ATAD3B Mediates the Clearance of Oxidative Stress-induced Damaged mtDNA by Acting as a Mitophagy Receptor

Li Shu, Chao Hu, Meng Xu, Jianglong Yu, He He, Jie Lin, Hongying Sha, Bin Lu, Simone Engelender, Minxin Guan, and Zhiyin Song

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Appendix Figure Legends

Appendix Figure S1. Autophagy contributes to the clearance of H_2O_2-induced damaged mtDNA.

A. HeLa, MEF, and HEK293 were used for the extraction of total DNA. DNA samples were used for amplification of 8.9 kb mtDNA(human), 12.2 kb nDNA(human), 10 kb mtDNA (mouse), or 6.6 kb nDNA (mouse) fragments, and PCR products were run in agarose gel electrophoresis (AGE).

B. Control or shATG5 HEK293 cells were treated with DMSO, 100 μM H_2O_2 or 200 μM H_2O_2 for 2 h. After the treatment, cells were immediately 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 8.9 kb mtDNA fragment using quantitative PCR and quantitated by PicoGreen staining using Micro Plate Reader. Error bars are presented as mean ± SD (n = 3 independent experiments), statistical significance was assessed by two-tailed student's t-test, N.S., not significant, *$p < 0.05$, **$p < 0.01$.

C. Control or shATG5 HEK293 cell lysates were analyzed by Western blotting with anti-ATG5 or anti-Tubulin antibodies.

D-F. HEK293 (D), HeLa (E) and MEF (F) were treated with DMSO or 200 μM H_2O_2 for 2 h. After the treatment, 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 human 12.2 kb nDNA segments (HEK293 and HeLa) or mouse 6.6 kb nDNA segments (MEFs) using quantitative PCR and quantitated by PicoGreen staining using Micro Plate Reader. Error bars are presented as mean ± SD (n = 3 independent experiments), statistical significance was assessed by two-tailed student's t-test, N.S., not significant, *$p < 0.05$.

G. HeLa cells were incubated with DMSO, 200 μM H_2O_2 or 4 mM 3-NPA for 2 h, and then stained with mitoSOX and analyzed by confocal microscopy. Quantification of relative MitoSOX intensity was shown at right panels. Error bars are presented as mean ± SD (n =6 pictures), statistical significance was assessed by a one-way ANOVA, N.S., not significant, ***$p < 0.001$. Scale bar, 25 μm.

H. HeLa cells were treated with DMSO or 200μM H_2O_2 for 2 h, and were imaged by transmission electron microscopy, the red arrowhead indicates autophagosome, and the yellow arrowheads indicate the mitochondria. Scale bar, 500 nm.

I. HeLa WT were treated with DMSO, 200 μM H_2O_2 or 10 μM FCCP for 2 h, and incubated with TMRM (600 nm, 25 min), then analyzed by confocal microscopy. Quantification of relative membrane potential was shown at right panels. Error bars are presented as mean ± SD (n= 3 independent experiments, 20 cells per experiment), statistical significance was assessed by a one-way ANOVA, ***p < 0.001. Scale bar, 25 μm.

Appendix Figure S2. Knockdown effect of mtDNA nucleoid-related proteins.

A-C. Control or mtDNA nucleoid-associated proteins (indicated proteins) were depleted in HeLa cells by short hairpin RNA interference. Cell lysates were analyzed by Western blotting using the indicated antibodies (A). The effect of POLMRT (B) or POLG2 (C) knockdown was analyzed by quantitative PCR.

Appendix Figure S3. Alignment and expression of human ATAD3A and ATAD3B.

A. Human ATAD3A and ATAD3B protein sequences were aligned by Clustal X (version 2.1) software.

B. Phylogenetic tree based on ATAD3A and ATAD3B protein sequences. ATAD3A and ATAD3B protein sequences were analyzed with Molecular Evolutionary Genetics Analysis (date from NCBI).

C. Expression levels of human ATAD3A and ATAD3B in different tissues (data from HumanProteome Map).

Appendix Figure S4. ATAD3B regulates mitophagy in a PINK1-independent manner.

