Expanded View Figures

Figure EV1. Effect of valinomycin treatment on the levels of NDP52 and mitochondrial proteins.

A HeLa-GFP-Parkin cells incubated with valinomycin for 0–24 h. Cell lysates were subjected to Western blotting using the indicated antibodies.

B HeLa-GFP-Parkin cells incubated with valinomycin for 3 h in the presence or absence of 100 nM bafilomycin A_1 or epoxomicin. Cell lysates were subjected to Western blotting using the indicated antibodies.

C HeLa-GFP-Parkin cells expressing mCherry-NDP52 were incubated with valinomycin. Cells were immunostained with anti-Tom20 antibody. Colocalization of GFP-Parkin, NDP52, and Tom20 was determined using Line Scan. Fluorescence intensities of each channel were measured along the white arrow. Scale bars, 10 μm.

D Mitophagy flux assay in FIP200 KD cells. Cells transfected with siFIP200 oligo were incubated with valinomycin for 6 h in the presence or absence bafilomycin A_1. Results are a summary of three independent experiments. Values are the means ± SEM. $^{**}P < 0.01$ compared with scrambled oligo-treated cells, determined with one-way ANOVA followed by the Student's *t*-test.

E mCherry-NDP52 was expressed in HeLa-GFP-Parkin cells and immunostained with anti-Lamp2 antibody. Scale bars, 10 μm.

Source data are available online for this figure.

