Expanded View Figures

Figure EV1. ATAD3B promotes oxidative stress-induced mitophagy.

A Control and ATAD3 DKO (ATAD3A and ATAD3B double knockout) HeLa cell lines were lysed and analyzed by Western blotting using anti-ATAD3 or anti-tubulin antibodies.

B ATAD3 DKO HeLa cells were infected with lentiviral particles containing control, ATAD3A-Flag, or ATAD3B-Flag. Cell lysates were analyzed by Western blotting with anti-ATAD3 or anti-tubulin antibodies.

C–F WT or ATAD3 DKO HeLa cells stably expressing mito-Keima were infected with lentiviral particles containing control, ATAD3A-Flag, or ATAD3B-Flag. Five days later, cells were treated with DMSO, 200 μM H_2O_2 (C), or 4 mM 3-NPA (E) for 2 h and imaged with 458 nm (measuring mitochondria with a neutral pH) and 561 nm (measuring mitochondria with an acidic pH) laser excitation for mito-Keima by confocal microscopy. Scale bar, 10 μm. The relative ratio of red to green fluorescence intensity (561 nm/458 nm) of DMSO, H_2O_2-treated (D), or 3-NPA-treated (F) cells were then quantified respectively by ImageJ software. Data are presented as mean ± SD ($n = 3$ independent experiments, 20 cells per experiment), and statistical significance was assessed by a two-way ANOVA, $^*P < 0.05$, $^{**}P < 0.01$.

G MEFs stably expressing mito-Keima were infected with lentiviral particles containing control or ATAD3B-Flag and further cultured for 5 days. Cells were then incubated with DMSO or 200 μM H_2O_2 for 2 h and imaged with 458 nm (measuring mitochondria with a neutral pH) and 561 nm (measuring mitochondria with an acidic pH) laser excitation for mito-Keima using confocal microscopy. Scale bar, 10 μm.

H Quantification of the relative ratio of red to green fluorescence intensity (561 nm/458 nm) of the cells described in (F). Data are presented as mean ± SD ($n = 3$ independent experiments, 20 cells per experiment), and statistical significance was assessed by two-tailed Student's *t*-test, N.S., not significant, $^{**}P < 0.01$.

Source data are available online for this figure.

