Expanded View Figures

Figure EV1. The C-terminal region of MITOL interacts with the RING2 domain of Parkin, related to Fig 1.

A Interaction of endogenous MITOL and Parkin in SH-SY5Y cells. SH-SY5Y cells were treated with DMSO or CCCP (10 μM) for 12 h and subjected to an IP-IB assay with the indicated antibodies.

B Parkin interacts with C-terminal region of MITOL. The schematic diagram shows the three truncated MITOL fragments that were fused to GST (Top). HeLa cells stably expressing HA-Parkin were treated with DMSO or CCCP (10 μM) for 4 h. Lysates of cells were performed GST pull-down assay and then subjected to an IB assay with anti-HA or anti-GST antibodies (Bottom). (a) N-terminal RING domain (1–90 aa); (b) second loop domain (160–209 aa); (c) C-terminal domain (254–278 aa); TM, transmembrane.

C The C-terminal deleted MITOL mutant failed to interact with Parkin. HeLa cells were transfected with the indicated vectors and treated with CCCP (10 μM) for 4 h. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies. ΔRING, 14–68 aa deletion. Δ2nd, 160–209 aa deletion. ΔC, 254–278 aa deletion.

D MITOL interacts with RING2 domain of Parkin. The schematic diagram shows the six truncated Parkin fragments that were fused to GST (Top). Mitochondrial fractions were isolated from HeLa cells and performed a GST pull-down assay. IB assay was subjected with anti-MITOL or anti-GST antibodies (Bottom). (a) UBL domain (1–76 aa); (b) RING0 domain (141–224 aa); (c) RING1 domain (225–326 aa); (d) IBR domain (327–377 aa); (e) REP domain (378–409 aa); (f) RING2 domain (410–465 aa).

E Parkin K211N fails to interact with MITOL. HeLa cells were transfected with the indicated vectors and treated with DMSO or CCCP (10 μM) for 4 h. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies.

F Parkin K211N mutant fails to translocate to the mitochondria in CCCP-treated cells. HeLa cells were transfected with the indicated vectors and treated with CCCP (10 μM) for 8 h. Cells were immunostained with indicated antibody. Scale bar, 10 μm.

