Expanded View Figures

Figure EV1. The FKBP8-ATG8 interaction is dependent on an intact LIR docking site (LDS).

A, B GST-pulldown assay using ^{35}S-labeled Myc-FKBP8 WT incubated with recombinant GST and GST-LC3A-WT or GST-LC3A-F52A mutation (LDS mutation) (A) or GST-LC3B or GST-LC3B indicated mutations (LDS predicted sites) (B). Bound FKBP8 was detected by autoradiography. The bar graphs in the top panel represent the mean values of percentage binding of *in vitro*-translated FKBP8 to GST-LC3A or GST-LC3B WT and mutant proteins relative to the 10% input with standard deviations based on three independent experiments.

C Alignment of core LIR motifs in the autophagy mediators p62, NBR1, FYCO1, and the identified mitophagy receptors as well as FKBP8. Color coding; aromatic residues (magenta), acidic residues (red), hydrophobic residues (turquoise), Ser and Thr residues (green), Gln and Asn (orange), and basic residues (blue).

D The transmembrane (TM) domain at the C-terminus of FKBP8 (depicted schematically) was swapped with the ActA sequence and the cytochrome b5 sequence to generate mitochondria- and ER-specific FKBP8, respectively. The amino acid sequence of the C-terminus of each construct is shown. The TM domain sequences from ActA and cytochrome b5 are indicated in red with the important basic residue(s) in blue.

E HeLa cells were co-transfected with EGFP-FKBP8-Mito and dsRed2-Mito or EGFP-KDEL (ER marker) and mCherry-FKBP8-ER. Transfected cells were imaged by a Leica SP5 fluorescence confocal microscope to confirm co-localization. Scale bars, 10 μm.