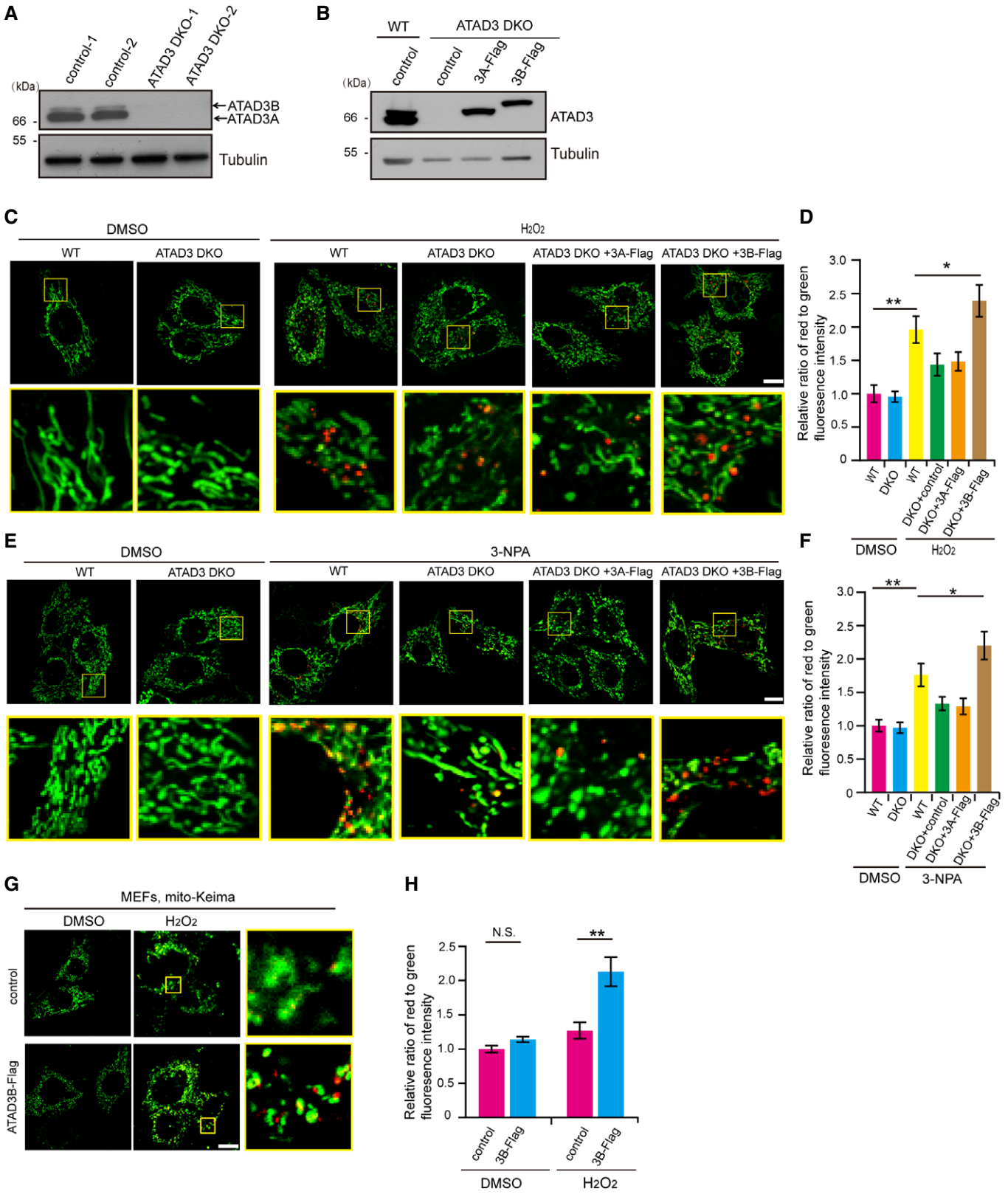
A
control-1
control-2
ATAD3 DKO-1
ATAD3 DKO-2
(kDa)
66
55
ATAD3B
ATAD3A
Tubulin
B
WT
ATAD3 DKO
control
control
3A-Flag
3B-Flag
(kDa)
66
55
ATAD3
Tubulin
C
DMSO
H2O2
WT
ATAD3 DKO
WT
ATAD3 DKO
ATAD3 DKO +3A-Flag
ATAD3 DKO +3B-Flag
D
Relative ratio of red to green fluoresence intensity
3.0
2.5
2.0
1.5
1.0
0.5
0
**
*
WT
DKO
WT
DKO+control
DKO+3A-Flag
DKO+3B-Flag
DMSO
H2O2
E
DMSO
3-NPA
WT
ATAD3 DKO
WT
ATAD3 DKO
ATAD3 DKO +3A-Flag
ATAD3 DKO +3B-Flag
F
Relative ratio of red to green fluoresence intensity
3.0
2.5
2.0
1.5
1.0
0.5
0
**
*
WT
DKO
WT
DKO+control
DKO+3A-Flag
DKO+3B-Flag
DMSO
3-NPA
G
MEFs, mito-Keima
DMSO
H2O2
control
ATAD3B-Flag
H
Relative ratio of red to green fluoresence intensity
2.5
2.0
1.5
1.0
0.5
0
N.S.
**
control
3B-Flag
control
3B-Flag
DMSO
H2O2

Figure EV1.