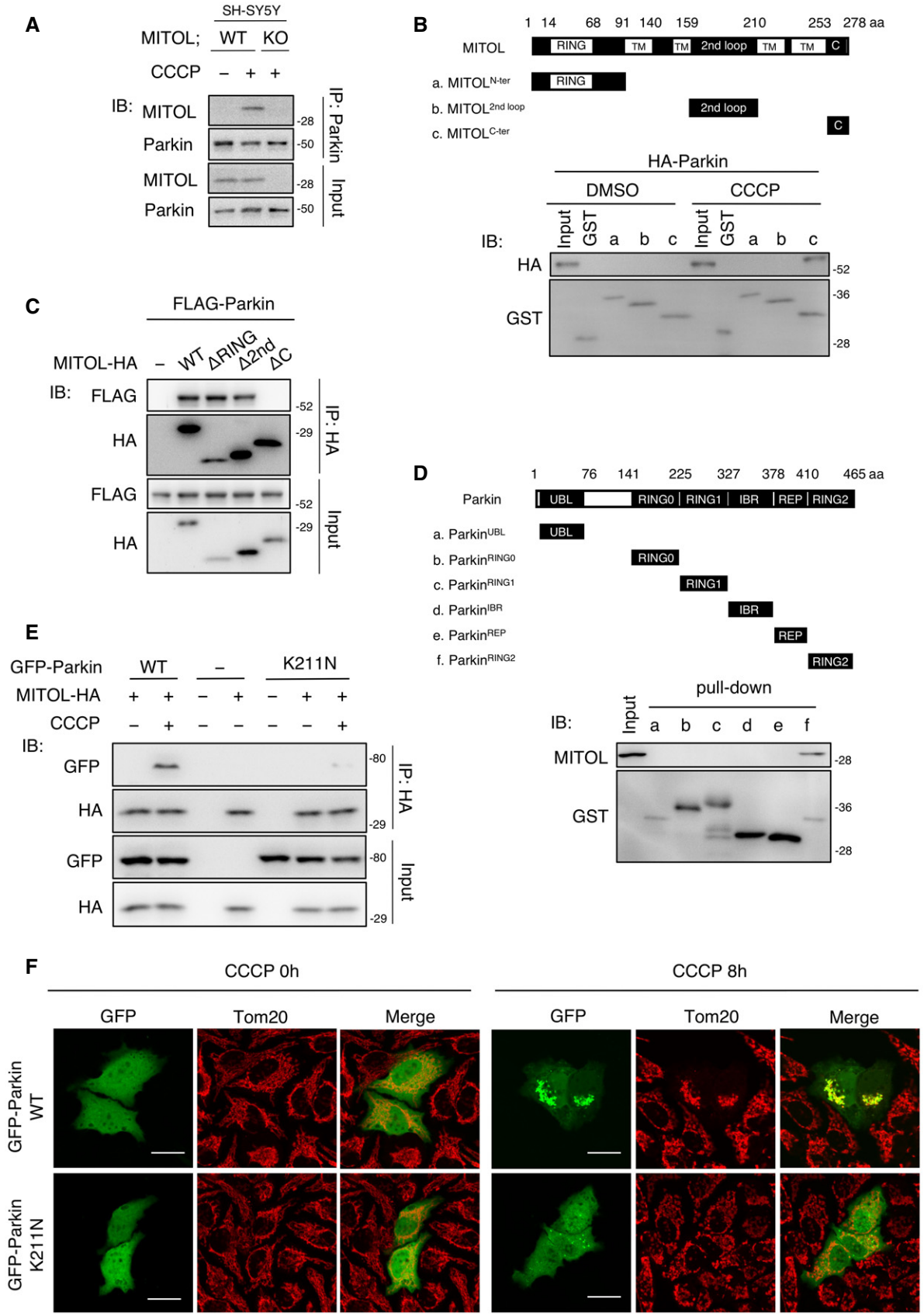

Figure EV1.