A. PINK1 KO 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, H_2O_2 (200 μM, 2 h), or OA (2.5 mM oligomycin plus 250 nM antimycin A, 4 h). Cells were then 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 (561nm/458nm) of the cells described in (A). Error bars are presented as mean ± SD (n= 3 independent experiments, 20 cells per experiment), statistical significance was assessed by two-tailed student's t-test, N.S., not significant, *p < 0.05, **p < 0.01.

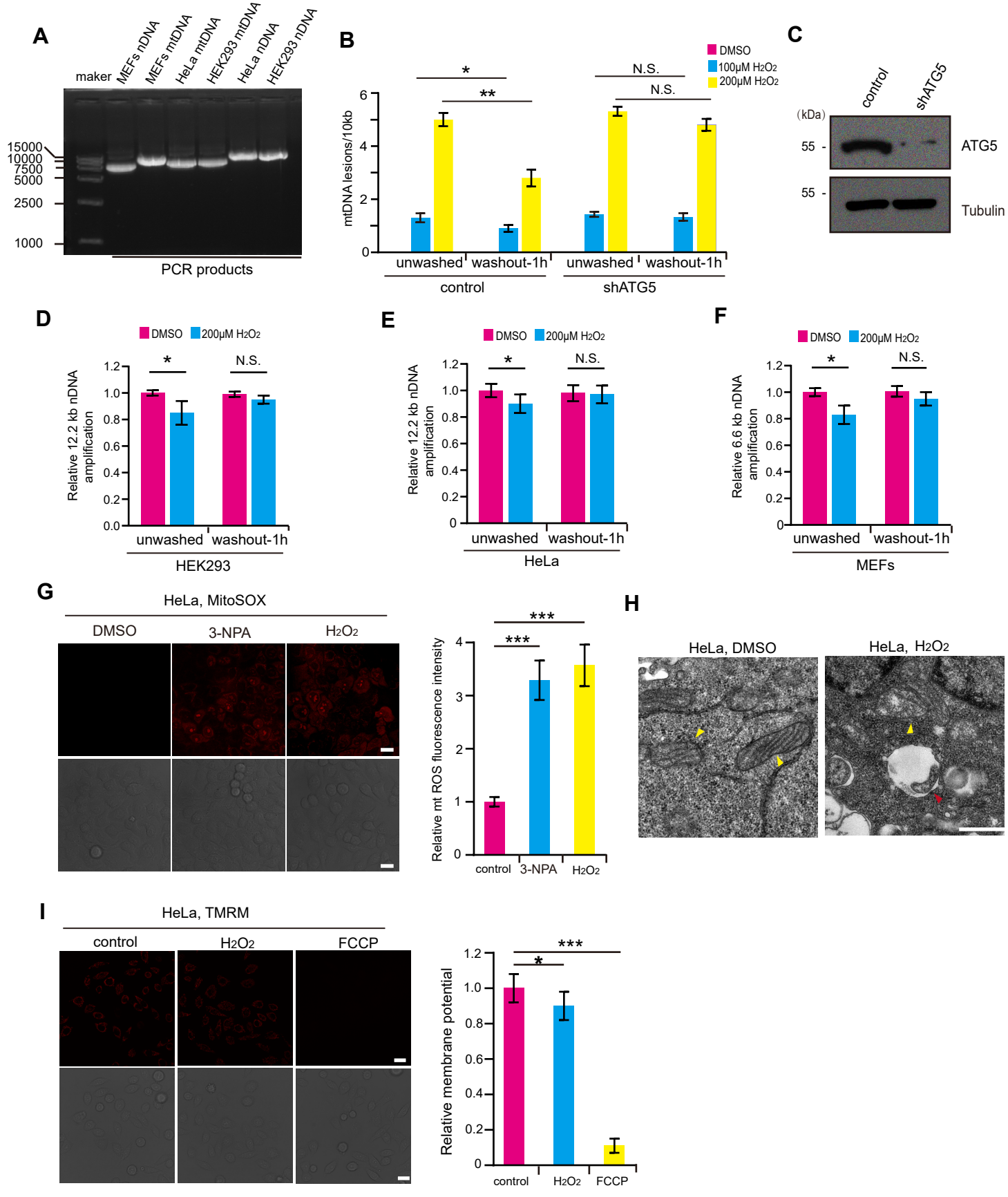
C. PINK1 KO HeLa cells expressing mt-Keima were infected with lentiviral particles containing control, ATAD3A-Flag or ATAD3B-Flag. Five days later, cell lysates were analyzed by Western blotting with anti-ATAD3 or anti-Tubulin antibodies.