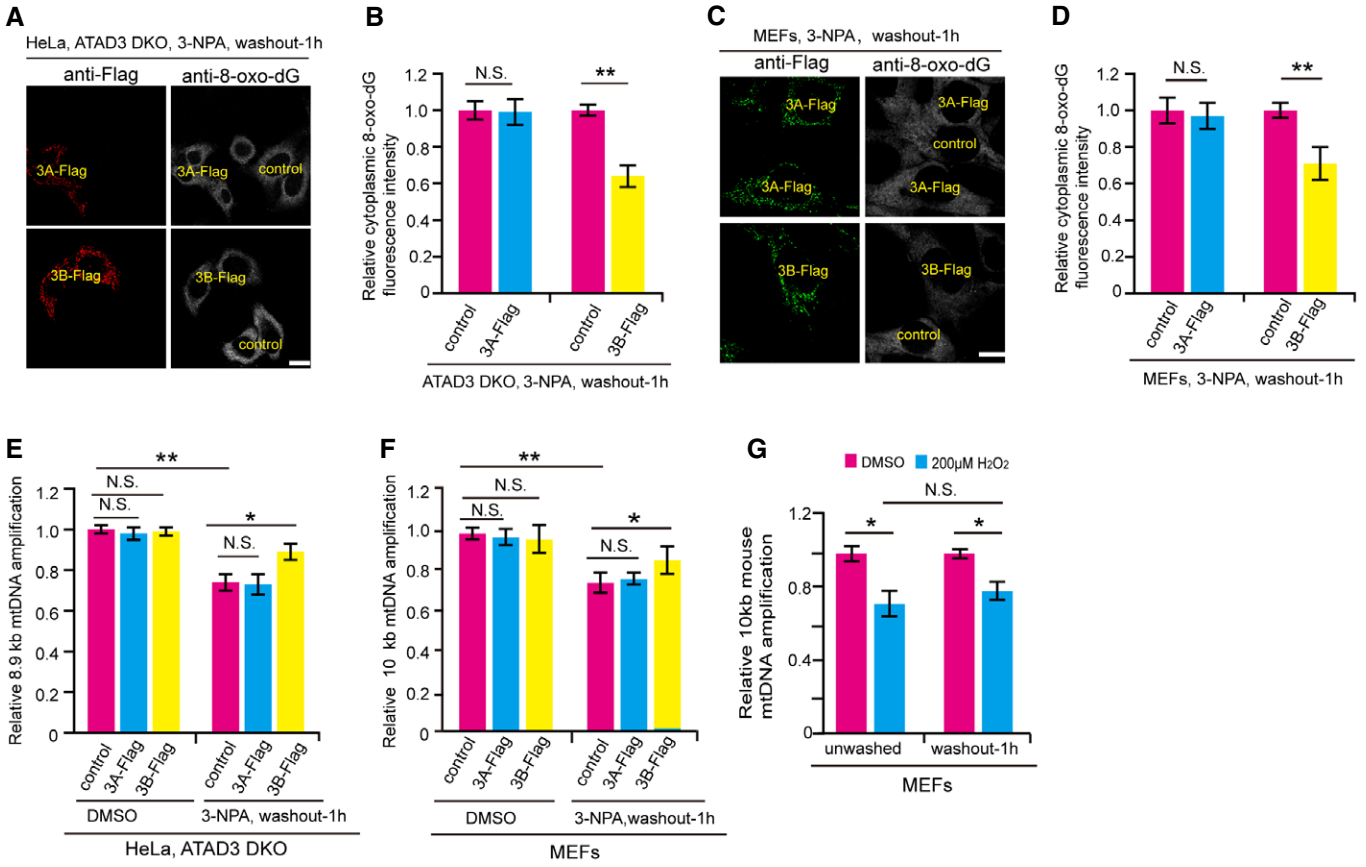

Figure EV2. ATAD3B promotes the clearance of damaged mtDNA.

A ATAD3 DKO HeLa cells stably expressing ATAD3A-Flag or ATAD3B-Flag were mixed with control ATAD3 DKO HeLa cells, respectively, and cultured for 24 h. After treated with 4 mM 3-NPA for 2 h, cells were washed with fresh medium and incubated for another 1 h. Cells were then fixed and immunostained with anti-Flag and anti-8-oxo-dG antibodies and were analyzed by confocal microscopy. Scale bar, 10 μm.

B Quantification of the relative 8-oxo-dG fluorescence intensity in cells described in (A). Data are presented as mean ± SD (n = 3 independent experiments, 20 cells per experiment), and statistical significance was assessed by individual two-tailed Student's t-test, N.S., not significant, $^{**}P < 0.01$.