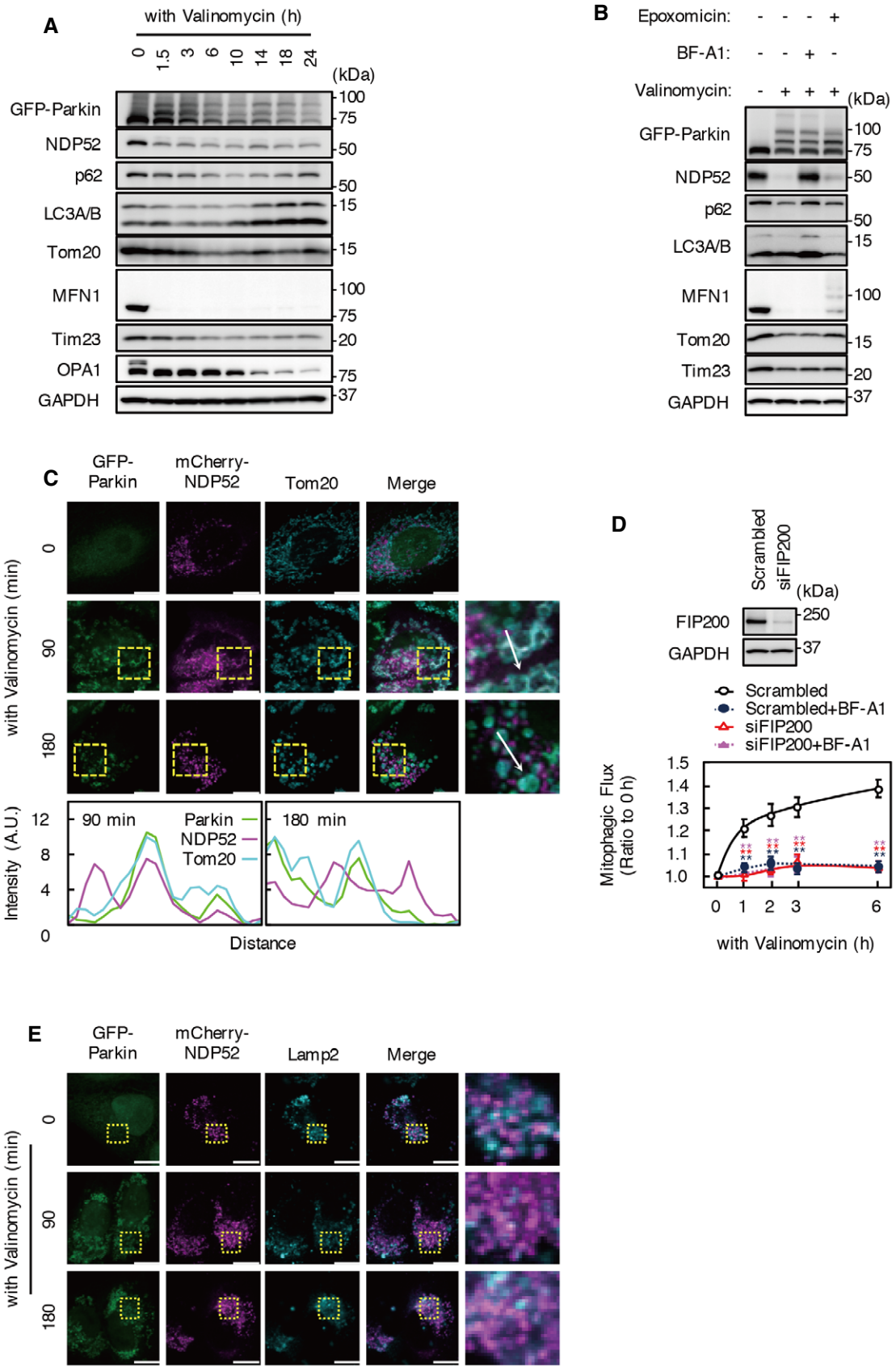

Figure EV1.

Figure EV2. Ubiquitin-binding-deficient mutants of NDP52 fail to localize at damaged mitochondria.

A Schematic structures of NDP52 truncated mutants used in this study.

B HeLa-GFP-Parkin cells expressing mCherry-NDP52 ΔSKICH, ΔCLIR, or ΔLIM-L were immunostained with anti-Tom20 antibody. Scale bars, 10 μm. Colocalization of GFP-Parkin, mCherry-NDP52 mutant, and Tom20 was determined using Line Scan. Fluorescence intensities of each channel were measured along the white arrow.

C HeLa-GFP-Parkin cells expressing mCherry-NDP52 or ΔCLIR were incubated with valinomycin and bafilomycin A_1 for 3 h. Cell lysates were subjected to immunoprecipitation using an anti-RFP antibody. Immunoprecipitates were analyzed by Western blotting.

D HeLa-GFP-Parkin cells expressing mCherry-NDP52 mutants were incubated with valinomycin and bafilomycin A_1 for 3 h. Cell lysates were subjected to immunoprecipitation using an anti-RFP antibody. Immunoprecipitates were analyzed by Western blotting.

E HeLa-GFP-Parkin cells expressing mCherry-NDP52ΔZF2 or D439R/C443K were incubated with valinomycin for 2 h. Immunostaining was performed with anti-Tom20 antibody. Scale bar, 10 μm. Colocalization of GFP-Parkin, mCherry-NDP52 mutant, and Tom20 was assessed using Line Scan.

F Mitophagic flux in NDP52 KD cells transfected with siRNA-resistant NDP52 or NDP52 mutants. Cells were incubated with valinomycin for 3 h. Results are a summary of three independent experiments. Values are the means ± SEM. $^{**}P < 0.01$ vs. scrambled oligo-transfected cells; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. empty vector-transfected cells, determined with one-way ANOVA followed by the Tukey–Kramer *post hoc* test.

G mCherry-NDP52 was expressed in TBK1 KD cells. Cells were incubated with 1 μM valinomycin for 2 h and immunostained with anti-Tom20 antibody. Scale bars, 10 μm. Colocalization of GFP-Parkin, mCherry-NDP52 mutant, and Tom20 was determined using Line Scan.

Source data are available online for this figure.