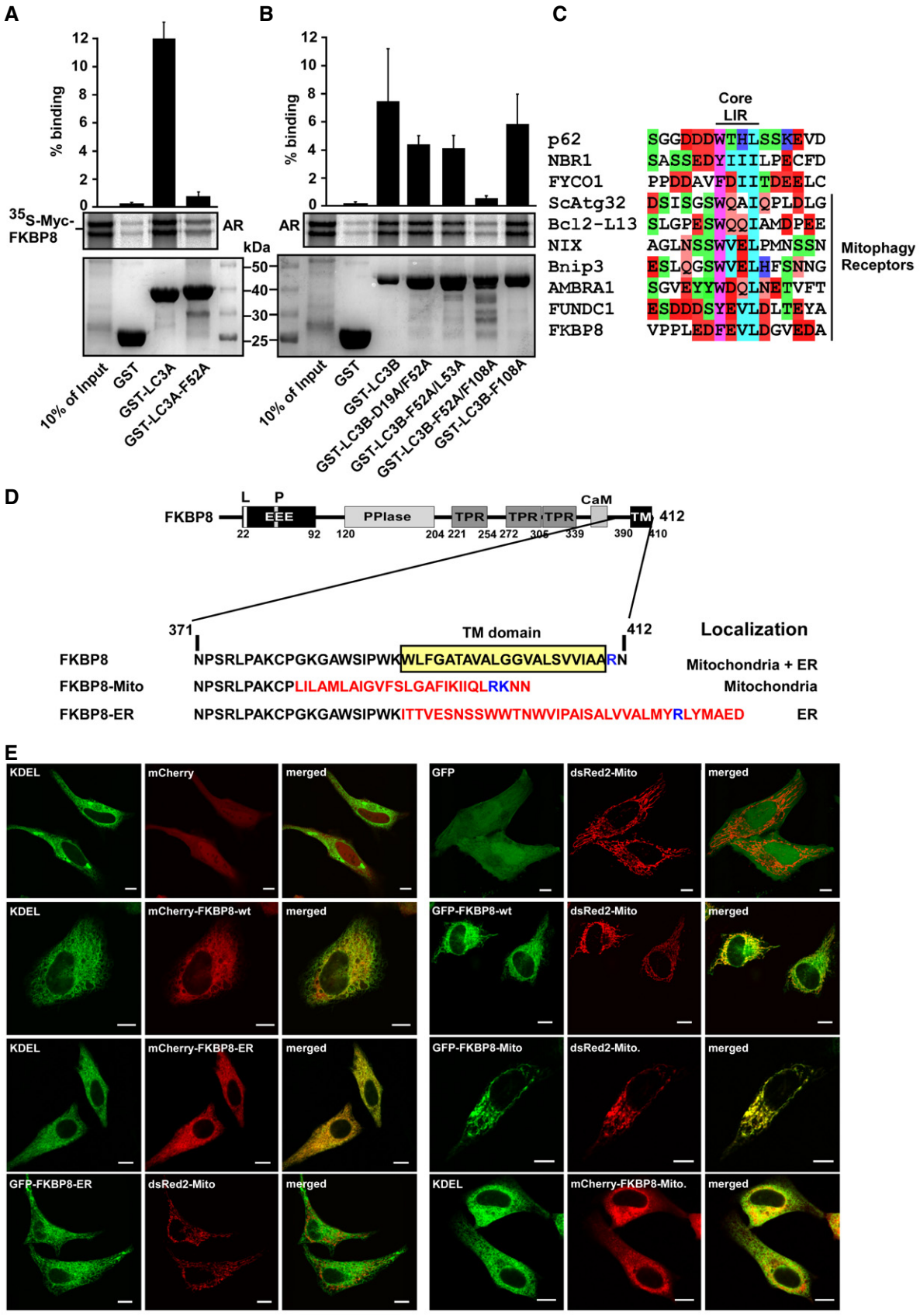

Figure EV1.

Figure EV2. CRISPR-mediated gene editing for generation of FKBP8 knockout clones in Flp-In-HEK293 and HeLa cell lines.

A Western blot analysis of cell extracts (40 μg) from HEK293, HeLa, and SH-SY5Y cells with anti-FKBP8 and anti-LC3A antibodies.

B Western blot analysis of CRISPR/Cas9 HeLa clones with anti-FKBP8 antibody in comparison with the mother cell line Flp-In T-REx HeLa.

C Immunostaining analyses of the wild-type Flp-In T-REx HeLa and knockout clones A4 and B1 with anti-FKBP8 antibody. The nuclei were stained with DAPI. Scale bars, 10 μm.

D Western blot analysis of CRISPR/Cas9 HEK293 clones with anti-FKBP8 antibody in comparison with the mother cell line Flp-In T-REx HEK293.

E Immunostaining analysis of the wild-type Flp-In T-REx Hek293 and knockout clones C3 and C4 with anti-FKBP8 antibody. The nuclei were stained with DAPI. Scale bars, 10 μm.

F, G FKBP8 overexpression in Flp-In T-REx HEK293 FKBP8 KO cells significantly increases the amount of lipidated Flag-LC3A in the mitochondrial fraction. Flp-In T-REx HEK293 WT and CRISPR/Cas9-generated KO clone C3 cells were transfected with Flag-LC3A constructs or Flag-LC3A and Myc-FKBP8 expression constructs and treated with DMSO or CCCP (30 μM; 4 h). The amounts of Flag-LC3A in the mitochondrial fraction (40 μg) and FKBP8 in the total fractions (30 μg) were analyzed by Western blotting. Ponceau S-stained blots are used as loading controls. The graph in (G) represents the average of quantitated Flag-LC3A II/Flag-LC3A I values of three independent experiments performed as in (F) (n = 3). Error bars represent the s.d. The P-values were calculated using paired Student's t-test and are indicated above the bars.