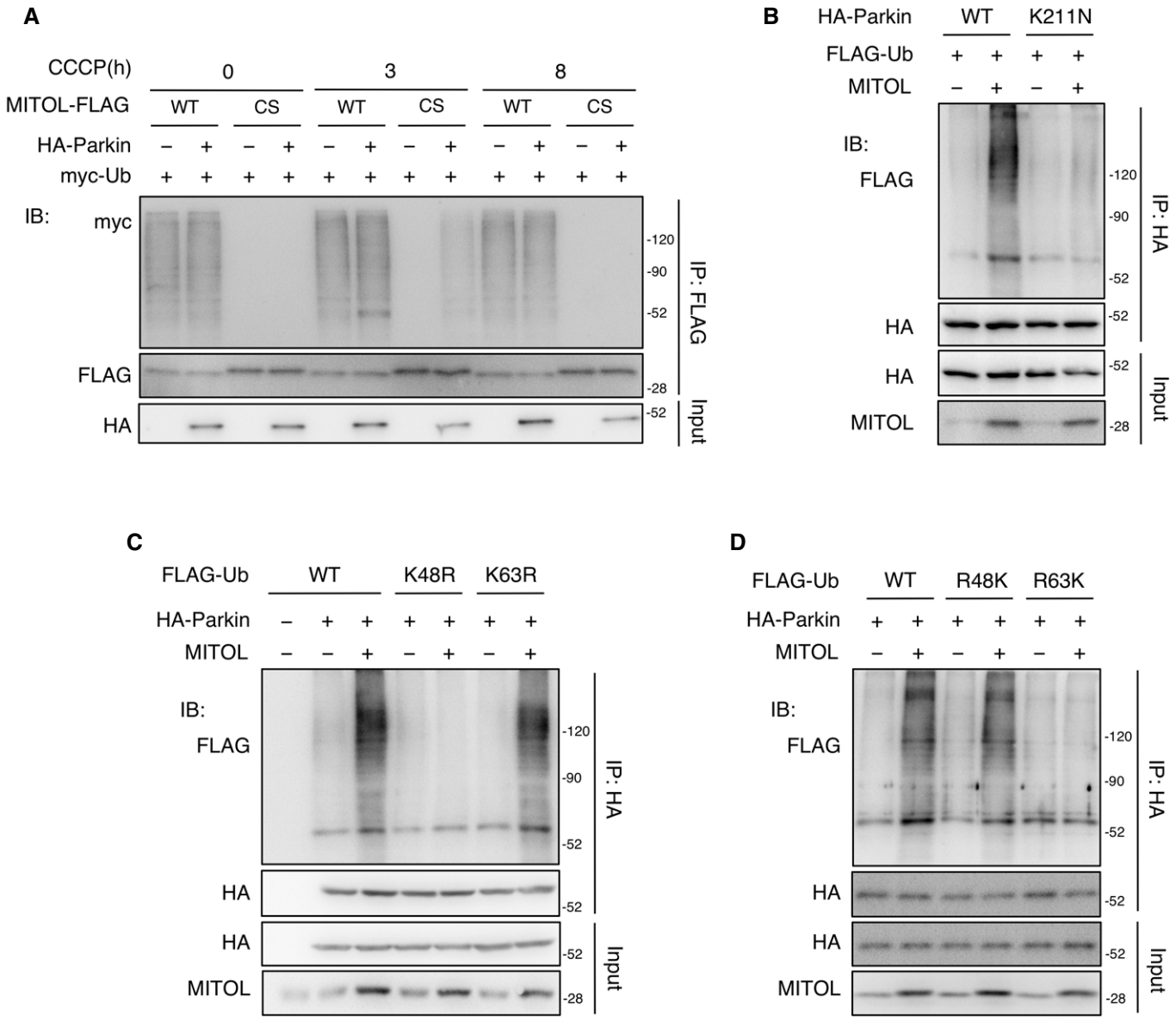

Figure EV2. MITOL mediated the K48-linked polyubiquitination of Parkin in mitophagy, related to Fig 1.

A Ubiquitylation of MITOL occurs in the early phase but not the late phase of mitophagy. HeLa cells were transfected with the indicated vectors and treated with DMSO or CCCP (10 μM) for indicated times. MG132 (30 μM) was added 3 h after each treatment. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies. WT, wild-type; CS, MITOL C65/67S mutant lacking ubiquitin ligase activity.

B MITOL fails to ubiquitinate the Parkin K211N mutant. HeLa cells were transfected with the indicated vectors and treated with DMSO or CCCP (10 μM) for 8 h. MG132 (30 μM) was added 3 h after each treatment. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies and compared ubiquitination levels of WT and K211N HA-Parkin. Parkin K211N mutant; impaired mitochondrial translocation.

C, D MITOL mediates K48-linked polyubiquitination of Parkin. HeLa cells stably expressing HA-Parkin were transfected with the indicated vectors and treated with DMSO or CCCP (10 μM) for 8 h. MG132 (30 μM) was added 3 h after each treatment. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies, and compared the effect of various KR FLAG-ubiquitin (Ub) mutants (C) or RK FLAG-Ub mutants (D) on MITOL-mediated Parkin ubiquitination. KR ubiquitin mutant, turn single lysine to arginine (K48R or K63R); RK ubiquitin mutant, turn all lysine to arginine and then put back single lysine (R48K or R63K).

Figure EV3. MITOL degrades Parkin and slightly effect for mitophagy, related to Fig 2.

