin and a DAG reporter RFP-DAGR, followed by CCCP treatment. Autophagosomes were assessed by immunostaining with an LC3 antibody. Note that the abundance of DAG-positive LC3 vesicles was reduced in EndoB1 knockdown cells. Scale bar = 25 μM.

C Control and EndoB1 KD cells were subjected to immunoblotting by EndoB1 and GAPDH antibodies to confirm knockdown efficiency.

D Mitochondria purified from control, and CCCP treated (5 h) Parkin-expressing HeLa cells were subjected to the lipidomic analysis by LC/MS. CCCP treatment resulted in the increase and alteration of fatty acyl compositions of mitochondrial DAGs. Most notably, the levels of DAGs containing longer, polyunsaturated fatty acids (e.g., C38:4 and C38:3 as well as 36:2 and 36:1) are drastically increased after CCCP treatment. Negative ion mass spectra showing the $[M + Cl]^-$ ions of DAGs in mitochondria isolated from cells treated with either DMSO or CCCP. The numbers of total acyl chain carbon atoms and double bonds of DAGs are denoted in parentheses.