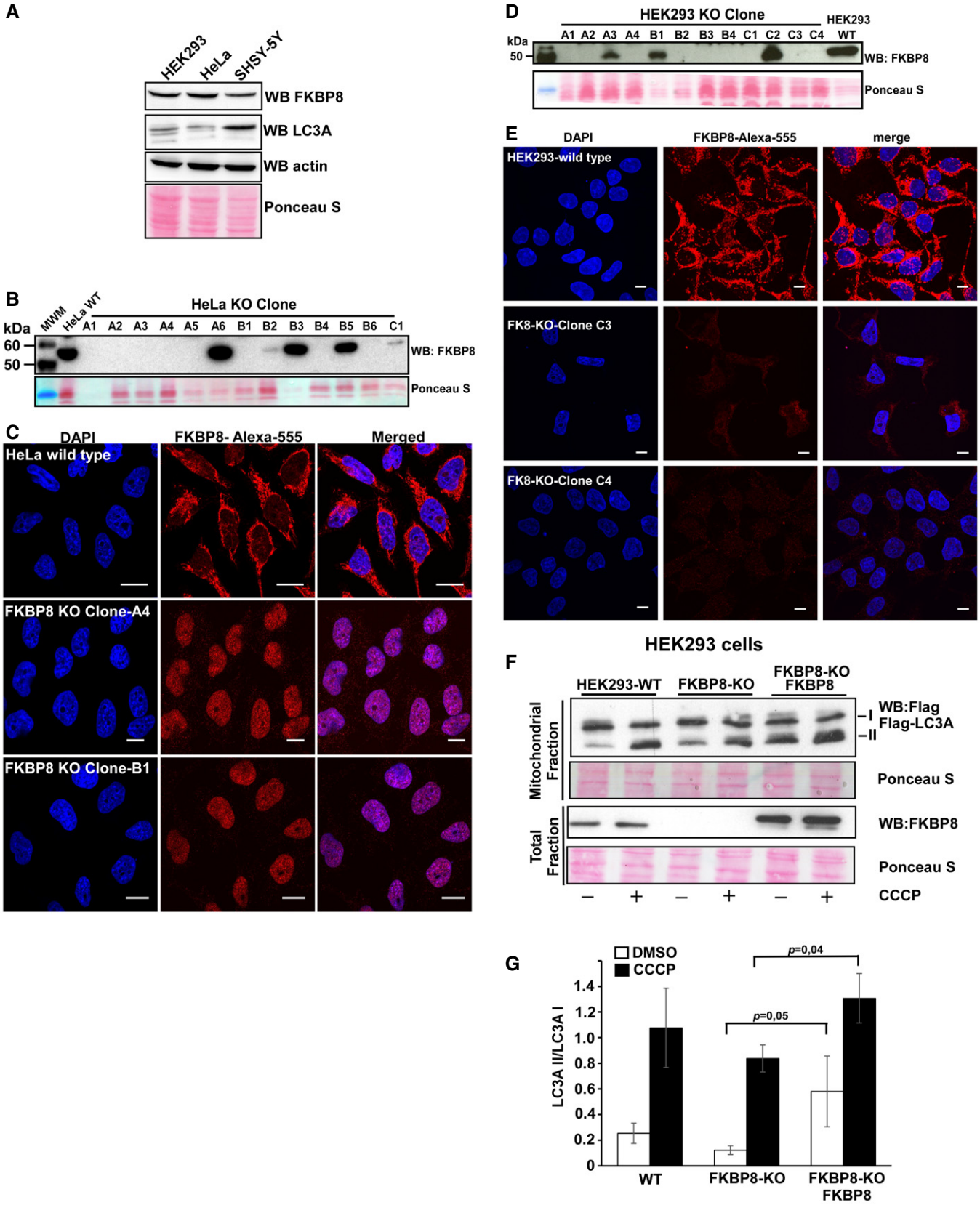

Figure EV2.

Figure EV3. FKBP8-ER recruits LC3A to the ER upon CCCP treatment.