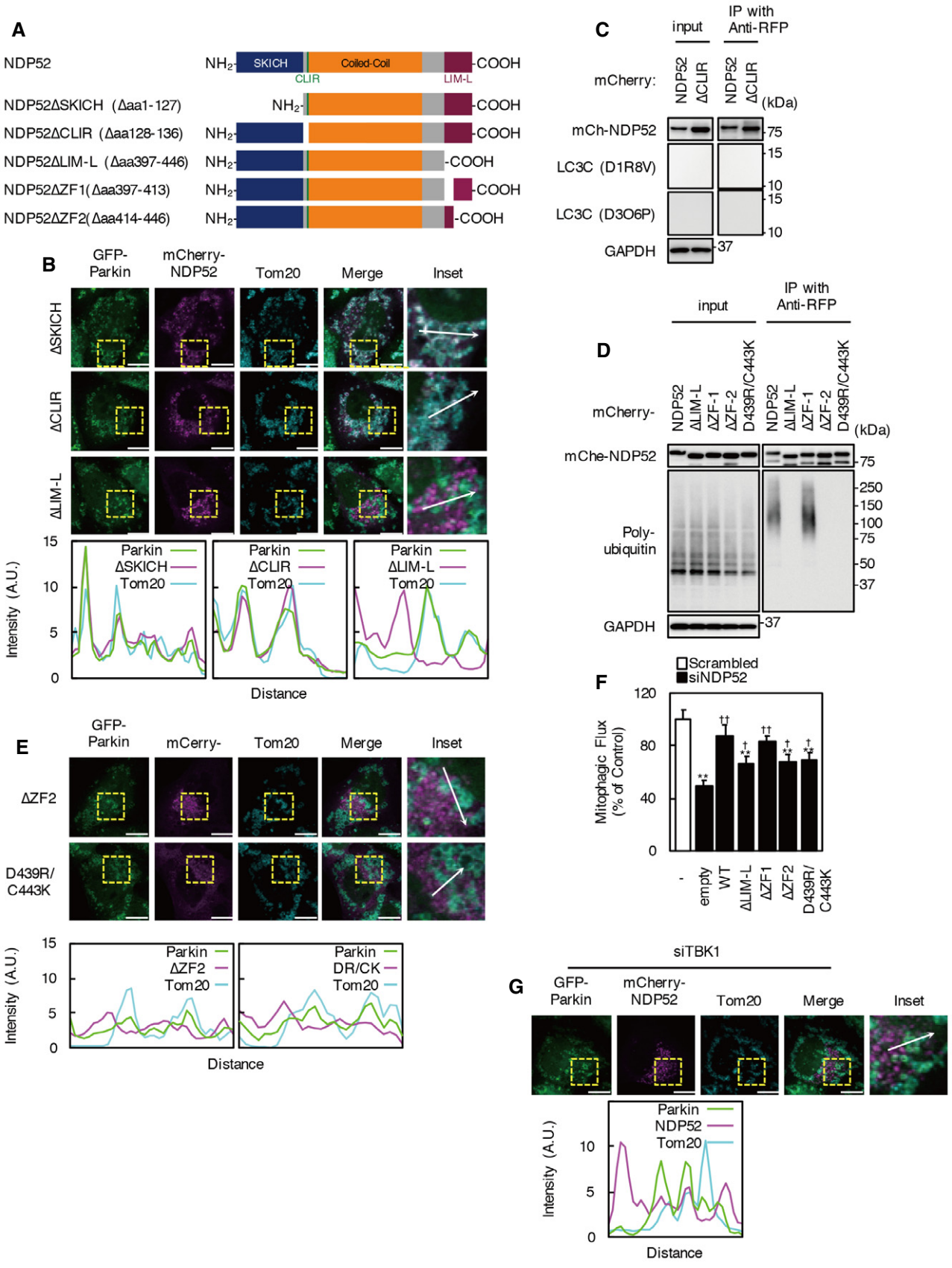

Figure EV2.