C MEF cells stably expressing ATAD3A-Flag or ATAD3B-Flag were mixed with control MEF cells, respectively, and cultured for 24 h. After treated with 4 mM 3-NPA for 2 h, cells were washed with fresh medium and incubated for another 1h. Cells were then fixed and immunostained with anti-Flag and anti-8-oxo-dG antibodies and were analyzed by confocal microscopy. Scale bar, 10 μm.

D Quantification of the relative 8-oxo-dG fluorescence intensity in cells described in (C). Data are presented as mean ± SD (n = 3 independent experiments, 20 cells per experiment), and statistical significance was assessed by individual two-tailed Student's t-test, N.S., not significant, $^{**}P < 0.01$.

E, F ATAD3 DKO HeLa or MEF cells stably expressing control, ATAD3A-Flag, and ATAD3B-Flag were treated with DMSO or 4 mM 3-NPA for 2 h and washed with fresh medium and incubated for another 1 h. Cells were then harvested and used for total DNA extraction. Extracted DNA samples were used for amplification of human 8.9 kb mtDNA (homo) fragment or mouse 10 kb mtDNA fragment using quantitative PCR and were normalized to amplification of a 221 bp (homo) or 117 bp (mus) mtDNA fragment. PCR products were quantitated by PicoGreen staining using Micro Plate Reader. Data are presented as mean ± SD (n = 3 independent experiments), and statistical significance was assessed by a two-way ANOVA, N.S., not significant, $^{*}P < 0.05$, $^{**}P < 0.01$.

G MEFs were incubated with DMSO or 200 μM H_2O_2 for 2 h. Cells were then harvested or washed with fresh medium and incubated for another 1 h. Cells with or without washout were used for extracting total DNA. All DNA samples were used for amplification of 10 kb mouse mtDNA fragment using quantitative PCR and were normalized to amplification of a 117 bp mtDNA fragment. PCR products were quantitated by PicoGreen staining using Micro Plate Reader. Data are presented as mean ± SD (n = 3 independent experiments), and statistical significance was assessed by a two-way ANOVA, N.S., not significant, $^{*}P < 0.05$.

Figure EV3. ATAD3B binds to LC3B and in a PHB2-independent manner.

A 293T cells were transfected with GFP-LC3B, in the presence of control, ATAD3A-Flag, or ATAD3B-Flag. Forty-eight hours after transfection, cell lysates were immunoprecipitated (IP) with anti-Flag M2 affinity gel, followed by Western blotting using anti-Flag or anti-GFP antibodies.

B Control or Prohibitin2 knockdown (siPHB2) 293T cells were treated with OA (2.5 mM oligomycin, 250 nM antimycin A) for 4 h. Cell lysates were then incubated with GST-ATAD3B(265-648aa) (expressed in *E. coli*) coupled to glutathione agarose beads for GST pull-down assay. Eluted protein samples were analyzed by Western blotting using antibodies against GST, LC3, or PHB2.

C HeLa expressing GFP-LC3 monoclonal cell line was infected with lentivirus particles containing control, ATAD3B-Flag, or ATAD3B (mLIR, Y604A/L607A)-Flag. Five days later, cells were treated with DMSO, or 200 μM H_2O_2 for 2 h, then fixed and immune-stained with anti-Tom20 or anti-Flag antibodies. Cells were analyzed by confocal microscopy. The white arrows indicate the LC3 punta colocalizing or contacting with Tom20 (mitochondria). Scale bar, 10 μm.

D Quantification of the GFP-LC3 puncta colocalized or contacted with Tom20 in cells described in (C). Data are presented as mean ± SD ($n = 30$), and statistical significance was assessed by a two-way ANOVA, N.S., not significant, ***$P < 0.001$.

Source data are available online for this figure.