A MITOL overexpression degrades Parkin on mitochondrial membrane. HeLa cells stably expressing HA-Parkin were transfected with the indicated vectors and treated with DMSO or CCCP (10 μM) for 12 h. Lysates of cells were fractionated into mitochondria-rich membrane fraction (Memb) and supernatant (Sup), and then subjected to an IB assay with the indicated antibodies. Loading was adjusted for approximately equal concentrations of VDAC in the membrane fraction (Memb).

B Endogenous MITOL attenuates mitophagy in CHX-treated cells. WT or MITOL KO HeLa cells stably expressing HA-Parkin were treated with DMSO or CCCP (10 μM) for 30 h. CHX (30 μM) was added 5 h after CCCP treatment. Lysates of cells were subjected to an IB assay with the indicated antibodies.

C MITOL has the specificity for the substrate. HeLa cells were transfected with the indicated vectors and treated with DMSO or CCCP (10 μM) for 8 h. MG132 (30 μM) was added 3 h after each treatment. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies and compared ubiquitination levels of HA-Parkin and MITOL-HA to Mito-GFP.

D Parkin K220R mutant normally translocated to the mitochondria in CCCP-treated cells. HeLa cells were transfected with indicated vectors and treated with CCCP (10 μM) for 3 h. Cells were immunostained with indicated antibody. Scale bar, 10 μm.

E Mt-Keima signal is responding to autophagy process. MITOL WT HeLa cells and MITOL KO HeLa cells expressing HA-Parkin and mt-Keima were treated with DMSO or CCCP (10 μM) for 8 h alone or either with bafilomycin A1 (10 μM). Then, mKeima was measured at 488 (pH 7) and 561 (pH 4) nm lasers using Flow Cytometer. Percentages of mitophagy were calculated from 30,000 cells.

F Parkin K220R mutant induces mitophagy even in the overexpression of MITOL. HeLa cells stably expressing mt-Keima were transfected with indicated vectors and treated with CCCP (10 μM) for 8 h. Then, mKeima was measured at 488 (pH 7) and 561 (pH 4) nm lasers using Flow Cytometer. Percentages of mitophagy were calculated from 30,000 cells in each independent experiment. Data represent the mean ± SD of three independent experiments. For statistical analysis, a one-way ANOVA with Tukey post-test was performed, $^{**}P < 0.01$.