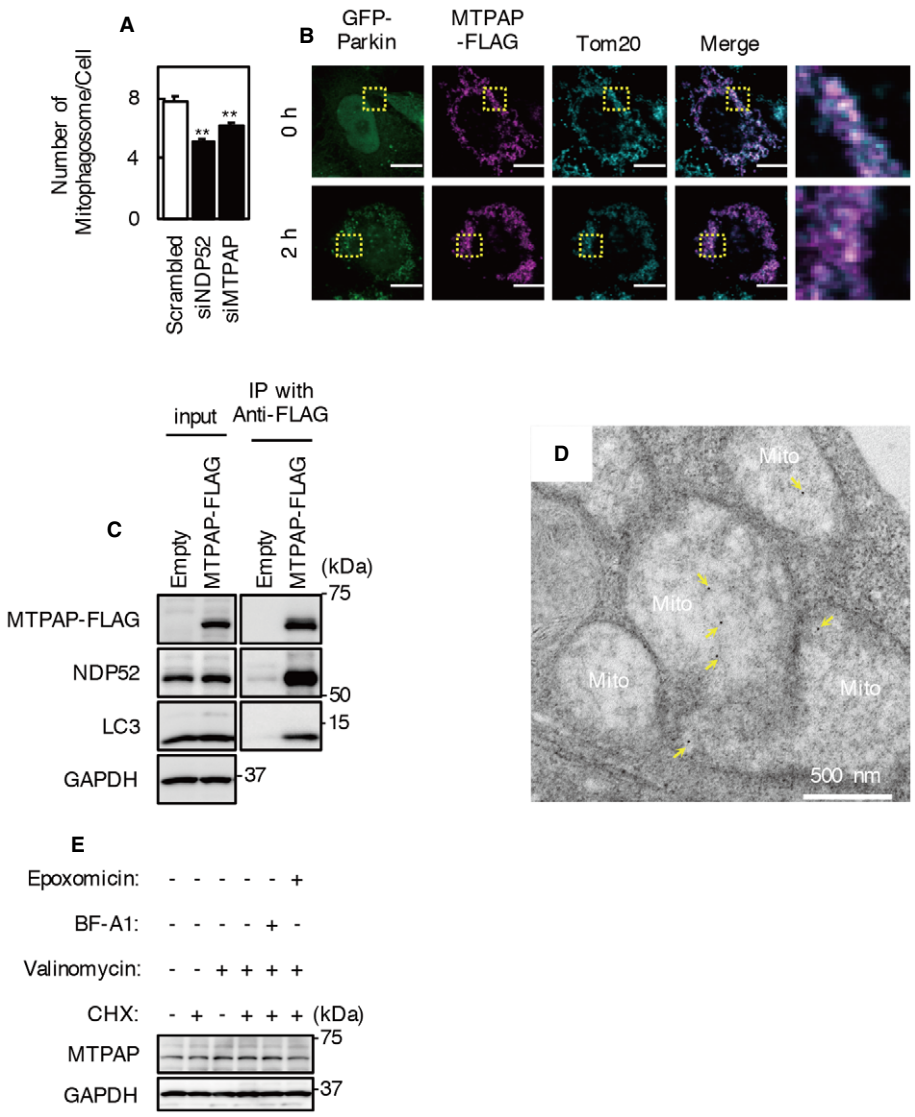

Figure EV3. Intra-mitochondrial localization of MTPAP does not change following valinomycin treatment.

A The number of mitophagosomes per cell was counted in NDP52 KD, MTPAP KD, and control cells. Results are a summary of three independent experiments. Values are the means ± SEM. $^{**}P < 0.01$ vs. control, determined with one-way ANOVA followed by the Tukey–Kramer *post hoc* test.

B MTPAP-FLAG was expressed in HeLa-GFP-Parkin cells. Cells were incubated with valinomycin for 0 or 2 h. Immunostaining was performed with anti-FLAG and anti-Tom20 antibodies. Scale bars, 10 μm.

C HeLa-GFP-Parkin cells transfected with pMTPAP-FLAG or empty vector were incubated with valinomycin and bafilomycin A_1 for 2 h. Cell lysates were immunoprecipitated with anti-FLAG magnetic beads.

D HeLa-GFP-Parkin cells expressing MTPAP-FLAG were incubated with 1 μM valinomycin for 2 h and then subjected to immunoelectron microscopy analysis using an anti-FLAG antibody. Arrow, immunogold-labeled FLAG-MTPAP. Scale bar, 500 nm.

E HeLa-GFP-Parkin cells were incubated with or without valinomycin and cycloheximide for 2 h, and Western blotting was performed.

Source data are available online for this figure.

Figure EV4. Knockdown of PHB2, Rab35, and TBK1 does not influence the interaction between NDP52 and MTPAP.

A Mitophagic flux in cells transfected with siNDP52, siPHB2, and a combination of siRNAs. Cells were incubated with valinomycin for 3 h. Results are a summary of three independent experiments. Values are the means ± SEM. *$P < 0.05$, **$P < 0.01$ vs. scrambled oligo-transfected cells; †$P < 0.05$ vs. siNDP52-transfected cells; §$P < 0.05$ vs. siPHB2-transfected cells, determined with one-way ANOVA followed by the Tukey–Kramer *post hoc* test.