D. Control or PINK1 KO HeLa cells were treated with or without OA (10 μM oligomycin, 4 μM antimycin A) for 4 h, and cell lysates were analyzed by Western blotting with anti-PINK1 or anti-Tubulin antibodies. The asterisk indicates an unspecific band.

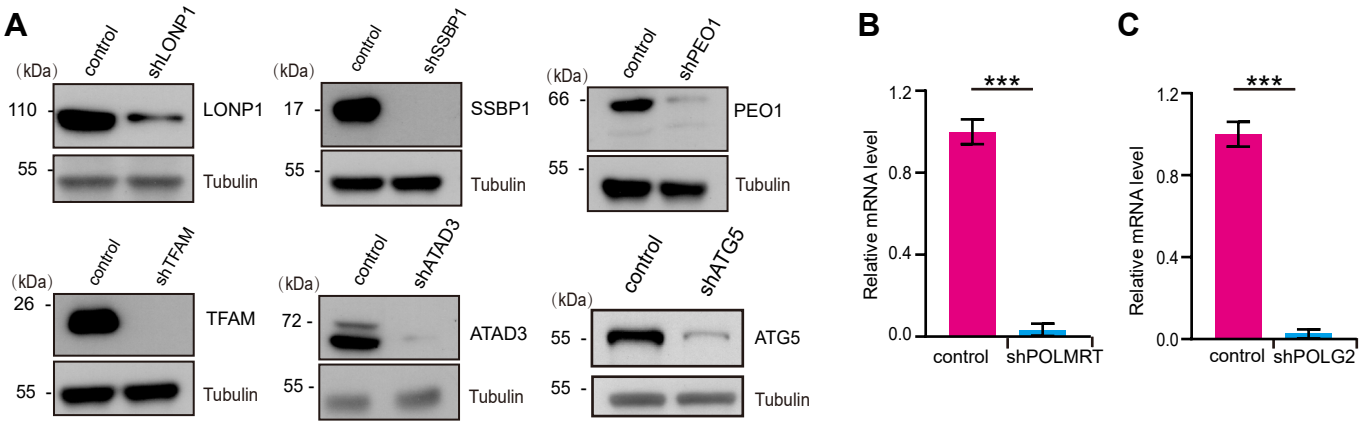
E. HEK293 or A549 were treated with DMSO, H_2O_2 (100 μM, 200 μM or 500 μM, 2 h), or OA (2.5 mM oligomycin plus 250 nM antimycin A, 4 h). Cell lysates were then analyzed by Western blotting with anti-PINK1 or anti-GAPDH antibodies. The asterisk indicates an unspecific band.

F. Control or shATAD3B PINK1 KO HeLa cell lines were lysed and analyzed by Western blotting with anti-ATAD3 or anti-Tubulin antibodies.