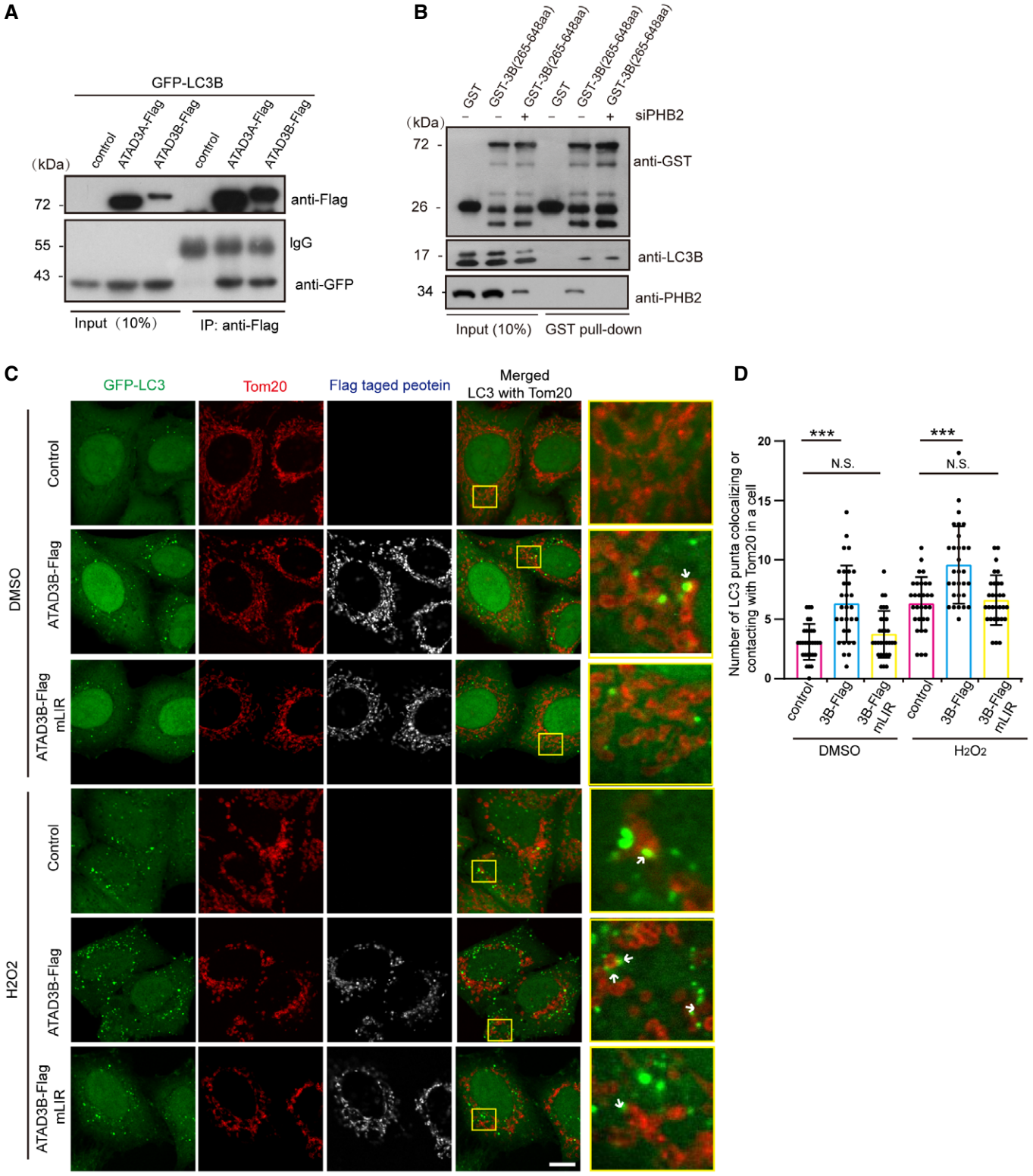

Figure EV3.

Figure EV4. Change of ATAD3B localization upon oxidative stress.

A Prediction of human ATAD3A and ATAD3B protein domains using TMPred and HMMTOP software. (PRM, proline-rich motif; CC1 and CC2, coiled-coil domains; TM, transmembrane domain).

B Mic10 KO COS7 cells stably expressing ATAD3A-Flag or ATAD3B-Flag were treated with DMSO or 4 mM 3-NPA for 2 h, and immunostained with anti-Tom20 or anti-Flag antibodies. Immunostained cells were then visualized and imaged by confocal microscopy. Scale bar, 10 μm.