B Images of PHB2 KD cells expressing mCherry-NDP52 under valinomycin treatment. The images are from the upper movie of Movie EV8. Cyan, GFP-Parkin; magenta, mCherry-NDP52. Scale bars, 10 μm.

C mCherry-NDP52 was expressed in MTPAP KD, PHB2 KD, and control cells. Lysates from cells treated with valinomycin and bafilomycin A_1 for 2 h were subjected to immunoprecipitation using anti-RFP beads. Immunoprecipitates were analyzed by Western blotting. The position of MTPAP is indicated by arrowheads.

D Mitophagic flux in cells transfected with combinations of siNDP52 and siRab35. Cells were incubated with valinomycin for 2 h. Results are a summary of three independent experiments. Values are the means ± SEM. *$P < 0.05$ vs. scrambled oligo-transfected cells; NS, not significant.

E Images of Rab35 KD cells expressing mCherry-NDP52 under valinomycin treatment. The images are from the lower movie of Movie EV8. Cyan, GFP-Parkin; magenta, mCherry-NDP52. Scale bars, 10 μm.

F mCherry-NDP52 was expressed in MTPAP KD, Rab35 KD, and control cells. Lysates from cells treated with 1 μM valinomycin and 100 nM bafilomycin A_1 for 2 h were subjected to immunoprecipitation using anti-RFP beads. Immunoprecipitates were analyzed by Western blotting. The position of MTPAP is indicated by arrowheads.

G mCherry-NDP52 was expressed in MTPAP KD, TBK1 KD, and control cells. Lysates from cells treated with 1 μM valinomycin and 100 nM bafilomycin A_1 for 2 h were subjected to immunoprecipitation using anti-RFP beads. Immunoprecipitates were analyzed by Western blotting. The position of MTPAP is indicated by arrowheads.

Source data are available online for this figure.

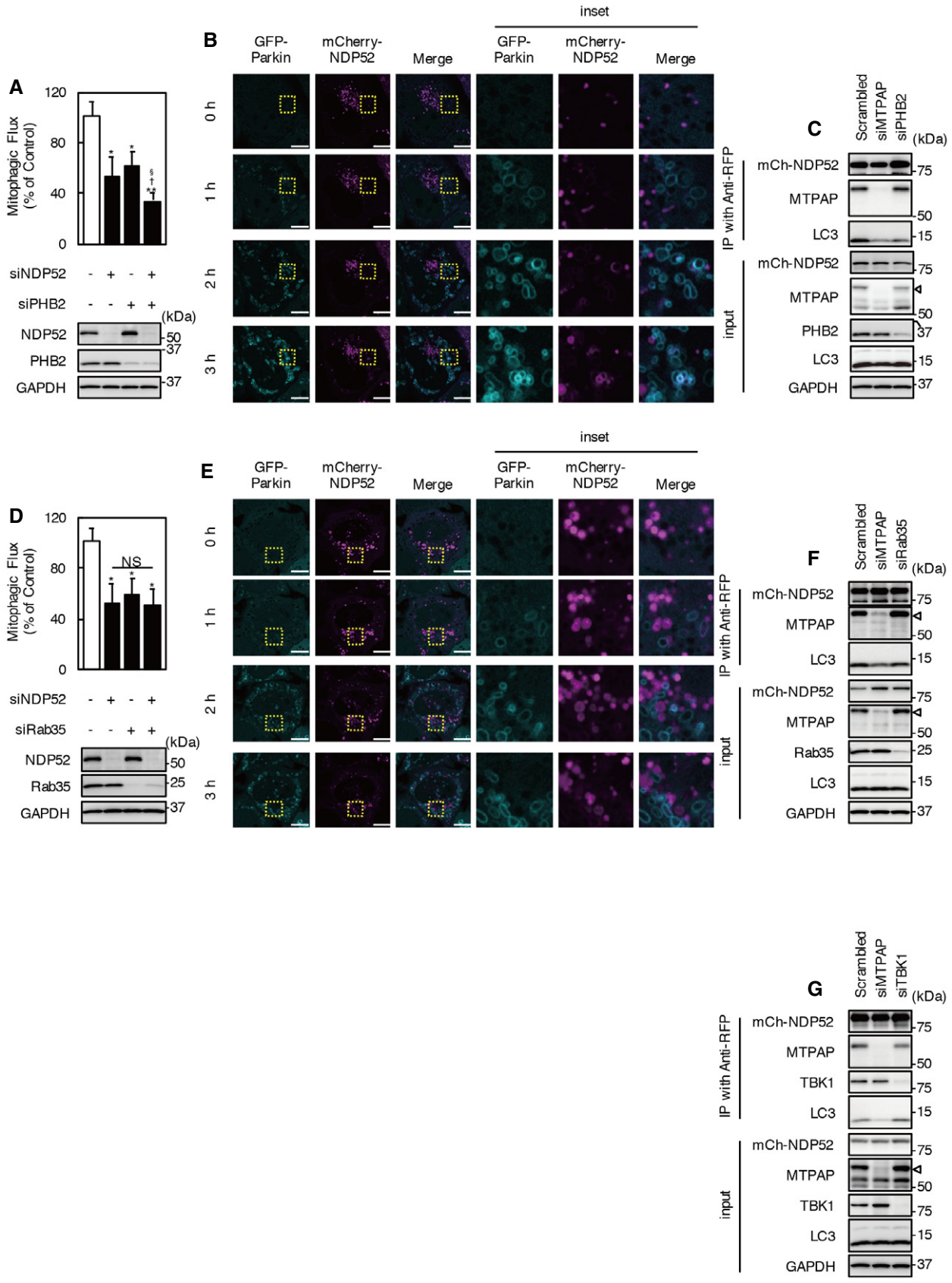
A
Mitophagic Flux (% of Control)
120
80
40
0
siNDP52 - + - +
siPHB2 - - + +
(kDa)
NDP52
PHB2
GAPDH
50
37
37
B
GFP-Parkin
mCherry-NDP52
Merge
inset
0 h
1 h
2 h
3 h
C
Scrambled
siMTPAP
siPHB2
(kDa)
IP with Anti-RFP
input
mCh-NDP52
MTPAP
LC3
PHB2
GAPDH
75
50
15
37
D
NS
siNDP52 - + - +
siRab35 - - + +
Rab35
25
E
F
siRab35
G
siTBK1
TBK1