Appendix Figure S1

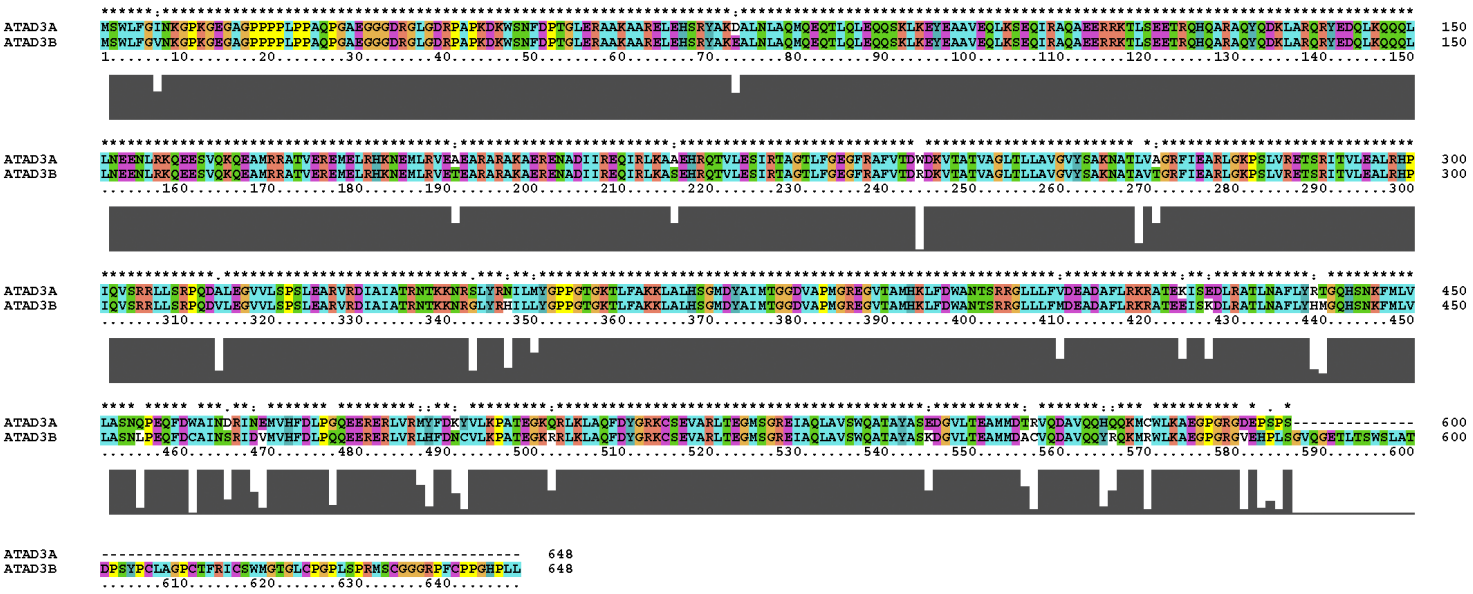


Appendix Figure S2



Appendix Figure S3

A



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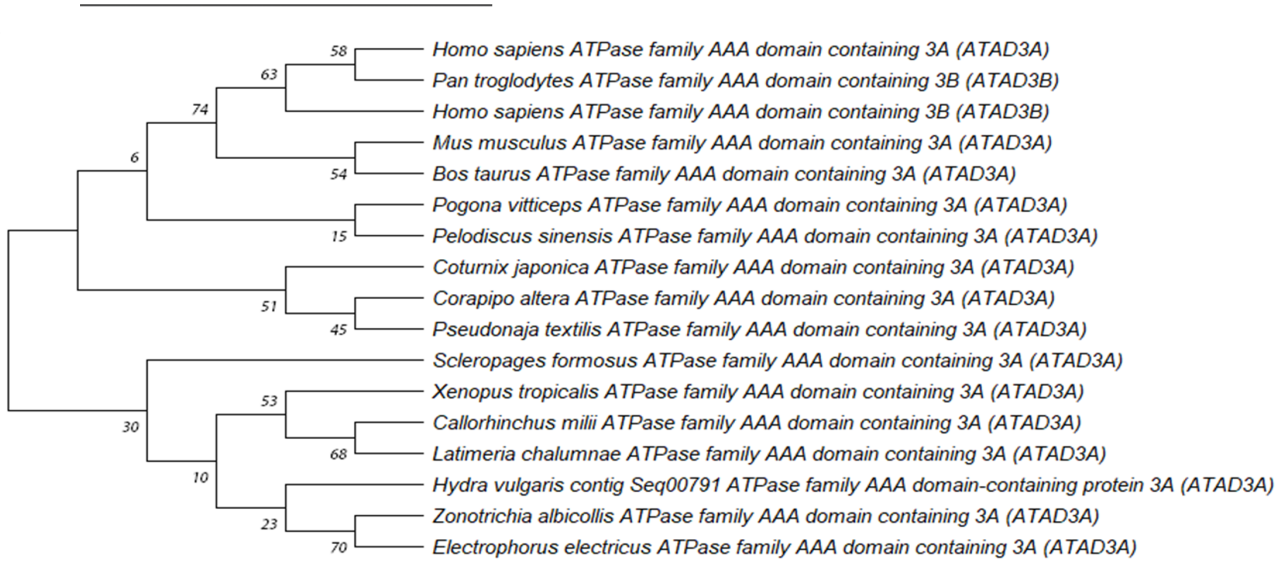