C–F 293T cells treated with DMSO (C) or H_2O_2 (200 μM, 2 h) (E) were harvested for mitochondrial isolation. Purified mitochondria were treated with the indicated gradient concentration of proteinase K (0, 20, 30, 40, 50, 60 μg/ml) for 20 min on ice and then were analyzed by Western blotting with anti-ATAD3, anti-Tom20 (OMM), anti-Tim23 (IMS), and anti-HSP60 (matrix). Relative protein levels of proteins in (C) or (E) were further evaluated by densitometry analysis using ImageJ software. Relative trends of proteolysis of indicated mitochondrial proteins from DMSO- or H_2O_2-treated cells were shown (D and F). Data are presented as mean ± SD (n = 3 independent experiments).

G 293T cells were transiently transfected with control, ATAD3A-Flag or ATAD3B-Flag. Forty-eight hours after transfection, cells were collected for CHIP-Seq with anti-Flag M2 affinity gel. Fold enrichment of the targeted genomic sequences over IgG was calculated as: $2^{-(Ct_{CHIP}-Ct_{IgG})}$, where Ct_{CHIP} and Ct_{IgG} are the mean threshold cycles of triplicate PCRs of DNA samples. Data are presented as means ± SD of three independent experiments. Statistical significance was assessed by Student's t-test, $^{**}P < 0.01$.

H Representative structural modeling of ATAD3A, ATAD3B, and ATAD3A-ATAD3B complex. The structures of ATAD3A and ATAD3B were predicted using I-TASSER (Iterative Threading ASSEmbly Refinement). The RMSD (Root mean square deviation) between structures of ATAD3A and ATAD3B was calculated by Rosetta software. The binding sites were analyzed based on the first 10 low-energy-score-optimized models.

I 293T cells transiently transfected with control or ATAD3B-Flag were treated with or without 200 μM H_2O_2 for 2 h. Cell lysates were immunoprecipitated (IP) with anti-Flag M2 affinity gel, followed by Western blotting with anti-Flag or anti-ATAD3 antibodies.

J Quantification of the relative protein level of ATAD3A to ATAD3B-Flag described in (E). Data are presented as mean ± SD (n = 3 independent experiments), and statistical significance was assessed by two-tailed Student's t-test, $^{**}P < 0.01$.

K HeLa cells were treated with DMSO, 200 μM H_2O_2, or 200 μM H_2O_2 plus 10 mM NAC for 2 h. Mitochondria isolated from cells were subjected to blue native PAGE (BN–PAGE) and were analyzed by Western blotting with anti-ATAD3 or SDHA (loading control). SDS–PAGE and followed Western blotting data were shown at the right panel.

Source data are available online for this figure.