Figure EV4.

A

MOTIF	START	END	LIR sequence	PSSM score
xLIR	245	250	DMFLDL	13 (7.9e-02)
WxxL	96	101	SQFGPI	5 (1.0e+00)
WxxL	111	116	GLYAVV	4 (1.4e+00)
WxxL	146	151	SRFFNL	3 (1.9e+00)
WxxL	175	180	QLFELL	6 (7.4e-01)
WxxL	235	240	NTFGKL	5 (1.9e+00)
WxxL	341	346	YIYGAL	1 (3.6e+00)
WxxL	496	501	QKFVDL	8 (3.9e-01)
WxxL	523	528	RPWGLV	10 (2.0e-01)

B

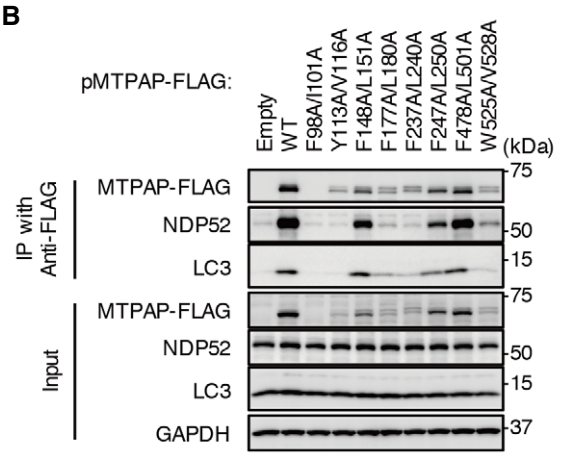

Figure EV5. xLIR motif and WXXL motifs in human MTPAP.

A One xLIR motif and nine WXXL motifs of human MTPAP were identified by the iLIR online server.

B HeLa-GFP-Parkin cells expressing xLIR mutant and WXXL mutants of MTPAP-FLAG incubated with valinomycin and bafilomycin A1 for 3 h. Cell lysates were subjected to immunoprecipitation with anti-FLAG magnetic beads. Immunoprecipitates were analyzed by Western blotting.