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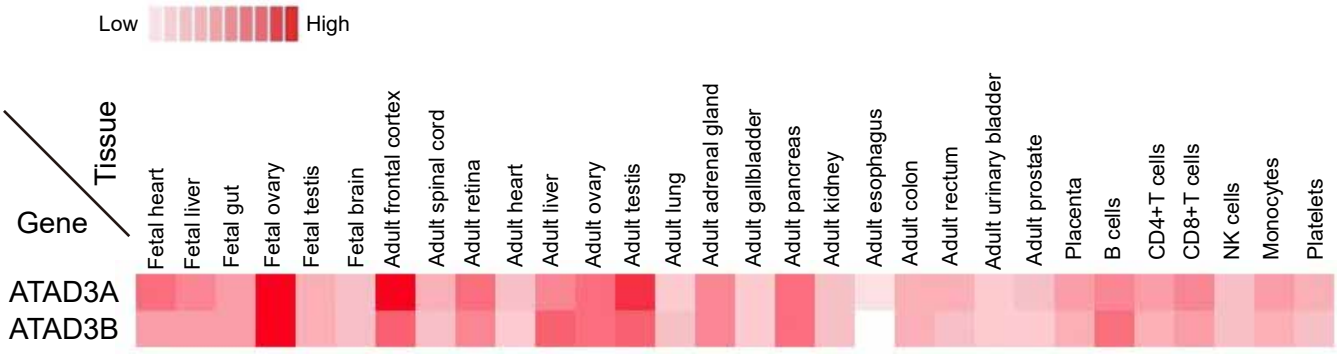
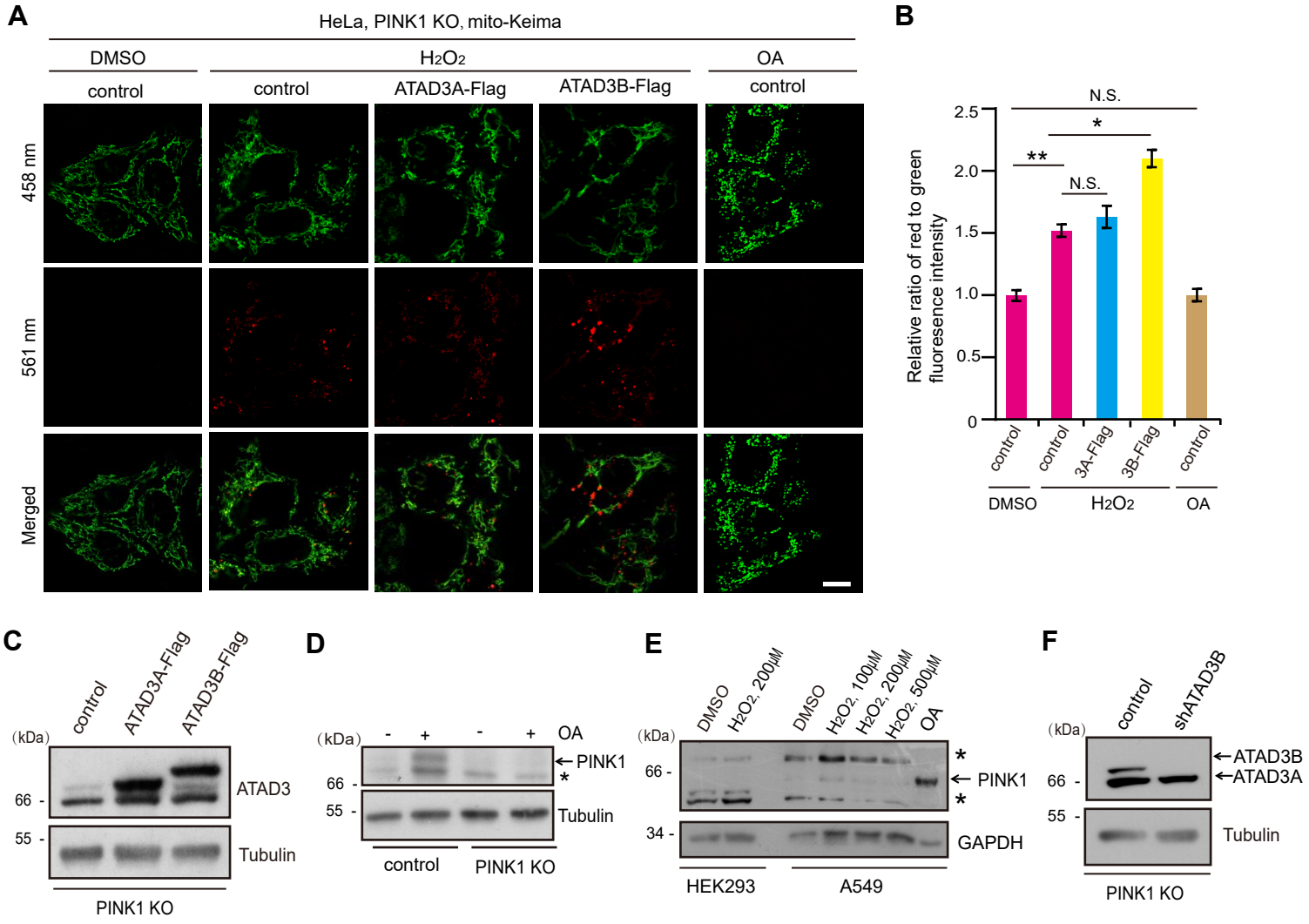