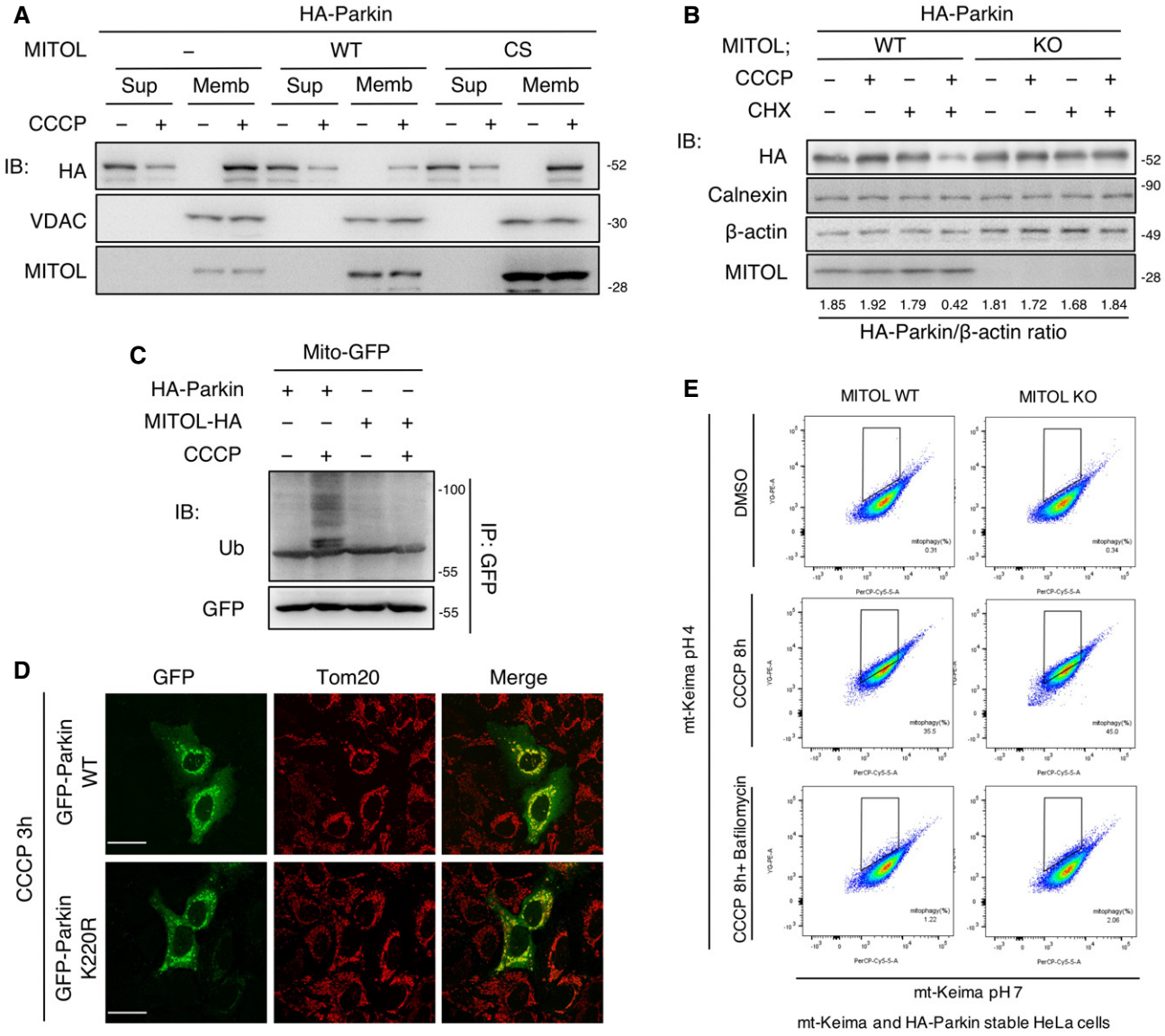
A
HA-Parkin
MITOL
–
WT
CS
Sup
Memb
CCCP
IB:
HA
VDAC
MITOL
-52
-30
-28
B
HA-Parkin
MITOL;
WT
KO
CCCP
CHX
IB:
HA
Calnexin
β-actin
MITOL
-52
-90
-49
-28
1.85 1.92 1.79 0.42 1.81 1.72 1.68 1.84
HA-Parkin/β-actin ratio
C
Mito-GFP
HA-Parkin
MITOL-HA
CCCP
IB:
Ub
GFP
-100
-55
-55
IP: GFP
D
GFP
Tom20
Merge
CCCP 3h
GFP-Parkin WT
GFP-Parkin K220R
E
MITOL WT
MITOL KO
DMSO
CCCP 8h
CCCP 8h+ Bafilomycin
mt-Keima pH4
mt-Keima pH7
mt-Keima and HA-Parkin stable HeLa cells
F
mt-Keima stable HeLa cells
Parkin WT
Parkin WT + MITOL^WT
Parkin K220R
Parkin K220R + MITOL^WT
DMSO
CCCP 8h
mt-Keima pH4
mt-Keima pH7
DMSO
CCCP
Mitophagy (%)
Parkin WT
Parkin WT + MITOL^WT
Parkin 220R
Parkin K220R + MITOL^WT

Figure EV3.