A, B HeLa cells were co-transfected with Cerulean vector control (A) or Myc-FKBP8-ER (B), EYFP-LC3A, and mCherry-OMP25TM expression constructs. The cells were treated with DMSO or 10 μM CCCP for 4 h and imaged by a Leica SP5 fluorescence confocal microscope. Scale bars, 10 μm; in zoomed images, 5 μm. Line-profile co-localization plots of Cerulean or FKBP8 ER (blue line), EYFP-LC3A (green line), and OMP25TM (red line) were made using the line-profile quantification tool in the Leica imaging software (LAS-AF). The vertical axes represent measurements of fluorescent intensity and the horizontal axis the drawn distances.

C A HeLa cell line stably expressing GFP-LC3A was transfected with plasmids expressing the indicated mCherry-fusion proteins imaged by a Leica SP5 confocal microscope. Scale bars, 10 μm. Line-profile co-localization of plots of GFP-LC3A (green line) and mCherry vector control, mCherry-FKBP8, mCherry-NIX, or mCherry-BNIP3 (red lines) were made using the line-profile quantification tool in Leica imaging software. The measurements of the intensity (*y*-axis) and distance (*x*-axis in μm) are plotted on the graph.

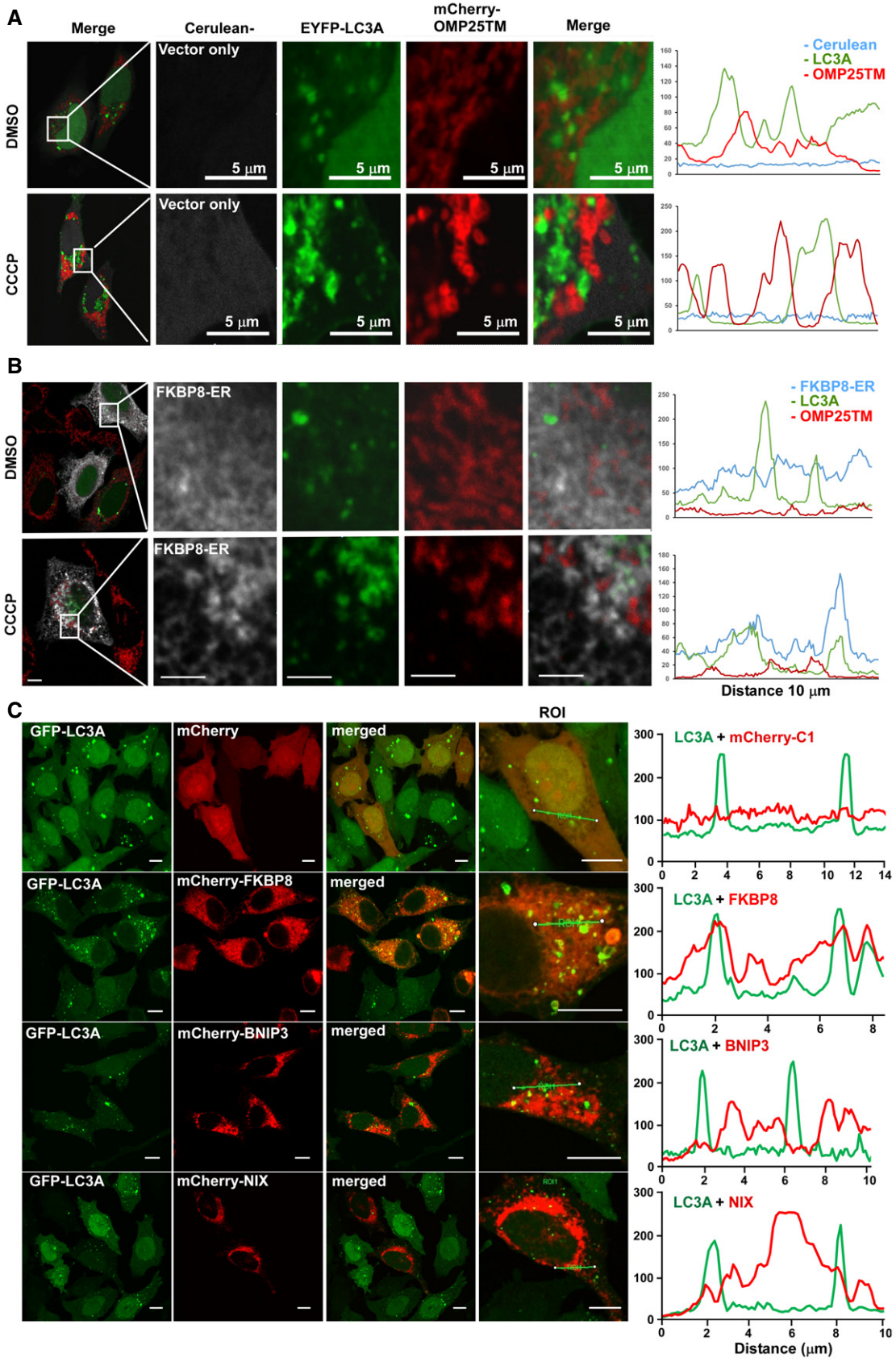
A
Merge
Cerulean-
EYFP-LC3A
mCherry-OMP25TM
Merge
DMSO
CCCP
Vector only
5 μm
- Cerulean
- LC3A
- OMP25TM
B
FKBP8-ER
- FKBP8-ER
- LC3A
- OMP25TM
Distance 10 μm
C
ROI
GFP-LC3A
mCherry
merged
mCherry-FKBP8
mCherry-BNIP3
mCherry-NIX
LC3A + mCherry-C1
LC3A + FKBP8
LC3A + BNIP3
LC3A + NIX
Distance (μm)