Appendix Figure S4



Supplemental Experimental Tables

Appendix Table S1. List of primers for construction of knockdown (KD) or knockout (KO) plasmids.

Targeted gene sequences for knockdown were listed in italic, bold and underline.

Target gene	**Oligonucleotides sequence**
Homo ATAD3A (ATAD3A KO-1)	F 5′ -CACCGAATGAGATGCTGCGAGTGG-3′ R 5′ -AAACCCACGCGCAGCATCTCATTC-3′
Homo ATAD3A (ATAD3A KO-2)	F 5′ -CACCGCCCCAAGGGTGAAGGCGCG-3′ R 5′ -AAACCGCGCCTTCACCCTTGGGGC-3′
Homo ATAD3B (ATAD3B KO-1)	F 5′ -CACCGGCGGCGGGGACCGCGGTTT-3′ R 5′ -AAACAAACCGCGGTCCCCGCCGCC-3′
Homo ATAD3B (ATAD3B KO-2)	F 5′ -CACCGCCGGCCGGTCTCCCAAACCG-3′ R 5′ -AAACCGGTTTGGGAGACCGGCCGGC-3′
Homo ATAD3 (ATAD3 KD-1, targeting both ATAD3A and ATAD3B)	F5′-GATCCCC***CGTGGCTCTTCGGCATTAA***TTCAAGAGA TTAATGCCGAAGAGCCACGTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAACGTGGCTCTTCGGCATTAA TCTCTTGAATTAATGCCGAAGAGCCACGGGG-3′
Homo ATAD3 (ATAD3 KD-2, targeting both ATAD3A and ATAD3B)	F5′-GATCCCC***GCAGCAGCGACTTCTCAAT***TTCAAGAGA ATTGAGAAGTCGCTGCTGCTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAAGCAGCAGCGACTTCTCAAT TCTCTTGAAATTGAGAAGTCGCTGCTGCGGG-3′
Homo ATAD3B (ATAD3B KD-1)	F5′-GATCCCC***GGCCAACACAGCAACAAAT***TTCAAGAGA ATTTGTTGCTGTGTTGGCCTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAAGGCCAACACAGCAACAAAT TCTCTTGAAATTTGTTGCTGTGTTGGCCGGG-3′
Homo ATAD3B (ATAD3B KD-2)	F5′-GATCCCC***GGGCCCAGTATCAAGACAA***TTCAAGAGA TTGTCTTGATACTGGGCCCTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAAGGGCCCAGTATCAAGACAA TCTCTTGAATTGTCTTGATACTGGGCCCGGG-3′
Homo ATG5 (ATG5 KD)	F5′-GATCCCC***CCTTTCATTCAGAAGCTGT***TTCAAGAGA ACAGCTTCTGAATGAAAGGTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAACCTTTCATTCAGAAGCTGT

	TCTCTTGAAACAGCTTCTGAATGAAAGGGGG-3′
Homo mtSSB (mtSSB KD)	F5′-GATCCCC***GGCATATCAATATGTGAAA***TTCAAGAGA TTTCACATATTGATATGCCTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAAGGCATATCAATATGTGAAA TCTCTTGAATTTCACATATTGATATGCCGGG-3′
Homo POLG2 (POLG2 KD)	F5′-GATCCCC***GGAGGAGTTTCAACAAGAT***TTCAAGAGA ATCTTGTTGAAACTCCTCCTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAAGGAGGAGTTTCAACAAGAT TCTCTTGAAATCTTGTTGAAACTCCTCCGGG-3′
Homo Twinkle (Twinkle KD)	F5′-GATCCCC***GGCGGCTGGAAGATCAACT***TTCAAGAGA AGTTGATCTTCCAGCCGCCTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAAGGCGGCTGGAAGATCAACT TCTCTTGAAAGTTGATCTTCCAGCCGCCGGG-3′
Homo LONP1 (LONP1 KD)	F5′-GATCCCC***GCACGTCATGGATGTTGTG***TTCAAGAGA CACAACATCCATGACGTGCTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAAGCACGTCATGGATGTTGTG TCTCTTGAACACAACATCCATGACGTGCGGG-3′
Homo POLMRT (POLMRT KD)	F5′-GATCCCC***GGAGCTGGTATATGTGTTA***TTCAAGAGA TAACACATATACCAGCTCCTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAAGGAGCTGGTATATGTGTTA TCTCTTGAATAACACATATACCAGCTCCGGG-3′
Homo TFAM (TFAM KD)	F5′-GATCCCC***GTGGCAGGTATATAAAGAA***TTCAAGAGA TTCTTTATATACCTGCCACTTTTTGGAAA-3′ R5′-AGCTTTTCCAAAAAGTGGCAGGTATATAAAGAA TCTCTTGAATTCTTTATATACCTGCCACGGG-3′
Homo PHB2 (siPHB2)	F5′ -AGAUAAACACCAACCCAGGAAUUCT-3′ R5′ -AGAAUUCCUGGGUUGGUGUUUAUCUCC-3′

Appendix Table S2. List of primers for quantitative real-time PCR.

Primes	**Oligonucleotides sequence**
8.9kb segments of human mitochondrial DNA	F5′ -TCTAAGCCTCCTTATTCGAGCCGA-3′ R5′ -TTTCATCATGCGGAGATGTTGGATGG-3′
12.2kb segments of human nuclear DNA	F5′ -CATGTCACCACTGGACTCTGAAC-3′ R5′ -CCTGGAGTAGGAACAAAATTGCT-3′
221bp fragment of the human mitochondrial DNA	F5′ -CCCCACAAACCCCATTACTAAACCCA-3′ R5′ -TTTCATCATGCGGAGATGTTGGATGG-3′
10kb segment of mouse mitochondrial DNA	F5′ -GCCAGCCTGACCCATAGCCATAATAT-3′ R5′ -GAGAGATTTTATGGGTGTAATGCGG-3′
Mouse 6.6kb fragment of the DNA polymerase beta gene	F5′ -TATCTCTCTTCCTCTTCACTTCTCCCCTGG-3′ R5′ -CGTGATGCCGCCGTTGAGGGTCTCCTG-3′
117bp fragment of mouse mitochondrial DNA	F5′-CCCAGCTACTACCATCATTCAAGT-3′ R5′-GATGGTTTGGGAGATTGGTTGATGT-3′
Taqman probe for 3243G of human mitochondrial DNA	F5′ -CCACACCCACCCAAGAACAG-3′ R5′ -TATGTTGTTAAGAAGAGGAATTGAACCT-3′ Probe, 5′ -FAM-TAAGATGGCAGGGC-MGB-3′
Tubulin (homo)	F5′-GACCTGACTGACTACCTCATGAAGAT-3′ R5′- GTCACACTTCATGATGGAGTTGAAGG-3′