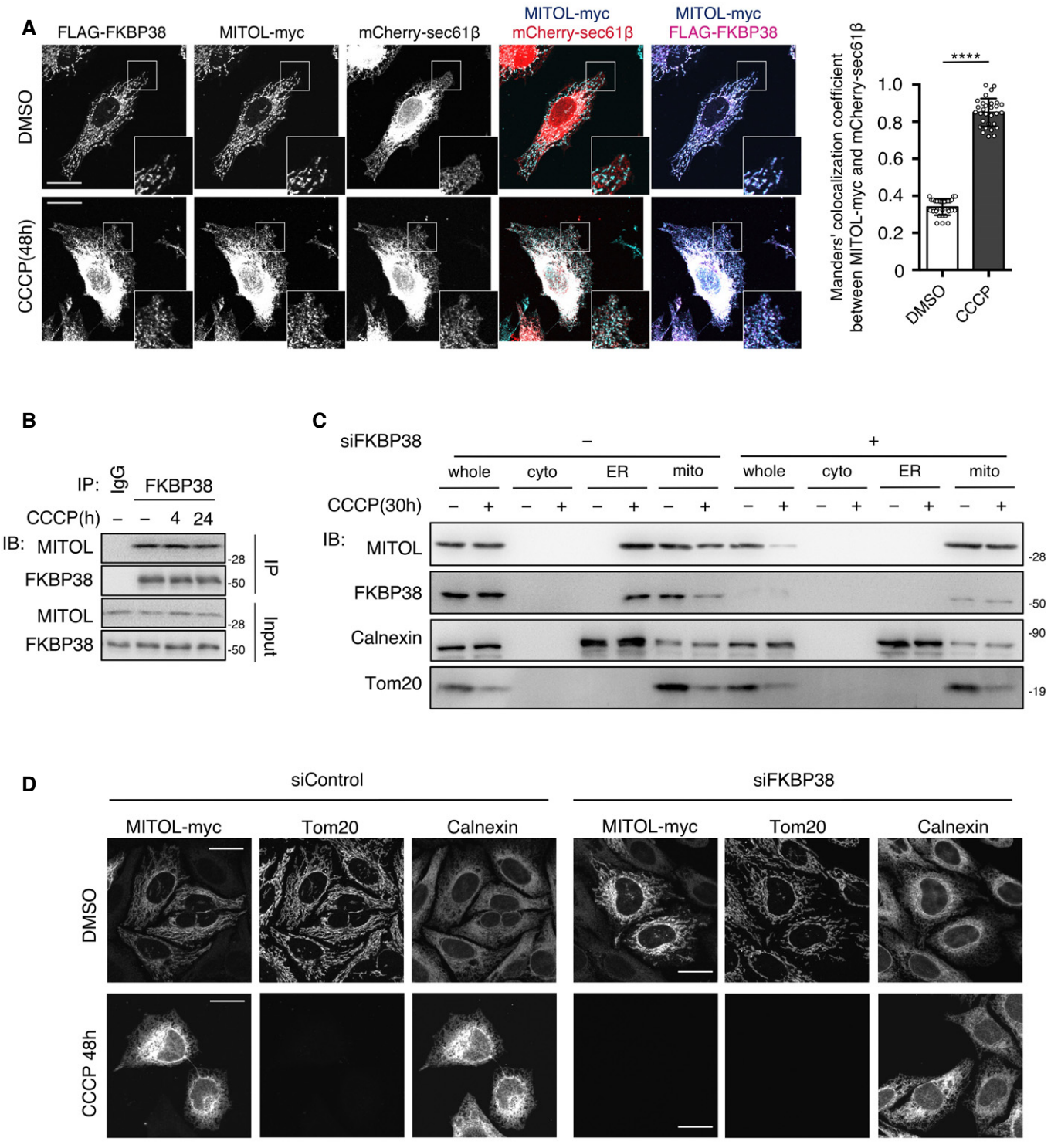

Figure EV4.

Figure EV4. MITOL translocates to the ER in an FKBP38-dependent manner, related to Fig 3.