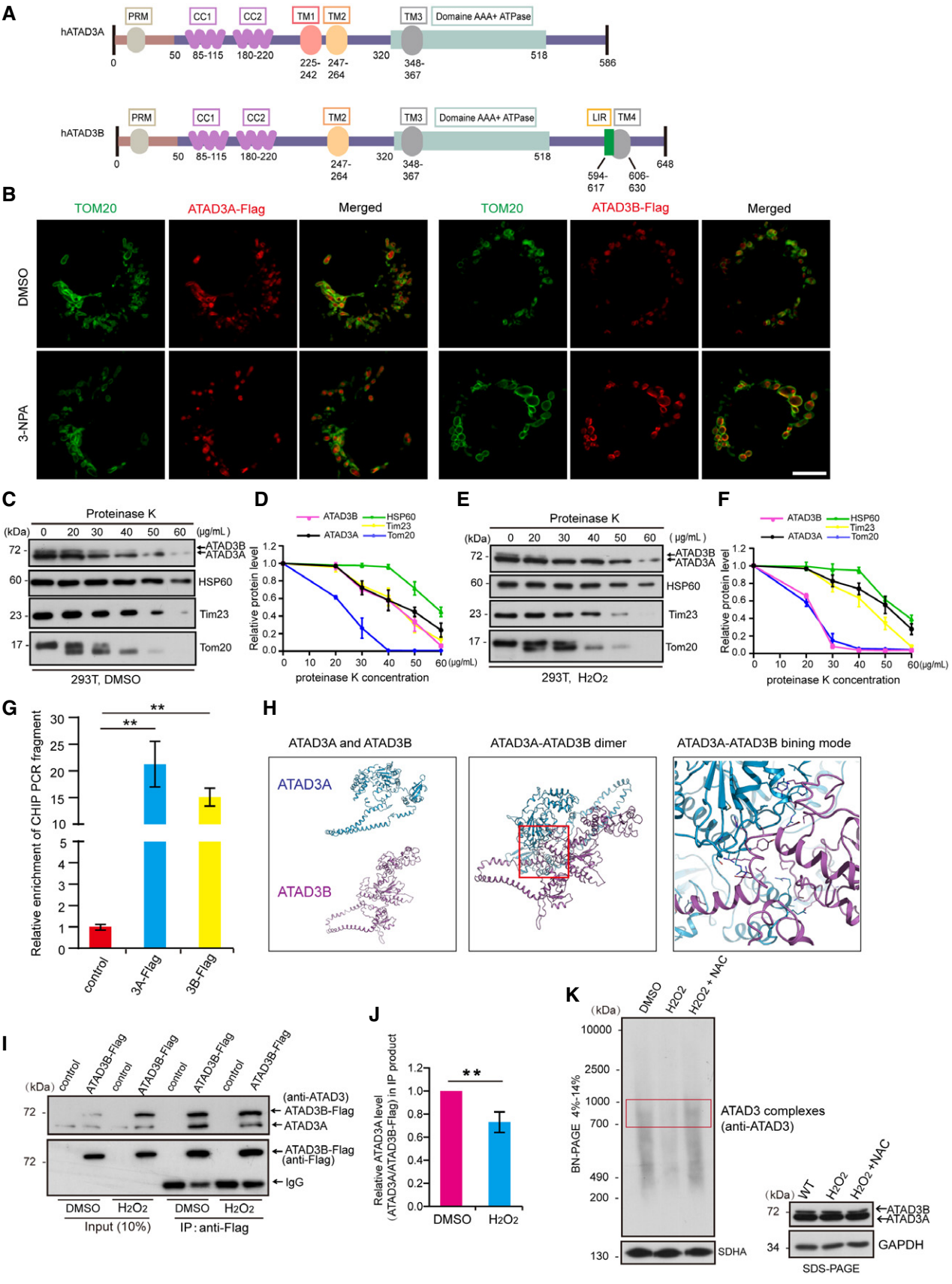

Figure EV4.

Figure EV5. ATAD3B promotes hypoxia-induced mitophagy.

A Control or ATAD3A KO HeLa cells stably expressing mito-Keima were treated with DMSO, 4 mM 3-NPA, or 200 μM H_2O_2 for 2 h. Then, cells were imaged with 458 nm (measuring mitochondria with a neutral pH) and 561 nm (measuring mitochondria with an acidic pH) laser excitation for mito-Keima by confocal microscopy. Scale bar, 10 μm.

B Quantification of the relative ratio of red to green fluorescence intensity (561 nm/458 nm) of the cells described in (A). Data are presented as mean ± SD ($n = 3$ independent experiments, 20 cells per experiment), and statistical significance was assessed by two-tailed Student's *t*-test, $^*P < 0.05$.

C, D HeLa cells were cultured in normoxia or hypoxia (1% O_2) for 12 h. Then, cells were stained with mitoSOX and analyzed by confocal microscopy (C). DIC, Differential Interference Contrast. Quantification of the MitoSOX fluorescence intensity was shown in the right panel (D). Data are presented as mean ± SD ($n = 3$ independent experiments, 20 cells per experiment), and statistical significance was assessed by Student's *t*-test, $^{***}P < 0.001$.

E HeLa cells stably expressing mito-Keima were infected with shATAD3B or ATAD3B-Flag. Then, cells were cultured in normoxia or in hypoxia (1% O_2) for 12 h. Cells were imaged with 458 nm (measuring mitochondria with a neutral pH) and 561 nm (measuring mitochondria with an acidic pH) laser excitation for mito-Keima by confocal microscopy. Scale bar, 10 μm.