Figure EV3.

Figure EV4. FKBP8 mutants that are not localized on mitochondria are unable to induce mitophagy.

A HeLa cells were transiently transfected with mCherry-GFP-OMP25-TM and 3×Flag-LC3A together with Myc-C1 as control and Myc-FKBP8 WT, Myc-FKBP8-ΔTM, or Myc-FKBP8-ER mutant expression constructs. The appearance of red-only structures indicates acidified mitochondria. Scale bars, 10 μm.

B HeLa cells were transiently overexpressing mCherry-GFP-OMP25-TM, 3×Flag-LC3A, together with Myc-tagged FKBP8 WT, FKBP8-ΔTM, or FKBP8-ER. The graph bars represent the percentage of transfected cells containing red-only dots indicative of mitophagy activity. Each bar shows the mean value with standard deviations from measurements on 50–70 cells obtained in three independent experiments.

C Western blot showing the expression levels of FKBP8 WT and mutant constructs, BNIP3, NIX, and the various ATG8 proteins in the cells used for experiments shown in Figs 5 and EV4B. Ponceau S staining is used as the loading control.

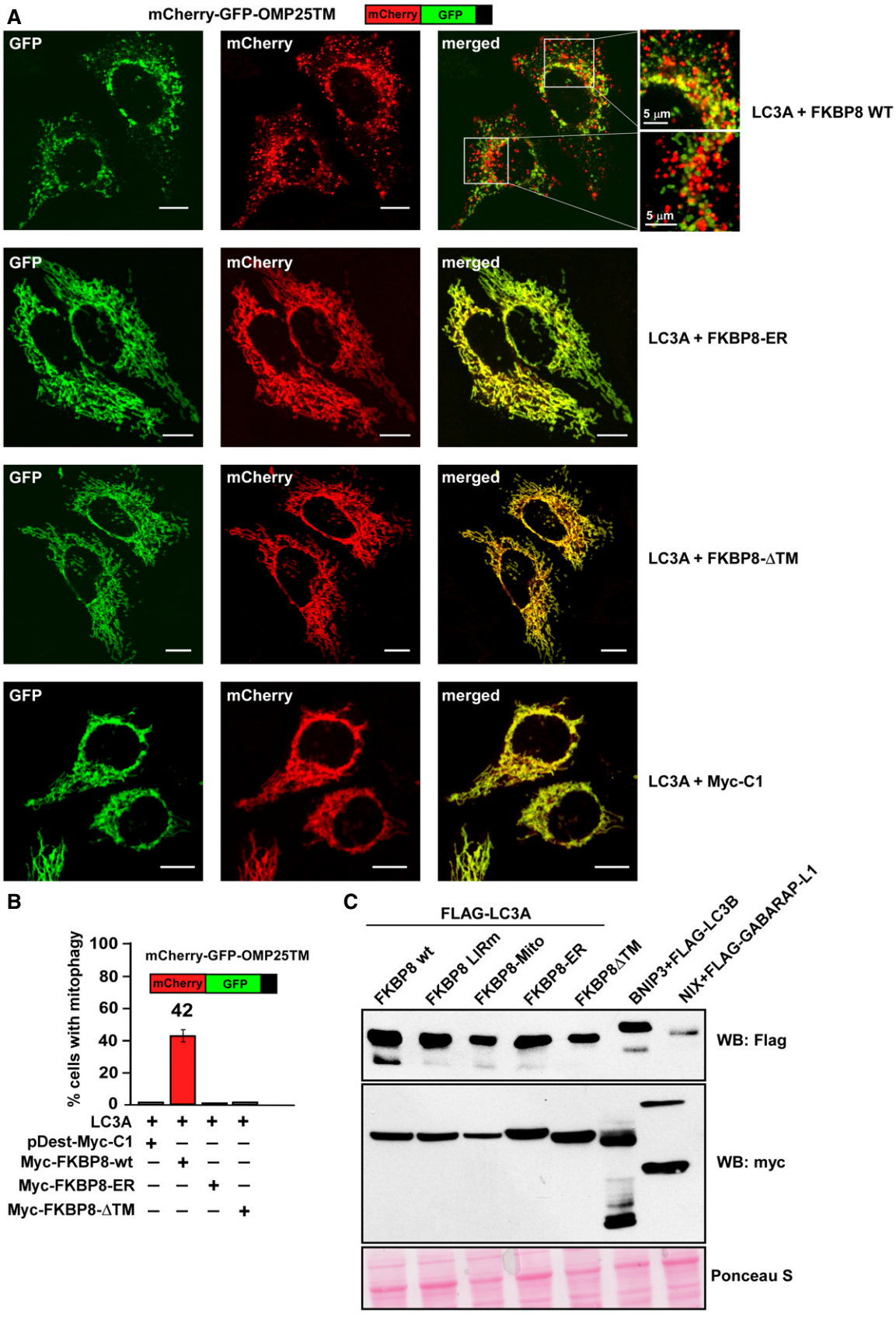

Figure EV4.