A MITOL translocates from the mitochondria to the ER in the late phase of mitophagy with FKBP38. HeLa cells stably expressing HA-Parkin were transfected with the indicated vectors and treated with DMSO or CCCP (10 μM) for 48 h. Cells were fixed, permeabilized, and subjected to immunofluorescence analysis with indicated antibodies. Colocalization was quantified by Manders's coefficient. Means ± SEM of more than 10 cells obtained from three independent experiments. For statistical analysis, Student's t-tests were performed, ****$P < 0.0001$. Scale bar represents 10 μm. Higher magnification images of the boxed regions are shown in the small panel.

B Interaction of endogenous MITOL and FKBP38. HeLa cells stably expressing HA-Parkin were treated with DMSO or CCCP (10 μM) for indicated times and subjected to an IP-IB assay with the indicated antibodies.

C, D MITOL translocation depends on FKBP38. HeLa cells stably expressing HA-Parkin were transfected with FKBP38 siRNA or control siRNA and treated with DMSO or CCCP (10 μM) for 30 h. Lysates of cells were fractioned into whole-cell, cytosolic, ER, and mitochondrial fractions and then subjected to an IB assay with the indicated antibodies (C). MITOL KO HeLa cells stably expressing HA-Parkin were transfected with MITOL-myc and treated with DMSO or CCCP (10 μM) for 48 h. Cells were immunostained with indicated antibodies (D). Scale bar, 10 μm.

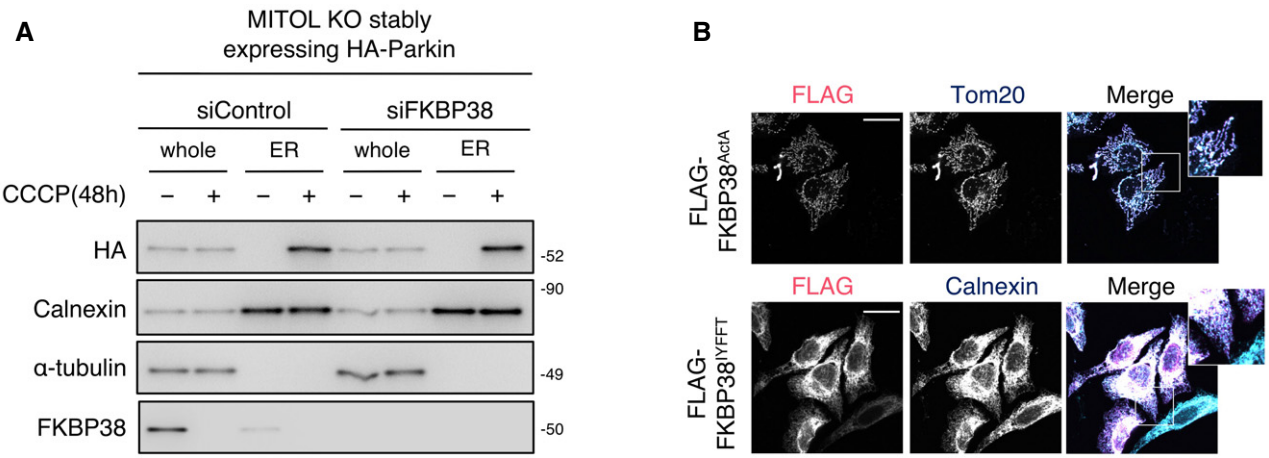

Figure EV5. Parkin translocation to the ER does not depend on FKBP38 and ubiquitination of FKBP38 needs Parkin E3 activity, related to Figs 4 and 5.

A Parkin accumulation in the ER does not depend on FKBP38. MITOL KO HeLa cells stably expressing HA-Parkin were transfected with FKBP38 siRNA or control siRNA and treated with CCCP (10 μM) for 48 h. Lysates of cells were fractionated into ER fractions and then subjected to IB assay with indicated antibodies.

B FKBP38^{IYFFT} and FKBP38^{ActA} are accurately localized in each targeting organelle. HeLa cells stably expressing HA-Parkin were transfected with indicated vectors and immunostained with indicated antibodies. Scale bar, 10 μm. Higher magnification images of the boxed regions are shown in the small panel.