F Quantification of the relative ratio of red to green fluorescence intensity (561 nm/458 nm) of the cells described in (A). Data are presented as mean ± SD ($n = 3$ independent experiments, 20 cells per experiment), and statistical significance was assessed by two-tailed Student's *t*-test, $^*P < 0.05$.

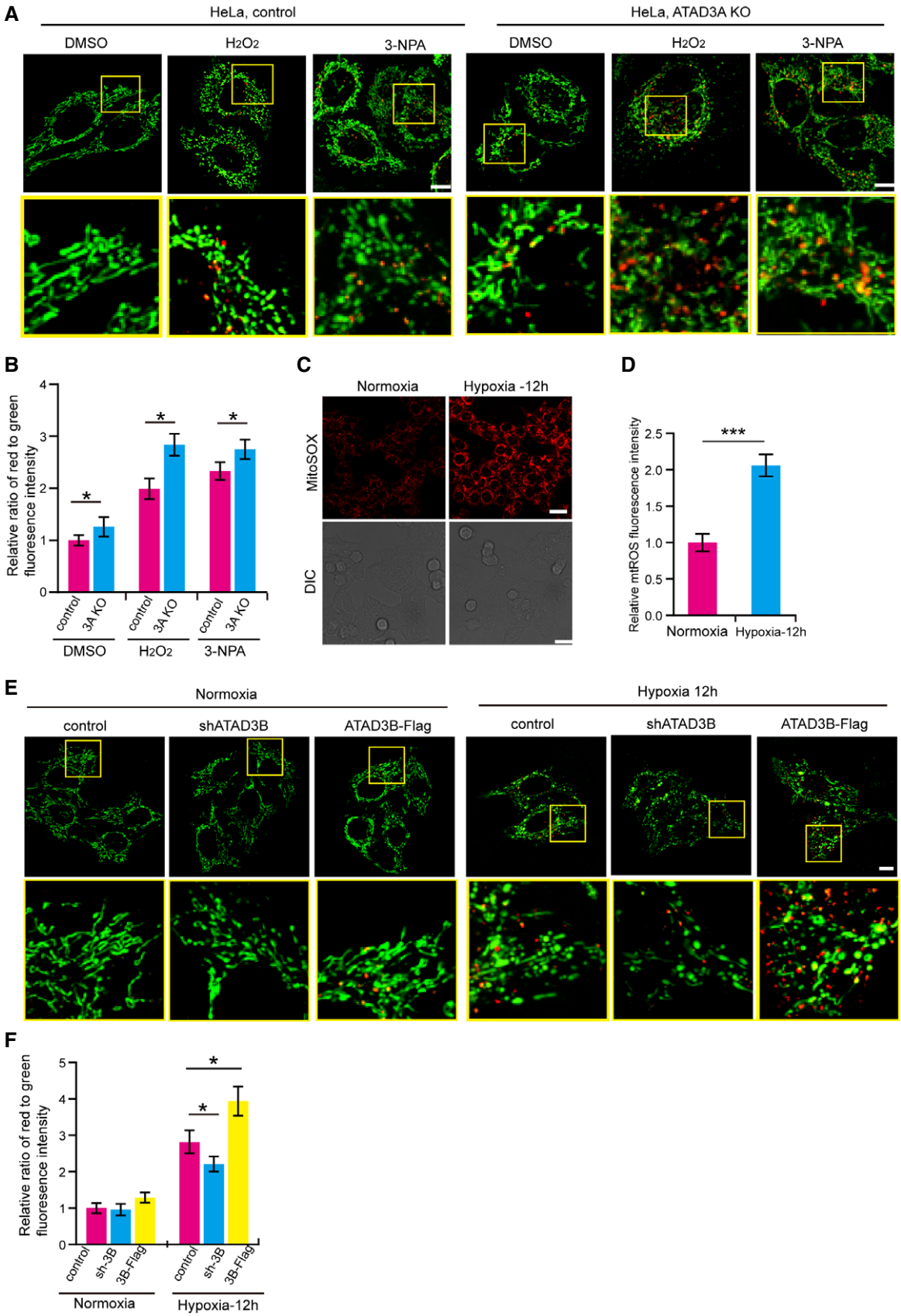

Figure EV5.