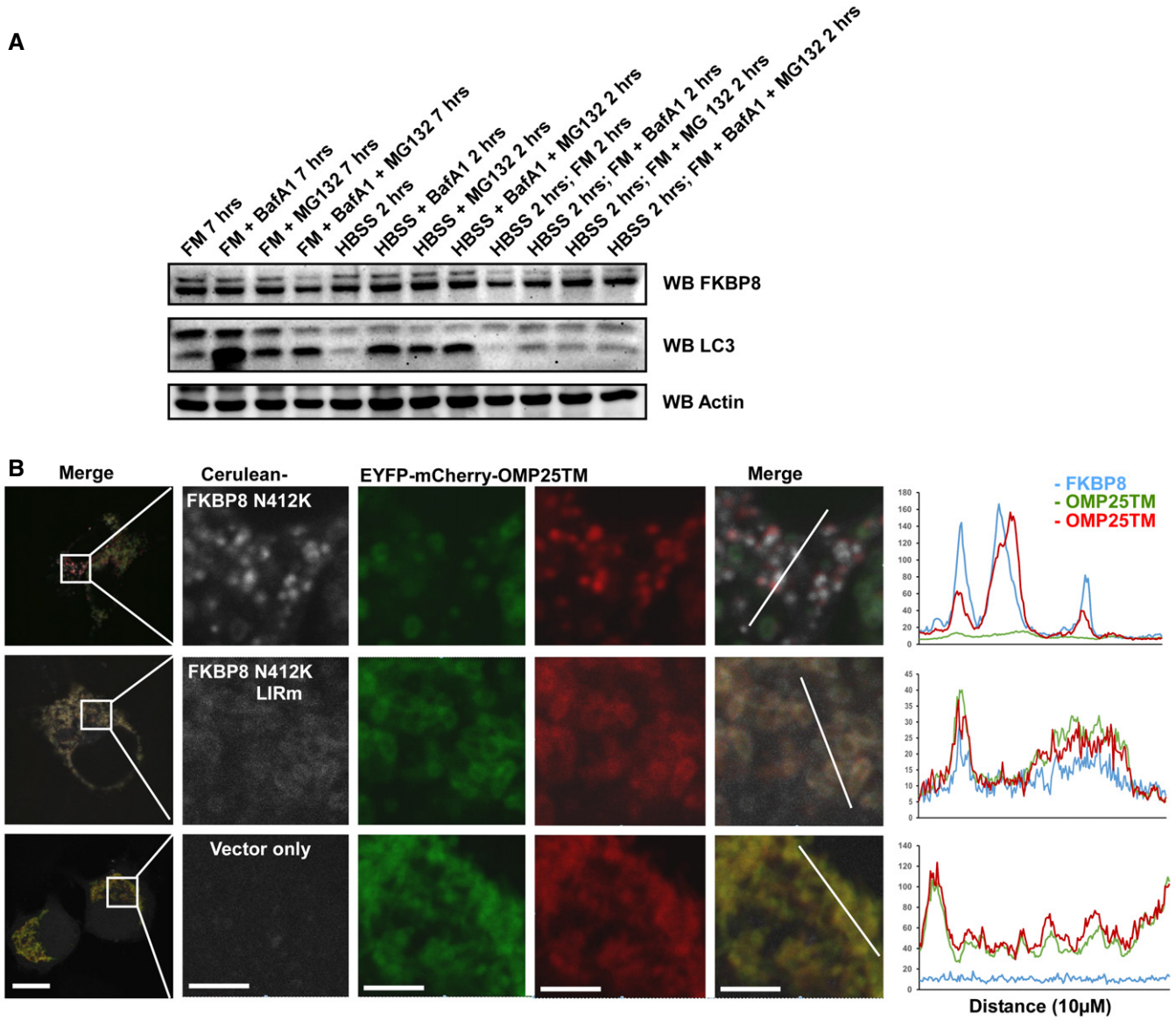

Figure EV5. Endogenous FKBP8 is not degraded during autophagy inducing conditions, whereas the FKBP8 N412K mutant is localized on acidified mitochondria.

A HeLa cells were treated with Baf-A1 or MG132 or starved with HBSS for the indicated times. Cells were harvested and subjected to Western blotting for analysis of endogenous FKBP8 and LC3B.

B HeLa cells were transiently transfected with mCherry-GFP-OMP25TM and Flag-LC3A expression constructs, together with Cerulean-FKBP8 N412K, Cerulean-FKBP8 N412K + LIRm, or Cerulean-only expression vectors. The cells were imaged using a Leica SP5 fluorescence confocal microscope. Scale bars, 10 μm; in zoomed images, 5 μm. Line-profile co-localization plots of Cerulean-FKBP8 mutants (blue line) and mCherry-GFP-OMP25TM (red and green lines) were made using the line-profile quantification tool in the Leica imaging software (LAS-AF). The vertical axes represent measurements of fluorescent intensity and the horizontal axis the drawn distances.